**Pharmacogn.** Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

# *In vitro* Antitumor Potential of Methanol Extract of *Mimosa pudica* in Human Breast Cancer Cell Lines

**Reni John, Bibu John Kariyil[1], P. T. A. Usha, S. Surya, G. Anu, Preethy John, S. Sujith, Aziz Zarina[2]**

Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, [1]Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode, Wayanad, [2]Department of Veterinary Physiology, College of Veterinary and Animal Sciences, Mannuthy, Thrissur, Kerala, India

Submitted: 26-Dec-2019 Revised: 26-Feb-2020 Accepted: 21-Apr-2020 Published: 28-Aug-2020

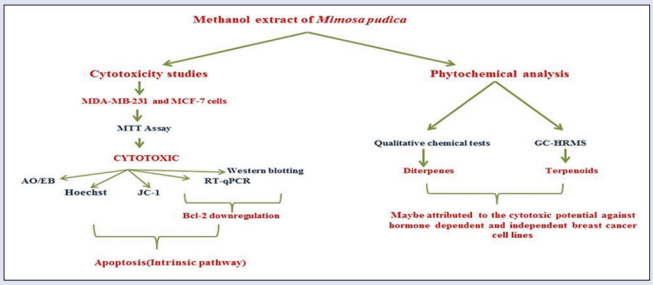
## ABSTRACT

**Background:** *Mimosa pudica* belonging to the family *Fabaceae*, is a small sized shrub, used traditionally for its anti-spasmodic, analgesic, anti-spasmodic, antibacterial, and antitumor activities. **Objectives:** The present study was conducted to assess the anticancer activity of methanol extract of whole plant of *M. pudica* (MMP) in MCF-7 and MDA-MB-231 breast cancer cell lines. **Materials and Methods:** The antitumor activity of MMP was analyzed using 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay in MCF-7 and MDA-MB-231 cell lines. The cytological and metabolic alterations due to MMP were evaluated using acridine orange/ethidium bromide dual staining, hoechst 33258 and fluoroprobe, benzimidazol-carbocyanine iodide 5, 5′, 6, 6′-tetrachloro-1, 1′, 3,3′-tetra ethyl (JC-1) staining. Real-time polymerase chain reaction and Western blotting were carried out to assess Bcl-2, the antiapoptotic gene, and protein expression, respectively. Qualitative analysis and gas chromatography high-resolution mass spectrometry were performed for the presence of various phytochemicals. **Results:** The methanol extract exhibited a potent antitumor activity in both the cell lines *in vitro*. **Conclusion:** The plant extract was showing the mode of action through intrinsic pathway of apoptotic cell death and may be studied further to develop a potent drug against breast cancer.

**Key words:** 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay, Bcl-2, gas chromatography high-resolution mass spectrometry, *Mimosa pudica*, phytochemical tests

## SUMMARY

- Phytochemical screening of the methanol extract of whole plant of *Mimosa pudica* revealed the presence of alkaloids, flavonoids, glycosides, steroids, phenolics, and diterpenes upon qualitative studies. Majority of compounds were belonging to terpenoids in the extract when assessed using gas chromatography high-resolution mass spectrometry. Since diterpenes were detected in phytochemical screening also, terpenoids might contributed for the obtained anticancer activity for the plant extract
- On the basis of $IC_{50}$ value calculated using 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide assay, the methanol *M. pudica* extract possessed potent cytotoxic action. Methanol extract of the plant was found to induce apoptosis in breast carcinoma cells through intrinsic pathway which was assessed through acridine orange/ethidium bromide dual staining, hoechst staining, and JC-1 staining. The antiapoptotic gene and protein expressions of Bcl-2 were found to be significantly down regulated after the treatment with the plant extract. Further research and fractionation are required to characterize the bioactive compounds responsible for the obtained potential of *M. pudica* as a novel source for antitumor drugs.




**Abbreviations used:** MMP: Methanol extract of whole plant of *Mimosa pudica*; MTT: 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; AO/EB: Acridine orange/ethidium bromide; JC-1: Benzimidiazol carbocyanine iodide 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetra ethyl; GC-HRMS: Gas chromatography high-resolution mass spectrometry; NISCAIR: National Institute of Science Communication and Information Resources; CSIR: Council of Scientific and Industrial Research; NCCS: National Centre for Cell Sciences; ER: Estrogen receptor; PR: Progesterone receptor, HER: Human epidermal growth factor receptor; FBS: Foetal bovine serum; $IC_{50}$: Half maximal inhibitory concentration; ANOVA: Analysis of variance; RT-qPCR: Real time-quantitative polymerase chain reaction; RIPA: Radioimmunoprecipitation assay; SDS-PAGE: Sodium dodecyl sulphate polyacrylamide gel electrophoresis; PVDF: Polyvinylidene difluoride; SAIF: Sophisticated Analytical Instrument Facility; IIT: Indian Institute of Technology; $\Delta\psi m$: Mitochondrial transmembrane potential; Bcl-2: B-cell lymphoma-2.

**Correspondence:**

Dr. Bibu John Kariyil,
Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Pookode - 673 576, Wayanad, Kerala, India.
E-mail: bibujohn@kvasu.ac.in
**DOI:** 10.4103/pm.pm_527_19

**Access this article online**
**Website:** www.phcog.com
**Quick Response Code:**

## INTRODUCTION

Herbal medicines have paved the way for the development of a huge number of drugs used for the cancer therapy nowadays. Recent research on cancer subtypes bring on evidence that medicinal plants and their derivatives could contribute to mainstream and supplementary therapeutic strategies in a tremendous manner. They are thought to augment therapeutic efficacy of conventional chemotherapy which may reduce their side effects in cancer patients.[1] Hence, the chemotherapeutic drug discovery field from herbal sources demand a sustained research to



**Cite this article as:** John R, Kariyil BJ, Usha PTA, Surya S, Anu G, John P, *et al*. *In vitro* antitumor potential of methanol extract of *Mimosa pudica* in human breast cancer cell lines. Phcog Mag 2020;16:S396-403.

isolate newer compounds with maximum efficacy and limited toxicity at therapeutic levels.

The study mainly aims at the development of plant based novel drug molecules which could serve as a reliable alternative source for cancer chemotherapy. *Mimosa pudica* belonging to the family *Fabaceae*, is a small-sized shrub, used traditionally for its anti-asthmatic, analgesic, anti-spasmodic, antibacterial, and antitumor activities.[2,3] Taking into consideration on the traditional knowledge, this study focusses on the antitumor potential of methanol extract of *M. pudica* whole plant and an insight toward the probable phytoconstituents contributing to the outcome.

## MATERIALS AND METHODS

### Collection of plant material and authentication

The whole plants of *M. pudica* were collected during June–July 2016 from the Aranmula region of Pathanamthitta district, Kerala, India. The plant materials were authenticated by the Raw material and herbarium Department, National Institute of Science Communication and Information Resources (NISCAIR), Council of Scientific and Industrial Research and the voucher specimen of the plant was deposited in the Department of Veterinary Pharmacology and Toxicology, College of Veterinary and Animal Sciences, Mannuthy with accession no. HERB/VPT/CVASMTY/2/2017.

### Cell lines and culture conditions

Adherent human breast adenocarcinoma cell lines, MDA-MB-231 and MCF-7, procured from National Centre for Cell Science, Pune, Maharashtra, India, was utilized for *in vitro* anticancer studies. MDA-MB-231 cell line was estrogen receptor (ER) –ve, progesterone receptor (PR) –ve, and human epidermal growth factor receptor (HER) –ve. MCF-7 cell line was ER +ve, PR +ve, HER –ve. These adherent cells were grown in RPMI-1640 fortified with 10% foetal bovine serum and 1% antibiotic antimycotic solution (penicillin-streptomycin and amphotericin B), which were maintained in a humidified incubator at 37°C with 5% $CO_2$. The cells were trypsinised using 0.25% trypsin/1 mM EDTA solution. Those cell suspensions having ≥95% viability (determined using trypan blue vital stain in automated cell counter (Countess™, Invitrogen, Van Allen Way, Carlsbad, California) were used for seeding in culture plates for various *in vitro* studies.

### Methanol extraction

The whole plant of *M. pudica* was washed, shade-dried, and powdered coarsely in a temperature controlled plant sample pulverizer. The powdered plant material (1 kg) was extracted with methanol in soxhlet extractor at room temperature., The methanol extract, obtained after exhaustive extraction, was filtered and concentrated in rotary vacuum evaporator (Evator, Equitron EV11.ABI.029, India) at 50°C and 30 mmHg pressure. A reddish-brown colored residue obtained was first kept open at room temperature for complete evaporation of solvent and then stored in the sealed airtight container in refrigerator for further use.

### Sample preparation

Stock solution of the methanol extract was prepared in 100% dimethyl sulfoxide (DMSO) at a concentration of 50 mg/mL. The filtered stock of extract was further diluted using phosphate buffered saline (PBS) to get the required concentrations of the extracts.

### Cytotoxic evaluation of methanol extract of whole plant of *Mimosa pudica in vitro*

*In vitro* cytotoxic potential of methanol extract of whole plant of *Mimosa pudica* (MMP) was assessed in MCF-7 and MDA-MB-231 breast cancer cell lines, using 3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide (MTT) reduction assay as per Riss *et al*.[4] The cell concentration was maintained at 10000 cells per well, in 200 µL medium were incubated overnight at 37°C in $CO_2$ incubator.

The extract stocks with concentrations ranging from 320, 160, 80, 40, 20, 10, 5, and 2.5 µg/mL were added to the cells for 48 h. After removing the extract added media, 20 µL of MTT (5 mg/mL prepared in Dulbecco's phosphate-buffered saline [DPBS]) was added and incubated at 37°C for 4 h in $CO_2$ incubator. After incubation, the media-containing MTT was removed. Added 200 µL of DMSO (cell culture grade) to dissolve the formazan crystals formed. The absorbance was measured using microplate reader (Varioskan Flash, Thermofischer scientific, Finland) at a wavelength of 570 nm.

The percent cell viability was calculated using the following formula:

Percent cell viability = (average absorbance of treated cells/average absorbance of untreated cells) × 100.

The net absorbance from the wells of control cells was taken as 100% viable. $IC_{50}$ (half maximal inhibitory concentration) value of extract was calculated by plotting the concentration against percentage cell viability using the online software "very simple $IC_{50}$ tool kit."

### Selection of concentrations

Three concentrations of the extract, i.e., one below $IC_{50}$, $IC_{50}$, and one above $IC_{50}$ were selected for the study, based on the MTT assay. Thus the concentrations that were used for the study were 10, 20 and 40 and 8, 16 and 32 µg/mL for MDA-MB-231 and MCF-7 cells, respectively.

### Microscopic studies

Trypsinized cells were seeded into 6 well cell culture plates at a cell concentration of $1 \times 10^6$ cells per well and incubated overnight. Cells were then treated with various concentrations of extracts for 24 h at 37°C and 5% $CO_2$. Untreated cells were used as control. Doxorubicin at a concentration of 0.58 µg/mL was adopted as positive control. After 24 h of incubation the cells were trypsinised, washed with 1X DPBS and fixed with 4% paraformaldehyde in 1X PBS for 30 min at room temperature. The cells were again washed and resuspended in 50 µL DPBS. Acridine orange/ethidium bromide (AO/EB), hoechst 33258 and fluoroprobe, benzimidazol-carbocyanine iodide 5,5',6,6×-tetrachloro-1,1',3,3'-tetra ethyl (JC-1) staining techniques were used.

### Dual acridine orange ethidium bromide staining

AO/EB staining was performed according to the method described by Kasibhatla *et al*.[5] in order to differentiate the live, apoptotic, and necrotic cells after treatment with the plant extract. Twenty-five microliters of cell suspension were mixed with 10 µL of AO/EB solution (1 part of l0 µg/mL acridine orange in PBS and 1 part of 10 µg/mL ethidium bromide in PBS) just before microscopy. Placed 10 µL of cell suspension on a microscopic slide, covered with cover slip and examined under trinocular Research Flourescent microscope (DM 200 LED Leica) with blue excitation (488 nm) and emission (550 nm) filters at 20×magnification.

### Hoechst 33258 staining

Nuclear changes of apoptosis were determined by Hoechst 33258 staining.[6] MCF-7 and MDA-MB-231 cells ($1 \times 10^5$ cells per well) were treated with the above-mentioned concentrations of MMP for 24 h. Cells were washed with PBS and fixed with methanol for 5 min. Cells were stained after fixation with Hoechsht 33258 stain in PBS (5 µg/mL) for 30 min at 37°C in the dark. Cells were thoroughly washed with PBS and examined under a fluorescence microscope (DM 200 LED Leica) with an excitation of 350 nm and emission of 460 nm filters at 200× magnification.

The percent apoptotic cells were assessed by counting the number of apoptotic cells in six different microscopic fields. The variation between the groups for apoptotic cell percent were assessed by the one-way analysis of variance followed by Duncan's multiple comparison test.

## JC-1 staining

MCF-7 and MDA-MB-231 cells were seeded in six well plates ($1 \times 10^5$ cells/well) and treated with MMP in the above-mentioned concentrations for 24 h. 5 μM JC-1 stain was added and incubated at 37°C for 30 min in the dark. The cells were evaluated using Trinocular Research Fluorescence microscope (DM 2000 LED, Leica). The filters used were blue and red excitation/emission of 540/570 nm and 590/610 nm.[7]

## *In vitro* Bcl-2 gene expression

The *Bcl-2* gene expression in cell culture samples were evaluated using the real time-quantitative polymerase chain reaction (RT-qPCR). Corresponding $IC_{50}$ concentrations of the extract were added to the cells for 24 h. The qRT-PCR was performed using Maxima SYBR green qPCR master mix (Thermo Scientific, USA) following the manufacturer's instructions. Reactions contained human Bcl-2 primer sets (Sigma). Human GAPDH served as a positive control. qRT-PCR was done on a Real time PCR cycler (Applied Biosystems, USA). The level of Bcl-2 expression was measured using the $2^{-\Delta\Delta CT}$ method[8] and presented as fold change of the gene relative to the control cells. Expression fold change in gene and protein expression was assessed using the one sample *t*-test.

## Western immunoblotting

Lysates of control and extract treated ($IC_{50}$ concentration) cells were prepared by homogenizing cells with radio immunoprecipitation assay buffer with protease and phosphatase inhibitors on ice for 1 hr after washing twice in 1X PBS followed by centrifugation at 18,728 g, 4°C for 15 min. Total protein concentration was estimated by taking an aliquot of the lysate using Lowry method (Genei kit protocol). Using 12% sodium dodecyl sulphate-polyacrylamide gel electrophoresis, proteins were separated and subsequently transferred to polyvinylidene difluoride membrane (Hoefer Semi dry transfer apparatus). β actin was used as internal control to ensure equal protein loading. The membranes were incubated with primary antibodies of Bcl-2 (1:1000, Sigma-Aldrich) and beta-actin (1:2000, Sigma-Aldrich). The binding of antibodies were visualized by incubating the blots with horse radish peroxidase-conjugated secondary antibody (Cell Signaling Technology) followed by colour reaction with DAB substrate buffer. The Western blotting band strength was determined by Image J density Measurement program (http://imagej.en.softonic.com).[9] Expression fold change in protein expression was assessed using the one sample *t*-test.

## Phytochemical screening

The methanol extract of *M. pudica* was subjected to preliminary phytochemical screening to study for the nature of various phytoconstituents.[10]

## Gas chromatography high-resolution mass spectrometry analysis

Gas chromatography high-resolution mass spectrometry (GC-HRMS) analysis of MMP was conducted in Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Mumbai, India. Gas chromatography (Agilent, USA, 7890) was used with a mass range of 10–2000 amu, mass resolution of 6000, FID detector, EI/CI source and time of flight analyser. The carrier gas used was helium at a flow rate of 1 ml/min. The oven temperature was increased to 200°C in 5 min after maintaining at 70°C for 1 min. The injector temperature was 250°C with a total analysis time of 50 min. After obtaining a clear baseline, 0.4 μL aliquots of extracts were injected into the chromatographic column. Interpretation on mass spectrum GC-MS and identification of major constituents were performed using mass spectrum library (NIST MS search 2.0 library).[11]

# RESULTS

## Cytotoxic evaluation of methanol extract of whole plant of *Mimosa pudica in vitro*

In both MDA-MB-231 and MCF-7 cancer cell lines, the percent cell viability after addition of MMP showed an abrupt reduction followed by a static range from 20 μg/mL onward. The $IC_{50}$ for MMP were obtained as 19.1 ± 8.28 and 16.07 ± 5.08 μg/mL for MDA-MB-231 cells and MCF-7 cells, respectively [Figure 1].

## Dual acridine orange ethidium bromide staining

Live, necrotic, early and late apoptotic cells were detected after the treatment with extract. The representative images of cells of treatments after AO/EB staining are given in Figures 2 and 3. In control cell population, cells were live showing greenish fluorescence with circular nucleus uniformly distributed in the center. Early apoptotic cells with localized crescent-shaped or granular yellow-green stained nucleus were seen in both cell lines after the treatment with below $IC_{50}$ concentrations of extract. Orange-to-red fluorescent cells in late apoptotic stage were seen with $IC_{50}$ and above $IC_{50}$ concentrations. Treated cells also showed obvious morphological changes such as membrane blebs, fragmentation of nuclei, chromatin condensation, and apoptotic bodies. Late apoptotic cells were seen mostly in doxorubicin-treated cells.

## Hoechst 33258 staining

In both control groups, uniform blue fluorescent live cells were obtained. Apoptotic characteristics, namely, nuclear fragmentation and marginalization, chromatin condensation were seen in extract treated and positive control cells [Figures 4 and 5]. Apoptotic cell percent showed a concentration-dependent significant increase ($P < 0.01$) [Figure 6].

## JC-1 staining

In control cells, JC-1 aggregates with reddish/orange fluorescence were observed suggestive of a higher mitochondrial membrane potential.

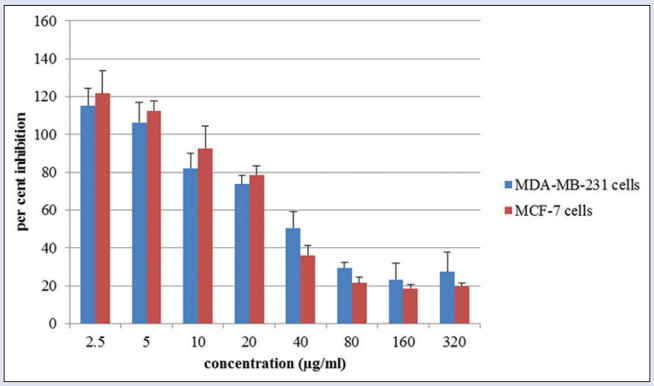



**Figure 1:** The percent cell viability of MDA-MB-231 and MCF-7 cells after 48 h treatment with methanol extract of *Mimosa pudica* determined by 3-(4,5-dimethyl thazol-2-yl)-2, 5-diphenyl tetrazolium bromide reduction assay. Values are expressed as mean ± standard error of the mean ($n = 3$)

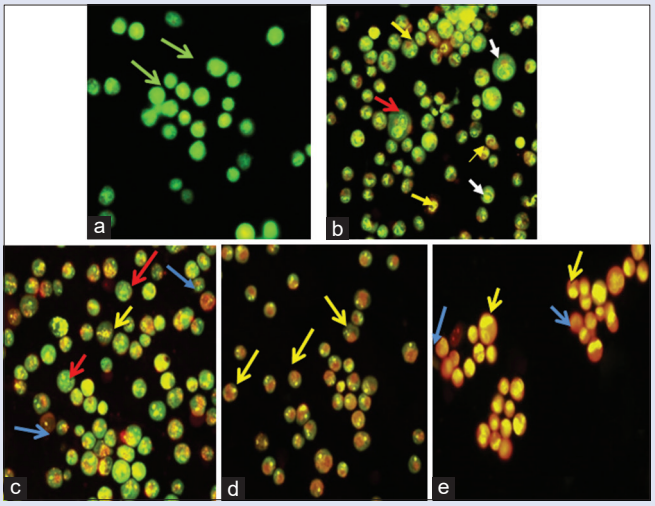



**Figure 2:** Morphological changes of MDA-MB-231 cells by acridine orange ethidium bromide staining, 200×. (a) Control cells; (b) Cells treated with doxorubicin 0.58 µg/mL; (c-e) Cells treated with methanol extract of *Mimosa pudica* at concentrations 10, 20, and 40 µg/mL, respectively. Green arrow-normal cells White arrow-early apoptotic cells, Yellow arrow-late apoptotic cells, Blue arrow indicates necrotised cells, Red arrow-nuclear fragmentation

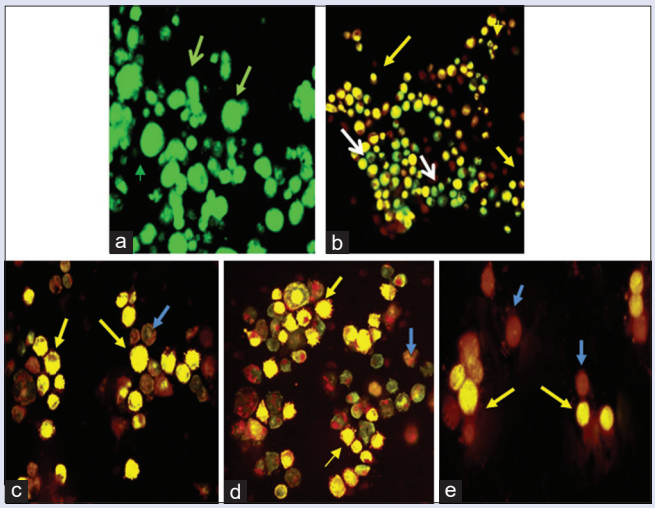



**Figure 3:** Morphological changes of MCF-7 cells by acridine orange ethidium bromide staining, 200×. (a) control cells; (b) Cells treated with doxorubicin 0.58 µg/mL; (c-e) cells treated with methanol extract of *Mimosa pudica* at concentrations 8, 16, and 32 µg/mL, respectively. Green arrow-normal cells, white arrow-early apoptotic cells, yellow arrow-late apoptotic cells, blue arrow indicates necrotised cells, red arrow-nuclear fragmentation

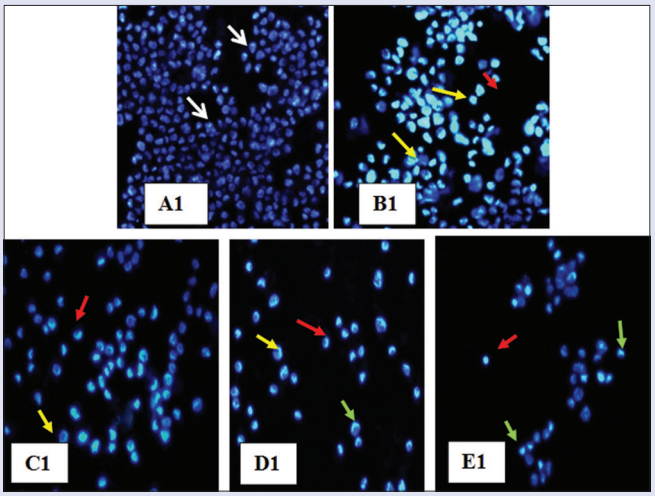



**Figure 4:** Morphological changes of MDA-MB-231 cells by hoechst staining, 200×. A1-control cells; B1-Cells treated with doxorubicin 0.58 µg/mL; C1, D1, E1-cells treated with methanol extract of *Mimosa pudica* at concentrations 10, 20, and 40 µg/mL, respectively. White arrow – live cells, Red arrow-apoptotic cells, Yellow arrow-chromatin condensation, Green arrow-marginalisation of nucleus, Orange arrow-fragmentation of nuclei

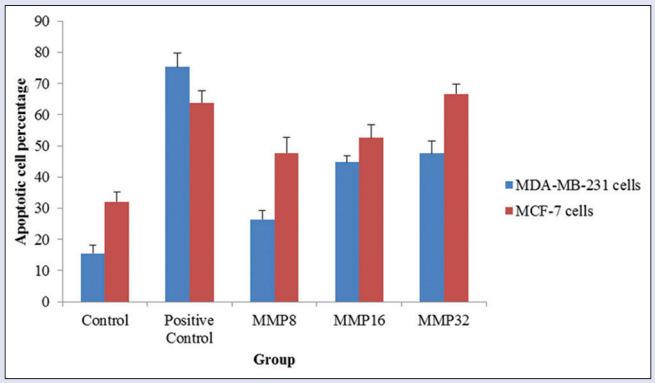



**Figure 5:** The apoptotic cell percent of MDA-MB-231 and MCF-7 cells after treatment with methanol extract of *Mimosa pudica* determined by Hoechst staining. Values are expressed as mean ± standard error of the mean ($n = 6$). Means with different superscripts differ significantly

A dose dependent shift from red-to-green fluorescence was obtained for MMP after 24 h in both the cell lines, depicting a concentration dependent lowering of mitochondrial membrane potential [Figures 7 and 8].

## *In vitro* Bcl-2 gene expression

The relative *Bcl-2* gene expression in the cell lines after MMP treatment is presented in Figure 9. The expression of Bcl-2 gene in the control cells was normalized to unity. In comparison to the control, Bcl2 gene expression after MMP treatment ($IC_{50}$) decreased statistically significantly ($P < 0.01$) in both the cells with 0.88 ± 0.02 and 0.83 ± 0.02 folds for MDA-MB-231 and MCF-7 cells, respectively.

## Western immunoblotting

The treatment of MDA-MB-231 and MCF-7 cells with MMP ($IC_{50}$ concentration) lowered the expression levels of anti-apoptotic protein Bcl-2 relatively [Figure 10]. Western blot images of β-actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 cancer cells are presented in Figure 11A-D, respectively. A significant decrease in Bcl-2 protein expression ($P < 0.01$) with 0.89 ± 0.002 and 0.83 ± 0.012 fold change was observed for MDA-MB-231 and MCF-7 cells when compared with the control.

## Phytochemical screening

The MMP shown the presence of alkaloids, flavonoids, glycosides, steroids, phenolics, and diterpenes upon analyzing phytochemical constituents using biochemical tests [Table 1].

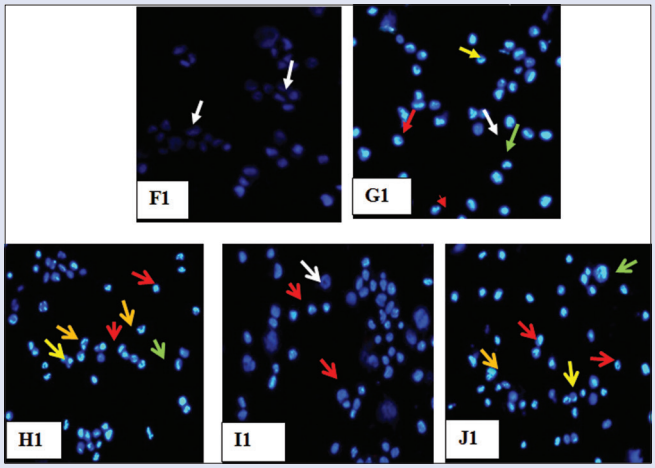



**Figure 6:** Morphological changes of MCF-7 cells by Hoechst staining, 200×. F1-control cells; G1-cells treated with doxorubicin 0.58 µg/mL; H1, I1, J1-cells treated with methanol extract of *Mimosa pudica* at concentrations 8, 16, and 32 µg/mL, respectively. White arrow - live cells, red arrow-apoptotic cells, yellow arrow-chromatin condensation, green arrow-marginalisation of nucleus, orange arrow fragmentation of nuclei

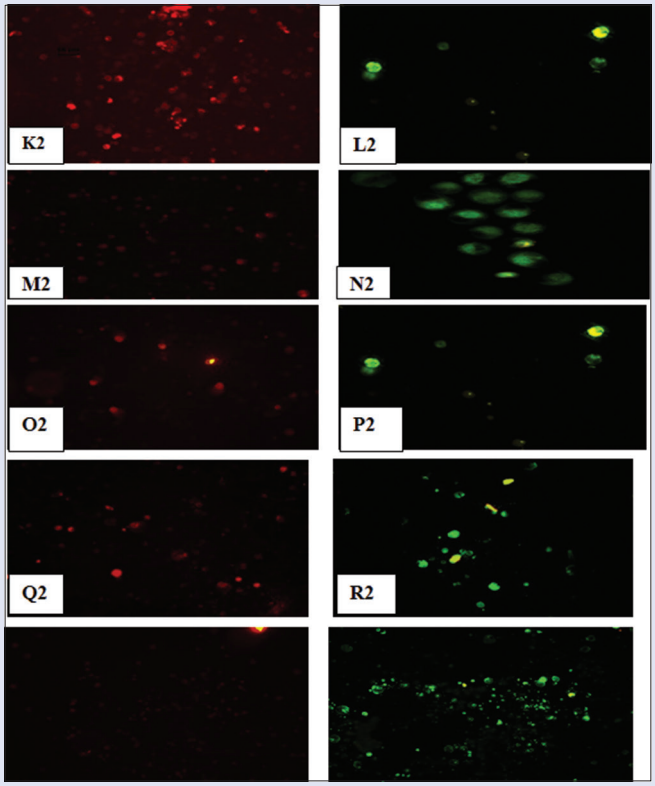



**Figure 8:** Morphological changes of MCF-7 cells studied by JC-1 staining, 200×. K2, M2, O2, Q2 and S2-JC-1 Red; L2, N2, P2, R2 and T2-JC-1 Green K2, L2-control cells; M2, N2-Cells treated with doxorubicin 0.58 µg/mL; O2, P2, Q2, R2, S2, T2-cells treated with methanol extract of *Mimosa pudica* at concentrations 8, 16 and 32 µg/mL, respectively

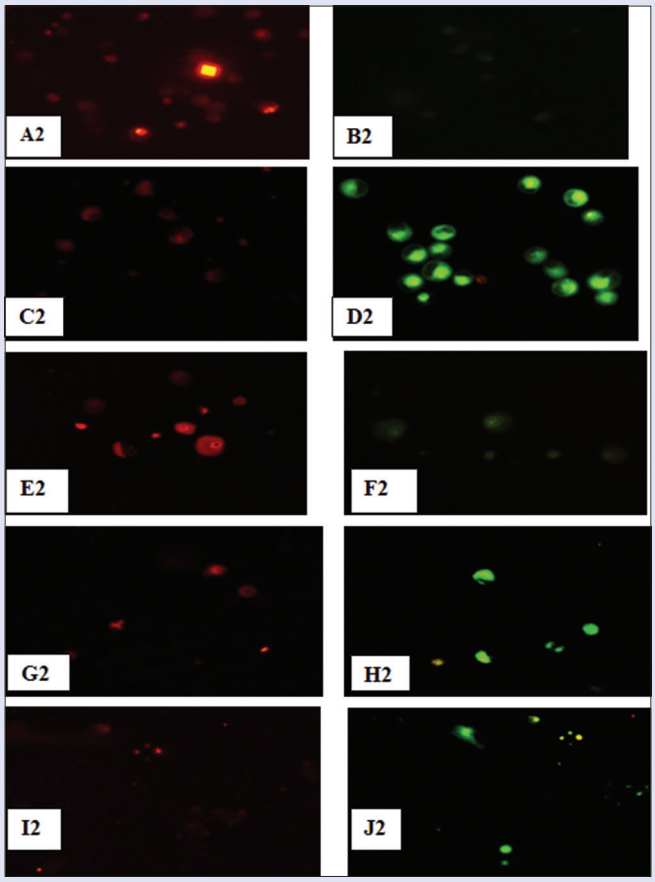



**Figure 7:** Morphological changes of MDA-MB-231 cells studied by JC-1 staining, for methanol extract of whole plant of *Mimosa pudica* 200×. A2, C2, E2, G2 and I2-JC-1 Red; B2, D2, F2, H2 and J2-JC-1 Green A2, B2-control cells; C2, D2-Cells treated with doxorubicin 0.58 µg/mL; E2, F2, G2, H2, I2, J2-cells treated with methanol extract of *Mimosa pudica* at concentrations 10, 20 and 40 µg/mL, respectively

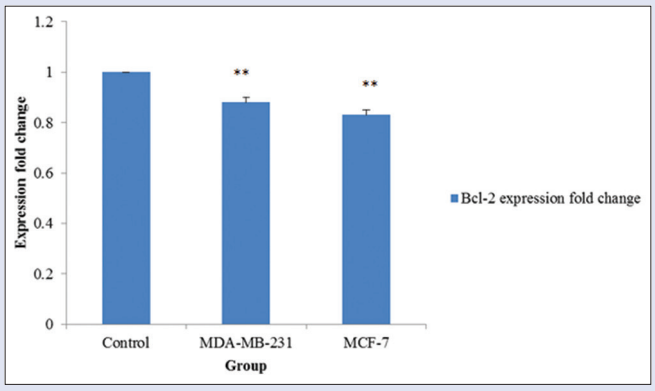



**Figure 9:** The relative Bcl-2 gene expression in MDA-MB-231 and MCF-7 cells in response to the treatment with methanol extract of *Mimosa pudica*. Values are expressed as mean ± standard error ($n = 3$) **denotes statistically significant ($P < 0.01$) difference compared with control

## Gas chromatography high-resolution mass spectrometry analysis

The GC-HRMS analysis of MMP showed chromatogram with major peaks obtained at 18.66, 21.87, 37.49, 39.82, 40.27, 41.28, 43.52, and 44.10 min retention times [Figure 12]. The major phytochemicals identified using mass spectrum library were 2,4-bis (1,1-dimethyl ethyl) phenol, undecanoic acid, 2-hexadecen-1-ol, 3, 7, 11, 15-tetramethyl, carboxylic acid 4-oxazole, methyl, ethyl ester, 4,5 dihydro-2-phenyl-, 9, 12, octadecadienoyl chloride (Z, Z'), phytol, 17-octadecynoic acid, oleic

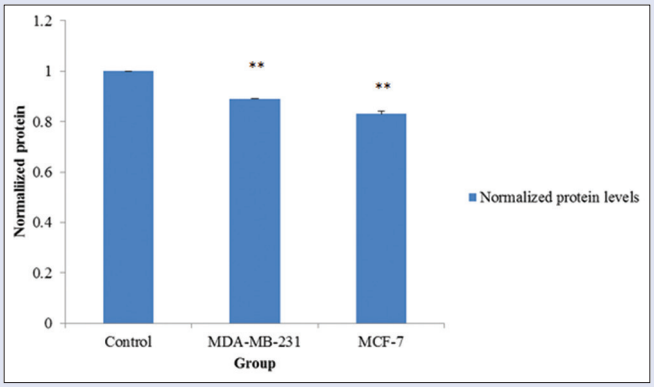



**Figure 10:** The relative Bcl-2 protein expression in MDA-MB-231 and MCF-7 cells in response to treatment with methanol extract of *Mimosa pudica*. Values are expressed as mean ± standard error ($n$ = 3) **denotes significant ($P$ < 0.01) difference compared with control

**Table 1:** Phytochemical analysis of methanol extract of *Mimosa pudica*

| Test | MMP |
|---|---|
| Steroids, Salkowski's test | + |
| Alkaloids | |
| Dragendorff's test | + |
| Mayer's test | + |
| Wagner's test | + |
| Hager's test | + |
| Glycosides, sodium hydroxide test | + |
| Tannins | |
| Ferric chloride test | - |
| Gelatin test | - |
| Flavonoids | |
| Lead acetate test | + |
| Ferric chloride test | + |
| Diterpene detection test | + |
| Triterpenes, Salkowski's test | - |
| Saponins, foam test | - |
| Phenolic compounds | + |

MMP: Methanol extract of whole plant of *Mimosa pudica*

acid, hexadecanoic acid, 3[(trimethyl silyl) oxy] propyl ester, oxirane tetradecyl, 9,12,15–octadecatrienoic acid, and Vitamin E which were mainly belonging to terpenoids, ether, fatty acid analogs, and sterols [Table 2].

## DISCUSSION

Breast cancer, being one of the most frequently occurring types of carcinomas in humans, is responsible for the majority of carcinoma-related deaths. Oestrogen and progesterone play a pivotal lead in the treatment of certain breast cancers based on the presence of the corresponding receptors, owing the therapy to be classified to hormone responsive and nonresponsive types. Treatment regimens effective in both hormone-mediated and nonmediated types are of paramount importance as non-mediated types are difficult to diagnose, aggressive, and invasive in nature. Thus, the present study aimed at the development of novel agents which could be utilized in both these types of breast cancers.

Although there were few reports on the antiproliferative potential of *M. pudica*, a detailed study on the mode of action of the plant has not been made till date.[12,13] MDA-MB-231 and MCF-7 cells were selected so that a compendious proposition on both hormone mediated and non-mediated carcinomas could be brought about from the current study.

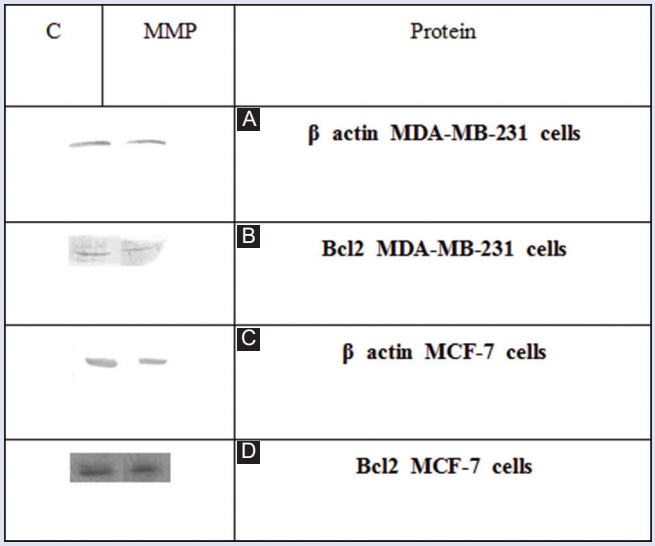



**Figure 11:** A, B, C and D, Western blot images of β-actin and Bcl-2 proteins in MDA-MB-231 and MCF-7 cells. C and methanol extract of whole plant of *Mimosa pudica* are control cells and cells after treatment with methanol extract of whole plant of *Mimosa pudica* at its respective $IC_{50}$ concentration

With the use of MTT assay, *in vitro* antitumor activity of MMP was determined preliminarily in both the cell lines. The cells in active metabolism alone could reduce MTT into purple formazan product which has an absorbance maximum at 570 nm owing to the direct proportionality of colour change to cell viability.[4] As per NCI guidelines, the $IC_{50}$ limit for selecting the plant extracts for anticancer studies is less than 30 μg/mL after 72 h of exposure.[14] Since $IC_{50}$ value obtained in both the cell lines lie in this range, the extract could be selected and studied in detail as a source for a potential antitumor compound. Earlier studies suggested higher cytotoxic activity for methanol extract than hydroalcohol extract as the use of methanol as solvent derives more amount of potent cytotoxic phytoconstituents.[12] Mimosine, obtained from *M. pudica* inhibits DNA replication of breast cancer cells by targeting ribonucleotide reductase and serine hydroxy methyl transferase enzymes involved in dNTP synthesis with iron chelation in the initiation phase.[15]

MTT assay is incapable of detecting whether the cell growth inhibition has occurred due to apoptosis or necrosis. Drugs cause destruction of cancer cells mostly by inducing apoptosis whose sensitivity is directly proportional to the apoptotic levels.[16,17] Dual AO/EB staining was used to assess apoptotic mode of cell death by morphological changes where a pronounced distinction between live, early, and late apoptotic cells and necrotic cells could be made. Cell penetration by AO stains the nuclei green by binding to DNA, especially normal and early apoptotic cells while EB stains the nucleus of late apoptotic and nerotic cells red whose plasma membrane integrity is lost by binding to DNA fragments and apoptotic bodies.[18,19]

The effect on morphological changes in nucleus due to MMP in MDA-MB-231 and MCF-7 cells was assessed using hoechst 33258 staining. Hoechst-33258, a nuclear counter stain, emits blue fluorescence when bound to double-stranded DNA by causing intercalation between adenine and thymine residues. Apoptotic cells could be differentiated from viable cells by the emission of bright blue instead of uniform blue fluorescence. Chromatin condensation, nuclear marginalization, early nuclear collapse, and nucleosomal ladder formation are the major nuclear changes observed during apoptosis due to the involvement of

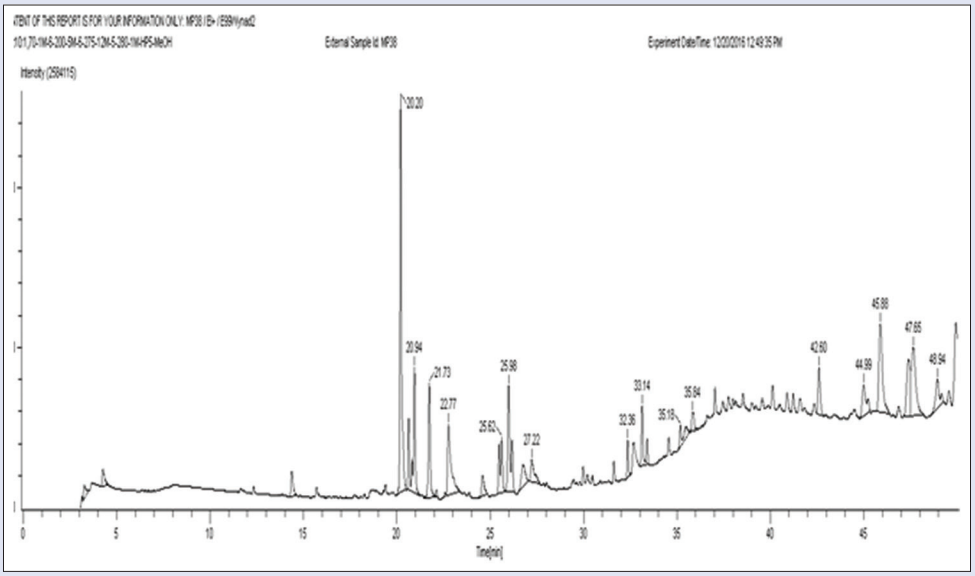


**Figure 12:** Gas chromatography high-resolution mass spectrometry chromatogram of methanol extract of whole plant of *Mimosa pudica*

**Table 2:** Gas chromatography - high-resolution mass spectrometry analysis of phytochemicals in the methanol extract of whole plant of *Mimosa pudica*

| RT (min) | Name of compound | Molecular formula | MW (g/mole) | Peak area (%) | Class | Probability (%) |
|---|---|---|---|---|---|---|
| 14.38 | Phenol-2,4- bis (1,1-Dimethyl ethyl) | $C_{14}H_{22}O$ | 206 | 1,206,037.08 | Alkylated phenol | 29.6 |
| 20.94 | 3, 7, 11, 15- Tetramethyl 2- hexadecen- 1- ol | $C_{20}H_{40}O$ | 296 | 7,613,199.64 | Acyclic diterpene alcohol | 17.7 |
| 24.59 | 4-Oxazole carboxylic acid, 4,5 dihydro-2-phenyl- methyl, ethyl ester | $C_{13}H_5NO_3$ | 233 | 1,332,483.6 | Heterocyclic aromatic compound | 36.7 |
| 25.62 | 9, 12, Octadecadienoyl chloride (Z, Z') | $C_{18}H_{31}ClO$ | 298 | 3,700,120.82 | Chloride salt of polyunsaturated omega 6 fatty acid | 13.2 |
| 25.99 | Phytol | $C_{20}H_{40}O$ | 296 | 6,560,747.13 | Acyclic diterpene alcohol | 39.3 |
| 26.74 | 17- octadecynoic acid | $C_{18}H_{34}O_2$ | 282 | 6,560,747.13 | Fatty acid analog | 22 |
| 27.22 | Oleic acid | $C_{18}H_{34}O_2$ | 282 | 1,788,551.88 | Fatty acid | 10.3 |
| 32.36 | Hexadecanoic acid 3[(trimethyl silyl) oxy] propyl ester | $C_{22}H_{46}O_3Si$ | 386 | 1,175,484.81 | Fatty acid analog | 15.6 |
| 33.14 | Oxirane tetradecyl | $C_{16}H_{32}O$ | 240 | 2,945,445.98 | Cyclic ether | 15.7 |
| 35.39 | 9,12,15 - Octadecatrienoic acid, 2[((trimethyl silyl) oxy) methyl] ethyl ester (Z, Z, Z') | $C_{27}H_{52}O_4Si_2$ | 496 | 1,595,026.19 | Fatty acid analogue | 42.0 |
| 35.84 | Oleic acid | $C_{18}H_{34}O_2$ | 282 | 1,022,646.82 | Fatty acid | 10.3 |
| 42.6 | Vitamin E | $C_{29}H_{50}O_2$ | 430 | 2,401,794.35 | Vitamin | 71.2 |

caspases and other mitochondrial factors. Our results suggest that MMP is inducing apoptosis being evident from the nuclear changes with a significant dose-dependent increase in apoptotic cell percent.[20]

Apoptotic protein in intrinsic (mitochondrial dependent) pathway, targets mitochondria, increase mitochondrial membrane permeability causing leakage of apoptotic effectors by a fall in the mitochondrial transmembrane potential (Δψm). Due to high Δψm, JC-1 accumulates in mitochondrial matrix and form fluorescent red aggregates on lowering causes a decrease in red-to-green signal ratio, with increase in green monomer percent.[21] In the current study, MMP induced a decrease in Δψm in a dose-dependent manner by a mitochondrial-specific cationic dye, JC-1 showing probability for mitochondria dependent intrinsic pathway of apoptosis.[22,23]

B-cell lymphoma-2 (Bcl-2) family proteins are significant in intermitochondrial membrane protein release. They could be classified into proapoptotic (Bax and Bak) and antiapoptotic (Bcl-2 and Bcl-$x_L$) molecules. Bcl-2 suppresses apoptosis by blocking the release of cytochrome c from mitochondria, thereby inhibiting the subsequent activation of caspases associated with apoptotic cell death.[24,25]

A significant reduction in Bcl-2 gene and protein expression was obtained due to MMP in MDA-MB-231 and MCF-7 cells.[26,27] The results represent the first report on the possible cellular mode of action of *M. pudica* extract on anticancer potential.

Alkaloids, flavonoids, glycosides, steroids, phenolics, and diterpenes were obtained on qualitative phytochemical analysis of MMP.[28] Previous studies suggested that flavonoids isolated from *M. pudica* present significant cytotoxic potential.[13] Phenolic acid exhibits marked antitumour and antimutagenic effect by hindering malignant tumor progression and flavonoids interfere in tumor development. Isoflavones often referred to as phytoestrogens, modulate estrogen levels, and thereby regulates hormone responsive tumor progression.[29]

GC-HRMS, a pivotal study for metabolic profiling, works on a combination of gas chromatography and mass spectrum division patterns with a database for detecting phytochemicals. Nowadays, GC-MS evolved as a key method for metabolic profiling.

Among the major compounds obtained with GC-HRMS, there are early records on the detection of 9, 12, octadecadienoyl chloride (Z, Z'), phytol, 17-octadecynoic acid, hexadecanoic acid, and Vitamin E.[28,30,31]

Phytol possess anticancer activity and act as precursor for Vitamin E, a well-known antioxidant.[32] Potent antitumor activity by terpenoids has also been reported.[33]

## CONCLUSION

Thus, the present study revealed that MMP possess antitumour activity *in vitro* against both MDA-MB-231 and MCF-7 cell lines. The extract was able to produce considerable cytotoxicity against these cell lines causing apoptosis of the cancer cells through intrinsic pathway as evidenced by AO/EB, Hoechst 33258, and JC-1 staining. The extract was causing a downregulation of the antiapoptotic gene and protein Bcl-2, which substantiate the potential to induce apoptosis *in vitro*. Terpenoids obtained on both phytochemical and GC-HRMS analysis may be a contributing factor for the obtained anticancer activity *in vitro*.

### Acknowledgements

The authors are thankful to Sophisticated Analytical Instrumental Facility, Indian Institute of Technology, Mumbai, India, for help in conducting GC-HRMS analysis.

### Financial support and sponsorship

The authors acknowledge the financial supports provided by Government of Kerala as State Plan project titled "Screening and evaluation of medicinal plants for anticancer activity" with grant No. RSP/16-17/VII-6 and Kerala Veterinary and Animal Sciences University research grant no. AD/2/96/MVM/2015/PH.

### Conflicts of interest

There are no conflicts of interest.

## REFERENCES

1. Qi F, Li A, Inagaki Y, Gao J, Li J, Kokudo N, *et al.* Chinese herbal medicines as adjuvant treatment during chemo-or radio-therapy for cancer. Bio Sci Trend 2010;4:297-307.
2. Chowdhury SA, Islam J, Rahaman MM, Rahman MM, Rumzhum NN, Sultana R, *et al.* Cytotoxicity, antimicrobial and antioxidant studies of different plant parts of *Mimosa pudica*. S J Pharm Sci 2008;1:80-4.
3. Ganguly M, Devi N, Mahanta R, Borthakur MK. Effect of *Mimosa pudica* root extract on vaginal estrous and serum hormones for screening of antifertility activity in albino mice. Contraception 2007;76:482-5.
4. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, *et al.* Cell viability assays. In: Sittampalam GS, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, *et al.*, editors. Assay Guidance Manual. Bethesda, MD: Eli Lily and Company and the National Centre for Advancing Translational Sciences; 2004.
5. Kasibhatla S, Amarante-Mendes GP, Finucane D, Brunner T, Bossy-Wetzel E, Green DR. Acridine orange/ethidium bromide (AO/EB) staining to detect apoptosis. CSH Protoc 2006;2006:pdb.prot4493.
6. Harada K, Kawaguchi S, Supriatno, Kawashima Y, Yoshida H, Sato M. S-1, an oral fluoropyrimidine anti-cancer agent, enhanced radiosensitivity in a human oral cancer cell line *in vivo* and *in vitro*: Involvement possibility of inhibition of survival signal, Akt/PKB. Cancer Lett 2005;226:161-8.
7. de Cordova CA, Locatelli C, Assunção LS, Mattei B, Mascarello A, Winter E, *et al.* Octyl and dodecyl gallates induce oxidative stress and apoptosis in a melanoma cell line. Toxicol *In Vitro* 2011;25:2025-34.
8. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 2001;25:402-8.
9. Shrivasthava S, Kulkarni P, Thummuri D, Jeengar MK, Naidu VG, Alvala M, *et al.* Piperlongumine, an alkaloid causes inhibition of PI3K/Akt/mTOR signaling axis to induce apoptosis in human triple negative breast cancer cells. Apoptosis 2014;19:1-17.
10. Harborne AJ. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd ed. New York: Springer; 1998.
11. Muthukrishnan S, Palanisamy S, Subramanian S, Selvaraj S, Mari KR, Kuppulingam R. Phytochemical profile of *Erythrina variegata* by using high-performance liquid chromatography and gas chromatography-mass spectroscopy analyses. J Acupunct Meridian Stud 2016;9:207-12.
12. Parmar F, Kushawaha N, Highland H, George LB. *In vitro* antioxidant and anticancer activity of *Mimosa pudica* Linn. extract and L-mimosine on lymphoma daudi cells. Int J Pharm Pharm Sci 2015;7:100-4.
13. Sudhakaran S, Jose J, Kumar ST, Jayaraman S, Variyar EJ. Evaluation of anticancer activities of flavonoids isolated from *Mimosa pudica*, *Aloe vera* and *Phyllanthus niruri* against human breast carcinoma cell lines (MCF-7) using MTT assay. Int J Pharm Pharm Sci 2014;6:319-22.
14. Abdel-Hameed ES, Salih A, Bazaid SA, El-Sayed MM, El-Wakil EA. Phytochemical studies and evaluation of antioxidant, anticancer and antimicrobial properties of *Conocarpus erectus* L. growing in Taif, Saudi Arabia. Eur J Med Plants 2012;2:93-112.
15. Oppenheim EW, Nasrallah IM, Mastri MG, Stover PJ. Mimosine is a cell-specific antagonist of folate metabolism. J Biol Chem 2000;275:19268-74.
16. Yamamoto M, Maehara Y, Oda S, Ichiyoshi Y, Kusumoto T, Sugimachi K. The p53 tumor suppressor gene in anticancer agent-induced apoptosis and chemosensitivity of human gastrointestinal cancer cell lines. Cancer Chemother Pharmacol 1999;43:43-9.
17. Thompson CB. Apoptosis in the pathogenesis and treatment of disease. Science 1995;267:1456-69.
18. Renvoizé C, Biola A, Pallardy M, Bréard J. Apoptosis: Identification of dying cells. Cell Biol Toxicol 1998;14:111-20.
19. Liu K, Liu PC, Liu R, Wu X. Dual AO/EB staining to detect apoptosis in osteosarcoma cells compared with flow cytometry. Med Sci Monit Basic Res 2015;21:15-20.
20. Yao J, Jiao R, Liu C, Zhang Y, Yu W, Lu Y, *et al.* Assessment of the cytotoxic and apoptotic effects of chaetominine in a human leukemia cell line. Biomol Ther 2016;24:147-55.
21. Sun Y, Zong W ×. Cellular apoptosis assay of breast cancer. Methods Mol Biol 2016;1406:139-49.
22. Afriyie DK, Asare GA, Bugyei K, Lin J, Peng J, Hong Z. Mitochondria-dependent apoptogenic activity of the aqueous root extract of *Croton membranaceus* against human BPH-1 cells. Genet Mol Res 2015;14:149-62.
23. Thamizhiniyan V, Young-Woong C, Young-Kyoon K. The cytotoxic nature of *Acanthopanax sessiliflorus* stem bark extracts in human breast cancer cells. Saudi J Biol Sci 2015;22:752-9.
24. Jacobson MD, Burne JF, Raff MC. Programmed cell death and Bcl-2 protection in the absence of a nucleus. EMBO J 1994;13:1899-910.
25. Luo X, Budihardjo I, Zou H, Slaughter C, Wang X. Bid, a Bcl2 interacting protein, mediates cytochrome c release from mitochondria in response to activation of cell surface death receptors. Cell 1998;94:481-90.
26. Birjandian E, Motamed N, Yassa N. Crude methanol extract of *Echinophora platyloba* induces apoptosis and cell cycle arrest at s-phase in human breast cancer cells. Iran J Pharm Res 2018;17:307-16.
27. Elkady AI, Abuzinadah OA, Baeshen NA, Rahmy TR. Differential control of growth, apoptotic activity, and gene expression in human breast cancer cells by extracts derived from medicinal herbs *Zingiber officinale*. J Biomed Biotechnol 2012;2012:614356.
28. Ramesh S, Chandran C, Venkatesan G. Phytochemical and GC-MS analysis of leaf extract of *Mimosa pudica* L. Int J Curr Res Dev 2014;2:78-87.
29. Okwu DE. Phytochemicals, vitamins and mineral contents of two Nigerian medicinal plants. Int J Mol Med Adv Sci 2005;1:375-81.
30. Saraswat R, Pokharkar R. GC-MS studies of *Mimosa pudica*. Int J Pharmtech Res 2012;4:93-8.
31. Sridharan S, Vaidyanathan M, Venkatesh K, Nayagam AA. GC-MS study and phytochemical profiling of *Mimosa pudica* Linn. J Pharm Res 2011;4:741-2.
32. Jenecius A, Mohan VR. GC-MS analysis of bioactive components on the stem extract of *Bacolepis nervosa* (wight and arn). Decne. Ex moq.(periplocaceae). World J Pharm Pharm Sci 2014;3:1044-59.
33. Li DL, Zheng X, Chen YC, Jiang S, Zhang Y, Zhang WM, *et al.* Terpenoid composition and the anticancer activity of *Acanthopanax trifoliatus*. Arch Pharm Res 2016;39:51-8.