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Methanol extract of *Pergularia daemia* (Forssk.) Chiov. leaves induce apoptosis in triple-negative breast cancer through intrinsic pathway

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Most traditional anticancer medications are ineffective in treating triple negative breast cancer (TNBC), which has enhanced resistance to chemotherapeutic agents. Herbs continue to be a key element in the discovery of anticancer medications. In the present study, we assessed the cytotoxicity of methanol extract of leaves of *Pergularia daemia* (MLPD) in MDA-MB-231 triple-negative breast cancer (TNBC) cell line. The cytotoxicity of MLPD was assessed using MTT assay which revealed significant concentration-dependent cytotoxicity with an IC_{50} value of 35.95±3.57 µg/mL. Morphological evaluation of MDA-MB-231 cells treated with MLPD showed cytotoxic changes like vacuole formation, altered morphology, and reduction in the number of cells with apoptotic bodies formation. Staining techniques, such as acridine orange and ethidium bromide (AO/EB) showed early apoptosis characterized by yellow-green fluorescence and crescent-shaped nucleus. Hoechst 33258 staining of MDA-MB-231 cells treated with MLPD showed nuclear marginalization indicative of nuclear apoptotic changes. To assess the mitochondrial membrane potential (MMP), we did JC-1 staining and the results revealed green fluorescence indicative of mitochondrial dependent apoptotic pathway. Comet assay confirmed significant DNA damaging property in MDA-MB-231 cells treated with MLPD. Western blotting analysis showed significant downregulation of Bcl-2 by 0.52±0.03 folds with no significant change in caspase-8 expression. Hence, the present study demonstrated that MLPD possessed potent cytotoxic effect against MDA-MB-231 TNBC cells through mitochondria mediated intrinsic pathway of apoptosis.

Keywords: Anticancer, Cytotoxicity, DNA strand breaks, Hair knot plant, Lupeol, MDA-MB-231, Stinking swallowwort, Trellis-vine, Whitlow plant

Triple-negative breast cancer (TNBC), an invasive ductal carcinoma with absence of estrogen, progesterone and HER2 receptors, is highly aggressive and characterized by proteolytic degradation of the extracellular matrix leading to extensive damage of breast tissue. Overall of 10% of breast cancer cases diagnosed are triple-negative in nature and nearly 20% of breast cancer cases are triple negative in African American women[1]. According to to the latest cancer statistics, 55,720 new cases of ductal carcinoma in situ in women has been estimated in 2023[2] and the invasive ductal carcinoma with triple negative phenotype has high rate of recurrence, poor prognosis with increased risk of metastasis to brain, bones and lungs[3].

The drugs used in the therapeutic strategy of TNBC include anthracyclines like doxorubicin, taxanes like paclitaxel, anti-metabolites like capecitabine and microtubule inhibitors like vinorelbine[4]. Use of these anticancer drugs has shown tremendous adverse effects and sometimes even found to be fatal. Additionally, the multifaceted chemoresistance of TNBC, based on tumour microenvironment, drug efflux and cancer stem cells, also presents a significant obstacle in the treatment of TNBC[5]. On the other hand, many researches showed promising results using natural products and extracts in treating TNBC *in vitro*[6]. It has been stated that a promising way to treat breast cancer with natural products is promoting apoptosis[7].

Pergularia daemia (Forssk.) Chiov., commonly known as hair knot plant, stinking swallowwort, trellis-vine and whitlow plant, is a foul-smelling perennial twinning herb with milky juice and hairy stem. Folklore people use the plant for the treatment of fever, jaundice and infantile diarrhoea whereas traditionally the plant is reported to have anthelminthic, expectorant and laxative properties[8]. Pharmacologically, the plant possesses

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antihypertensive[9], anti-inflammatory[10], antibacterial[11], hepatoprotective[12], anticancer and antioxidant activities[13]. *Pergularia daemia* showed cytotoxic potential against human epithelial ovarian cancer cell lines, OAW-42 and PA-1[14] and human oral epidermoid carcinoma cell line, KB[15]. However, its effect against triple-negative breast cancer has not yet been studied. Hence, in search of candidates which may further develop as novel chemotherapeutic agents against TNBC, here we assessed the cytotoxic potential and the mode of action of methanol extract of leaves of *P. daemia* in MDA-MB-231 TNBC cells.

Materials and Methods

Collection of plant sample

The whole plant of *Pergularia daemia* was collected from the campus of University of Agricultural Sciences, Raichur, Karnataka, India in February 2017. The leaves were authenticated by Raw Material Herbarium & Museum, National Institute of Science Communication and Information Resources (NISCAIR), New Delhi, India with accession number NISCAIR/RHMD/ Consult/2018/3281-82 and a voucher specimen was deposited at NISCAIR, New Delhi.

Preparation of methanol extract of *Pergularia daemia* leaves

The leaves of *P. daemia* were dried at room temperature (21°C) and coarsely powdered using an electric pulverizer. The powder obtained was extracted using Soxhlet apparatus with methanol at 67°C. The methanol extract of *P. daemia* leaves (MLPD) was then concentrated using a rotary vacuum evaporator under reduced pressure and temperature (40°C). The yield of the extract was calculated and kept under refrigeration in an airtight container after complete evaporation of the solvent until further use.

HPLC analysis of MLPD

HPLC technique for the quantitative detection of lupeol in MLPD was carried out using the method of Maji *et al.*[16] with slight modifications. The modifications were made in the mobile phase, flow rate and run time. The mobile phase containing 85:15 acetonitrile and 0.1% acetic water was used for the analysis of plant extract. The isocratic elution flow rate was fixed at 1.5 mL/min with Acclaim™ C18 column with 4.6×150 mm size, 120 Å pore size and 5 µm internal diameter column with temperature set to 25°C. Lupeol was detected using DAD set at 206 nm. The total run time was 25 min.

Cell line used for the study

Authenticated adherent human breast adeno-carcinoma cell line, MDA- MB- 231 procured from NCCS (National Centre for Cell Science), Pune, Maharashtra, India was used for *in vitro* anticancer studies. Cells were cultured in RPMI-1640 media supplemented with 10% FBS, 1% antibiotic-anti-mycotic solution containing penicillin-streptomycin and amphotericin B. The cells were maintained in a humidified incubator at 37°C with 5% carbon dioxide (CO_2). MDA-MB-231 cells were subcultured by enzymatic digestion with 1% trypsin/1 mM EDTA solution when they reached approximately 70-80% confluency and these trypsinized cells were used for the studies.

Sample preparation

The plant extract was solubilized in dimethyl sulfoxide (DMSO) at a concentration of 20 mg/mL; this stock solution was further diluted with RPMI media to required concentrations.

In vitro anticancer activity of MLPD

MTT assay

The cytotoxic changes produced by MLPD in MDA-MB-231 cells were evaluated using MTT (3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide) assay[17]. The MDA-MB-231 cells were seeded at a density of 5000 cells per well in 200 µL medium and were left overnight for attachment in a CO_2 incubator. Cells were treated with MLPD separately at concentrations of 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/mL for a period of 24 h. After treatment with plant extract, the media was removed and 20 µL of MTT (5 mg/mL) in 150 µL medium was added and incubated at 37°C for 4 h. The media containing 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 inhibition was calculated and IC_{50} value was calculated by plotting the concentration against per cent cell inhibition using Graphpad prism version 5.

Morphological assessment of cells treated with MLPD

Morphological alterations produced by MLPD in MDA-MB-231 cells after 24 h of exposure were assessed. The MDA-MB-231 cells (1×10^5) were incubated in a 6-well plate with MLPD at a concentration of IC_{50} value of MLPD for 24 h which was followed by microscopic observation at 40X magnification using a phase-contrast inverted microscope.

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

The MDA-MB-231 cells were seeded in a 6-well plate at a density of 1×10^5 cells and allowed to grow for 24 h. The IC_{50} concentration of MLPD was treated for 24 h to differentiate the live, apoptotic and necrotic cells. The spent media was discarded from the treated wells and 200 µL of acridine orange (10 µg/mL)/ ethidium bromide (10 µg/mL) was added and analyzed under Trinocular Research Fluorescence Microscope (Axio Vert. A1 FL-LED, Carl Zeiss, Jena, Germany) with blue excitation (488 nm) and emission (550 nm) filters at 20 and 40X magnification[18]. Doxorubicin at 0.58 µg/mL was used as a positive control.

Hoechst 33258 staining

The nuclear morphological changes produced by MLPD were assessed using hoechst 33258 staining using the modified method of Majumdar *et al*.[19]. The MDA-MB-231 cells were seeded in 6-well plates at a density of 1×10^5 cells per well and allowed to grow for 24 h followed by treatment with IC_{50} concentration of MLPD, and doxorubicin at 0.58 µg/mL was used as positive control, for 24 h. Hoechst 33258 staining was performed by adding 200 µL of hoechst 33258 stain (Sigma 0.5 µg/mL) for 30 min in the dark and images were captured by Trinocular Research Fluorescence Microscope (DM 2000 LED, Leica, Germany) with filters having blue excitation (352 nm) and emission (461 nm) at 20 and 40X magnification.

JC-1 staining

The JC-1 staining was conducted to assess the mitochondrial membrane potential change produced by the extract in MDA-MB-231 cells. The cells were plated at a concentration of 1×10^5 cells per well in six-well plates and were allowed to grow for 24 h. After 24 h of treatment with MLPD at IC_{50} concentration and doxorubicin at 0.58 µg/mL concentration, the cells were incubated with 5 µM fluoroprobe, 5,5ˈ,6,6ˈ-tetrachloro-1,1ˈ,3,3ˈ-tetra ethyl benzimidazole-carbocyanine iodide (JC-1) stain for 30 min at room temperature in the dark. The cells were analyzed using a trinocular fluorescent microscope with filters having blue excitation/emission of 540/ 570 nm and red excitation/emission of 590/610 nm (Axio Vert. A1 FL-LED, Carl Zeiss, Jena, Germany) at 40X magnification[20].

Comet assay

Comet assay or single-cell gel electrophoresis was performed to assess the DNA damaging property of the extract in MDA-MB-231 cells. The cells were seeded at a density of 1×10^5 cells in a six-well plate and were treated with IC_{50} concentration of MLPD and doxorubicin at 0.58 µg/mL for 24 h. The cells were then trypsinized and used for the basic alkaline comet assay[21]. Coating of slides with normal melting point agarose (NMPA) followed by a double coating of low melting point agarose (LMPA) containing approximately 2,000 cells was lysed in ice-cold lysis buffer. This was followed by subjecting the slides to electrophoresis. After electrophoresis, the slides were then treated with neutralization buffer and were allowed to dry and stained with ethidium bromide (20 µg/mL) and analyzed within one to two hours at 20X magnifications on a Trinocular Research Fluorescence Microscope (DM 2000 LED, Leica, Germany). Images of 100 randomly selected untreated MDA-MB-231 cells, MLPD treated cells and doxorubicin treated cells were analyzed from each sample and the distribution of DNA between tail and head of the comet was used to evaluate the degree of DNA damage. The quantification of the DNA strand breaks of the stored images was done using comet assay software project lab (CASP). Using this software, comet length, per cent DNA in tail, tail length, tail moment and olive tail moment were observed.

Western blot analysis

The MDA-MB-231 cells were treated with IC_{50} concentration of MLPD and doxorubicin at 0.58 µg/mL concentration for 24 h and were trypsinized followed by lysing the cells using CelLytic^M. Cell lysates were then centrifuged and the supernatant containing proteins were subjected to sodium dodecyl sulphated-polyacrylamide gel electrophoresis (SDS-PAGE) for protein separation. The separated proteins on the gel were transferred to polyvinylidene difluoride (PVDF) membrane (0.45 µm) using a Hoefer semi-dry transfer apparatus. The membrane was initially blocked with 5% bovine serum albumin (BSA) followed by treating the membrane with respective primary antibodies overnight at 4°C. The membrane was then incubated with horseradish peroxidase-conjugated secondary antibody for 1 h. Colour reaction was carried out with 3,3ˈ-diaminobenzidine (DAB) substrate buffer. 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)[22].

Statistical analysis

All results were expressed as Mean ± SE with 'n' equal to the number of replicates. All the statistical analysis was conducted using SPSS software version 24. Analysis of variance (ANOVA) in a completely randomized design followed by Duncan's multiple range tests was used to compare any significant differences among various concentrations of the extract. The same technique was used for comparing any significant difference among various parameters of the comet assay and also to compare the significant difference among normalized data of band intensity in western blotting.

Results and Discussion

Yield of MLPD

The yield of MLPD was 17.27% with reference to starting dry material. Extract yields are crucial in the computation of plant to extract ratio. In the present study, the plant to extract ratio is 5.79:1. It has been reviewed that the plant extract ratio for the majority of dry botanical materials extracted in aqueous or hydroethanolic solvents should be 10:1 and 4:1, respectively which is generally indicative of a targeted extraction procedure[23].

MTT assay

The per cent inhibition of cell proliferation as studied by MTT assay post 24 h of treatment with MLPD in MDA-MB-231 cell line is presented in (Table 1). The concentrations which showed cytotoxicity in the pilot study was taken for the main study. The extract showed concentration-dependent cytotoxicity at concentrations of 2.5, 5, 10, 20, 40, 80, 160 and 320 µg/mL in MDA-MB-231 cells with an IC_{50} value of 35.95 µg/mL

The MTT assay reduces the yellow-coloured tetrazolium dye MTT to insoluble purple formazan crystals with the help of nicotinamide adenine dinucleotide phosphate (NADPH)-dependent cellular oxidoreductase enzyme in the cells. When there is cytotoxicity, decrease in the number of cells causes decreased purple formazan development whose absorbance is measured by an ELISA plate reader. In the present study, MLPD inhibited the proliferation of MDA-MB-231 cells thereby causing reduced formation of purple formazan crystal which could be manifested to its cytotoxicity. Similar findings revealed the cytotoxicity of ethanol extract of leaf *P. daemia* on human cervical cancer cell lines, HeLa and human embryonic kidney cell lines, HEK 293 with IC_{50} values of 6.6 and 33.07 µg/mL, respectively[15]. The findings of the present study revealed that MLPD could be categorized as cytotoxic anticancer agents for TNBC [24,25].

Morphological assessment of cells treated with MLPD and HPLC analysis

The results of the morphological assessment of MDA-MB-231 cells treated with IC_{50} concentration of MLPD are depicted in Fig. 1. The results revealed that MLPD showed morphological alterations such as reduction in cell population, cell shrinkage, cell vacuolization, altered cell shape and apoptotic body formation.

Table 1 — The per cent cell viability of MDA-MB-231 cells after 24 h treatment with MLPD

Concentrations (µg/mL)	% Inhibition (MLPD)
2.5	12.85±1.69^{a}
5	15.41±1.62^{ab}
10	27.63±1.85^{bc}
20	22.65±1.72^{c}
40	37.21±1.83^{d}
80	51.38±3.03^{e}
160	58.99±3.81^{e}
320	58.86±4.22^{e}
IC_{50} (µg/mL)	35.95±3.57

[Values are expressed as Mean±SE (n=6). Means bearing the different superscript (a-e) vary significantly at P <0.05]

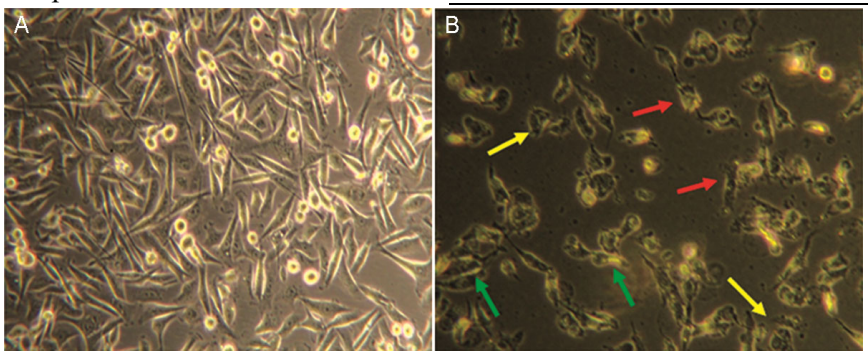

Fig. 1 — Morphological assessment of cells treated with MLPD, 20X. (A) Control cells; and (B) Cells treated with MLPD. [Green arrow indicates Cell vacuolization; Red arrow, Altered cell morphology; and Yellow arrow, Apoptotic body formation]

Bibu *et al*.[26] reported that chloroform soluble fraction of methanol extract of seeds of *Annona muricata* showed morphological changes like nuclear condensation, cell shrinkage, membrane blebbing and the presence of apoptotic- and pyknotic- bodies which revealed the apoptogenic property of the test substance. Thus, in the present study, the morphological alterations observed was indicative of the plant extract's apoptotic potential. These morphological alterations could be attributed to the presence of various anti-cancer phytochemicals.

A phytochemical analysis study performed using n-hexane, ethyl acetate and ethanolic extract of stem and leaves of *P. daemia* showed presence of flavonoids, steroid, terpenoids and glycosides[14]. Among the various phytochemicals, a pentacyclic triterpenoid, lupeol, is reported to exhibit potential anticancer activity against various cell lines[27]. HPLC analysis of MLPD in the present study also detected the presence of lupeol and it was found to be 3.13 µg/mL (0.31%) in 1 mg/mL of MLPD (Fig. 2). Thus, it could be suggested that lupeol could be one among the various phytochemicals attributing the anticancer activity of MLPD.

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

The results of apoptotic stages analyzed by AO/EB staining upon treatment of cells with MLPD and doxorubicin are depicted in Fig. 3A. Control cells emitted uniform green fluorescence with a circular nucleus in the centre whereas MLPD at its IC_{50} concentration treated cells showed early apoptosis featured by yellow-green fluorescence and crescent-shaped nucleus. The doxorubicin treated cells at 0.58 µg/mL exhibited late apoptosis emitting orange to red fluorescence. The apoptotic features like nuclear marginalization and condensation were observed in the apoptotic cells treated with MLPD.

Acridine orange dye diffuses into the cells and emits green fluorescence whereas ethidium bromide enters only those cells with reduced cell integrity as seen with apoptotic and necrotic cells[28]. Early apoptotic cells are characterized by granular, yellow-green, crescent-shaped marginalized nucleus while late apoptotic cells have concentrated or asymmetrically localized nucleus that stains orange in colour[29]. Necrotic cells are distinguished by increased cell volume in the disintegrating manner and uneven orange-red fluorescence at the periphery[19,20]. In the present study, MLPD showed early apoptosis whereas doxorubicin treated cells showed late apoptosis post 24 h of treatment in MDA-MB-231 cells[30,31]. Similar results were obtained by Mirunalini *et al*.[16] who studied the anti-cancer potential of methanol extract of *P. daemia* at IC_{50} concentration of 80 µg/mL against KB cells lines found that AO/EB staining showed uniform green fluorescence in control cells and plant extract treated KB cells produced orange fluorescence indicating late apoptosis. The difference in the stage of apoptosis found in the present and above-mentioned study could be due to the difference in the concentration of the extract and cancer cell line used.

Hoechst 33258 staining

The nuclear morphological changes analyzed by Hoechst 33258 staining upon treatment of cells with MLPD and doxorubicin are shown in the Fig. 3B. Control cells emitted uniform blue fluorescence whereas cells treated with IC_{50} concentration of

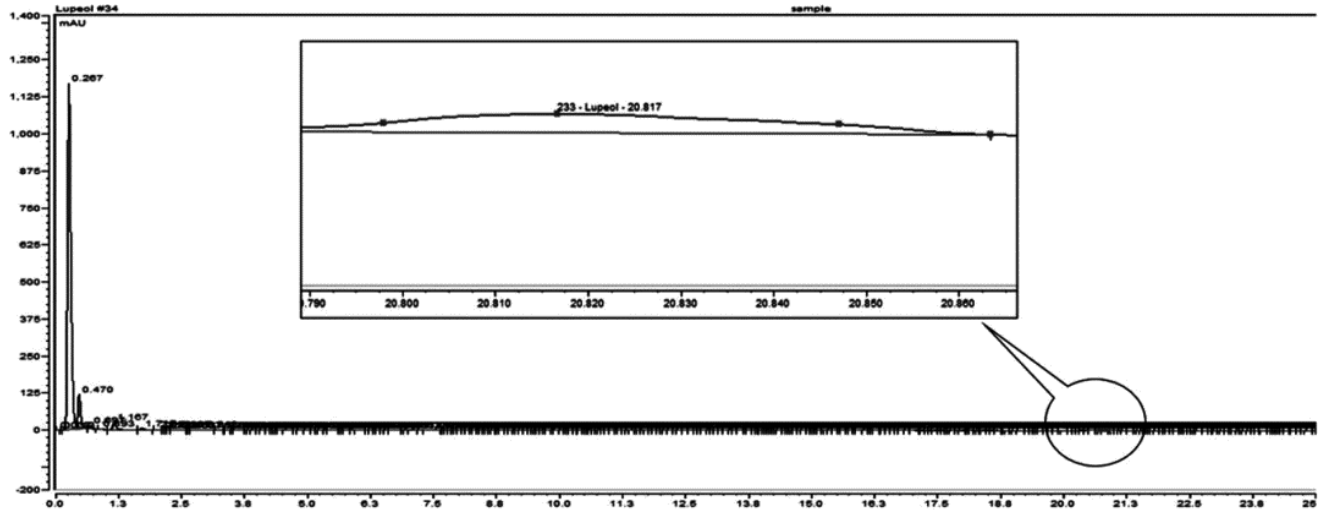

Fig. 2 — HPLC chromatogram of lupeol

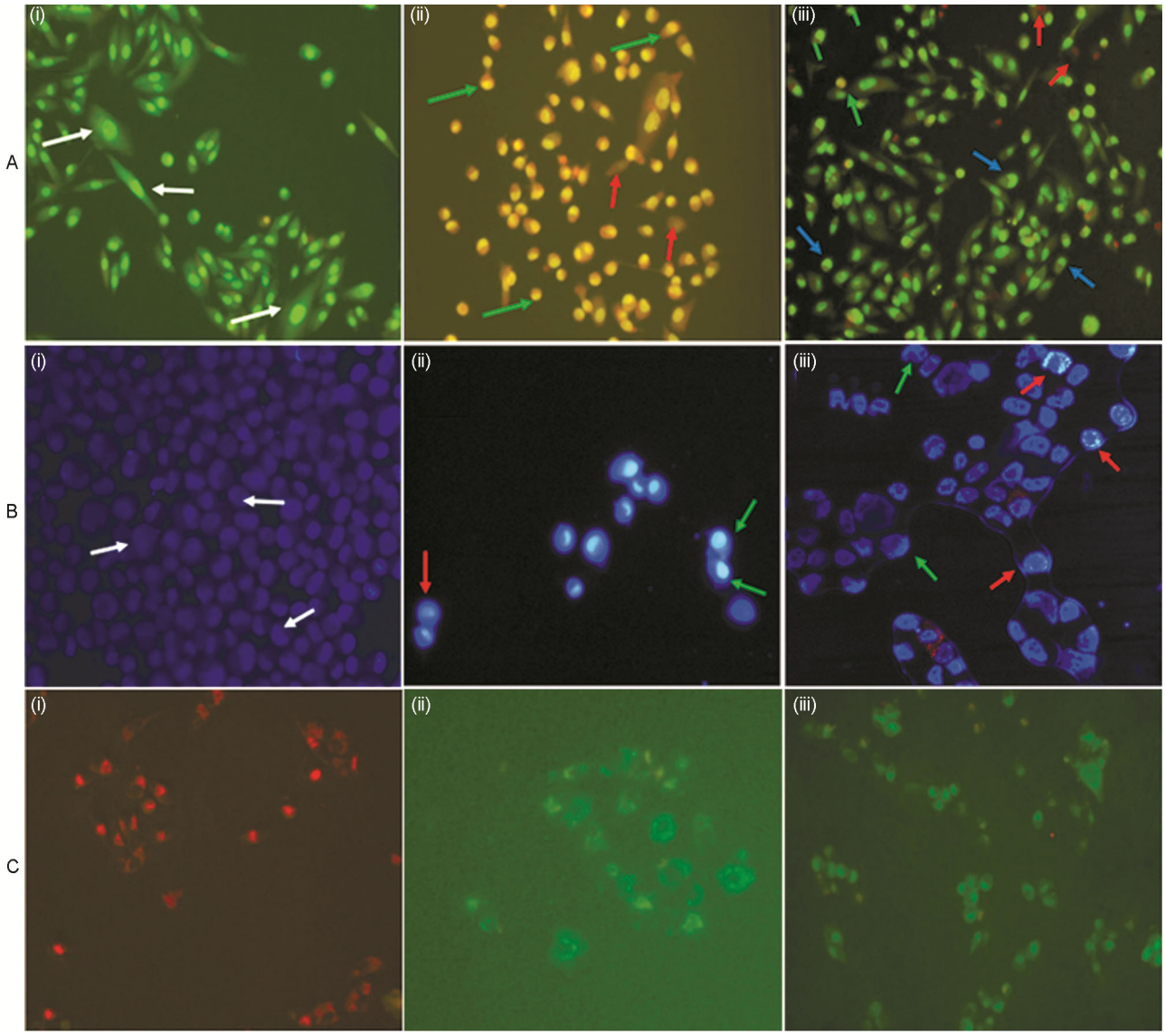

Fig. 3 — Morphological changes of MDA-MB-231 cells by (A) acridine orange/ethidium bromide staining, 20X; (B) hoechst staining, 40X; and (C) JC-1 staining, 40X. (i) Control cells, (ii) Cells treated with doxorubicin at 0.58 µg/mL, and (iii) Cells treated with MLPD. [A: White arrow indicates Normal cells; Blue arrow, Early apoptotic cells; Green arrow, Late apoptotic cells; and Red arrow, Necrotic cells. B: White arrow indicates Live cells; Red arrow, Fragmentation of nuclei; and Green arrow, Marginalization of nucleus]

MLPD and doxorubicin at 0.58 µg/mL showed bright blue fluorescence with apoptotic features like fragmentation and marginalization of nuclei.

Hoechst 33258 staining involves the binding of non-intercalating hoechst dye to A-T rich regions of the minor groove of double-stranded DNA producing blue fluorescence when excited by UV light (~360 nm). In normal cells, the dye is distributed evenly producing uniform blue fluorescence whereas in apoptotic cells, due to chromatin condensation and nuclear marginalization, the dye is concentrated producing intense bright blue fluorescence. In the present study also, similar features were observed in the apoptotic cells. Akhil *et al.*[32] demonstrated that apoptotic potential of methanol extract of germinated seeds of *Hordeum vulgare* using Hoechst 33258 staining and revealed that the plant extract produced nuclear changes characterized by nuclear marginalization and fragmentation. In the present study also, similar nuclear changes were observed. Hence, the results of the present study confirmed the apoptotic nuclear features exhibited by MLPD.

JC-1 staining

The results of mitochondrial membrane potential (MMP) evaluated by JC-1 staining upon treatment of cells with MLPD and doxorubicin are shown in

Fig. 3C. Cells treated with IC_{50} concentration of MLPD and doxorubicin at 0.58 µg/mL showed complete green fluorescence indicating a potential drop in MMP.

The JC-1 staining was performed to analyze the MMP of the cell. The JC-1 stain being a cationic dye particularly enters the mitochondria in normal cells due to high membrane potential, and produces intense red fluorescence as they form complexes known as J-aggregates. In apoptotic cells, there are alterations in electron transport and release of cytochrome C in mitochondria leading to depolarization of mitochondrial membrane causing DNA fragmentation and nuclear chromatin condensation ultimately leading to decreased MMP. This decrease in MMP causes the JC-1 dye to leak out from the mitochondria into the cytosol where it remains in its monomeric form producing green fluorescence[33]. In our study, MLPD produced green fluorescence indicating drop in MMP. Thus, it could be inferred that MLPD caused the translocation of mitochondrial protein, cytochrome C to cytosol thus inducing mitochondria mediated apoptosis. Similar results revealed the reduction in the MMP of KB cancer cells upon incubation with methanol extract of aerial parts of *P. daemia* at 160 µg/mL[16]. Hence, the findings of the present study support the fact that MLPD induced mitochondria mediated apoptosis.

Comet assay

The comet assay results of MDA-MB-231 cells treated with IC_{50} concentration of MLPD and doxorubicin at 0.58 µg/mL are shown in Table 2 and Fig. 4). The cells treated with MLPD showed comets when compared with control cells. Doxorubicin treated cells showed comets with a very long tail. The mean values of tail DNA per cent, tail length, tail moment and olive tail moment were estimated. All the comet assay parameters of MLPD treated cells were significantly different (P <0.01) from that of control and doxorubicin treated cells.

Comet assay is a popular technique to identify single and double-stranded breaks in DNA at an individual cell level. The parameters such as tail length (TL), tail moment and olive tail moment (OTM) of comet indicated the extent of DNA damage[34] and an increase in tail length and tail moment of the comets indicated frequent strand breakage leading to the formation of several small molecules of DNA. In the present study, a significant (P <0.01) increase in TL, tail moment and OTM was exhibited by MLPD in MDA-MB-231 cells indicating that the plant extract caused DNA strand breaks (Fig. 4 and Table 2). Similar results were obtained by the methanol extract of aerial parts of *P. daemia* against KB cancer cells wherein their results showed DNA damaging activity of the extract with an increased TL, tail moment and OTM at 160 µg/mL[16].

During apoptosis, the cleaving of DNA leading to the formation of double stranded breaks due to endogenous endonucleases occurs[35]. In the present study also MLPD produced DNA strand breaks and it could be inferred that MLPD induced apoptosis.

Western blot analysis

The relative expressions of Bcl-2 and caspase-8 in MDA-MB-231 cells on treatment with IC_{50}

Table 2 — Comet parameters of MDA-MB-231 treated with MLPD

	Tail DNA %	Tail Length (µm)	Tail Moment	Olive Tail Moment
Control	3.6±0.27[a]	0.09±0.04[a]	0.003±0.001[a]	0.05±0.03[a]
MLPD	19.95±1.48[b]	128.45±8.47[b]	27.40±3.61[b]	16.78±1.36[b]
Doxorubicin	68.48±2.32[c]	444.30±27.06[c]	313.91±27.65[c]	145.75±14.91[c]

[Values are expressed as Mean±SE (n=20). Means bearing the different superscript (a-c) vary significantly at P <0.05]

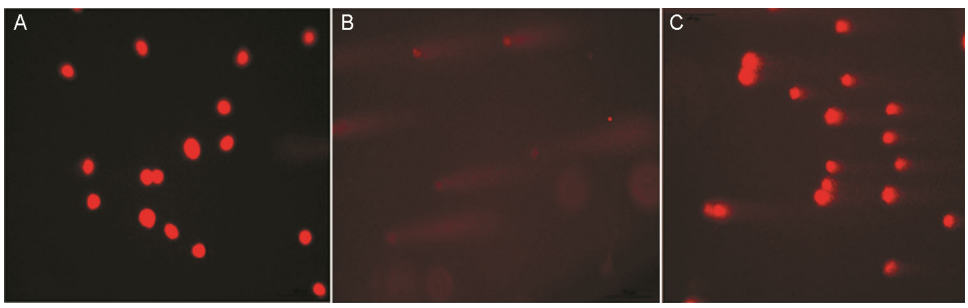

Fig. 4 — Assessment of DNA damage in MDA-MB-231 by comet assay. (A) Control cells; (B) Cells treated with doxorubicin at 0.58 µg/mL; and (C) Cells treated MLPD

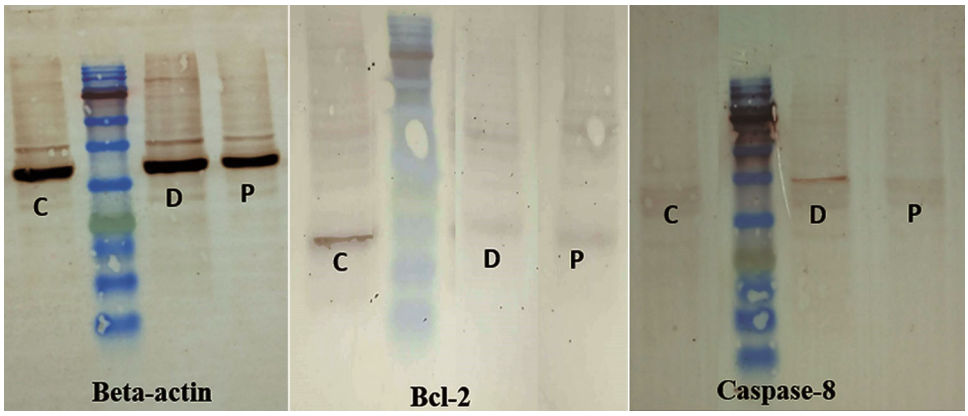

Fig. 5 — Western blot images of β-actin, Bcl-2 and Caspase-8 proteins in MDA-MB-231 cells. [C: Control cells; P: Cells treated with MLPD at its IC_{50} conc.; and D: Doxorubicin treated cells at 0.58 µg/mL]

Table 3 — Relative expression of Bcl-2 and Caspase-8 in MDA-MB-231 cells after treatment with MLPD

Treatment group	Normalised protein levels	
	Bcl-2	Caspase-8
Control cells	1	1
MLPD	0.52±0.03**	1.11±0.03
Doxorubicin	0.67±0.05**	3.77±0.38**

[Values are expressed as Mean±SE (n=3); ** denotes significant (P <0.01) difference compared with control]

concentration of MLPD and doxorubicin at 0.58 µg/mL were compared with control cells and the results are presented in Table 3 and Fig. 5. Bcl-2 expression was significantly (P <0.01) downregulated by 0.52±0.03 and 0.67±0.05 folds in MLPD and doxorubicin treated cells when compared with control cells whereas caspase-8 expression was significantly (P <0.01) upregulated by 3.77±0.38 folds in doxorubicin treated cells whereas no significant difference was noticed in MLPD and control cells.

Apoptosis is mediated through extrinsic and mitochondria mediated intrinsic pathways. Bcl-2 family proteins located in the outer mitochondrial membrane are found to be the negative regulators of apoptosis mediating the intrinsic pathway of apoptosis[27] whereas the extrinsic pathway of apoptosis is stimulated by caspase-8 which further cleaves and activates caspase 3, 6 and 7[34]. In the present study, downregulation of Bcl-2 protein and no change in the caspase-8 protein expression clearly indicated that MLPD induced intrinsic pathway of apoptosis.

The downregulation of Bcl-2 could be possibly due to the presence of lupeol, which could be supported by the studies of Eldohaji *et al.*[36] on Hep3B liver cancer cells. Additionally, molecular docking analysis of lupeol with different cancer targets revealed the best binding energy inhibition constant and ligand efficacy of lupeol with Bcl-2[37]. In a recent study conducted by Zhang *et al.*[38], lupeol inhibited the proliferation and migration of MDA-MB-231 breast cancer cells. Thus, the mitochondria mediated intrinsic pathway of apoptosis induced by MLPD in MDA-MB-231 cells could be attributed to the presence of lupeol. The roles of other phytochemicals present in MLPD in inducing apoptosis needs to be further investigated.

Conclusion

In this study, MLPD has shown significant concentration dependent cytotoxicity and morphological changes. Staining techniques such as acridine orange and ethidium bromide (AO/EB), hoechst 33258 staining and JC-1 revealed mitochondrial dependent apoptotic pathway. Comet assay confirmed the DNA damaging property of MLPD in MDA-MB-231 cells. MLPD treatment showed significant downregulation of Bcl-2 with no significant change in caspase-8 expression thereby exhibiting intrinsic pathway of apoptosis. Hence the results of the present study revealed the cytotoxic potential of MLPD against MDA-MB-231 TNBC cells. The cytotoxic activity of MLPD could be attributed to the presence of lupeol along with other phytochemicals.

Acknowledgment

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Conflict of interest

Authors declare no competing interests.

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