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Apoptogenic activity of ethyl acetate extract of leaves of *Memecylon edule* on human gastric carcinoma cells via mitochondrial dependent pathway

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ABSTRACT

Objective: To evaluate the anti-proliferative and apoptogenic activity of ethyl acetate extract from the leaves of *Memecylon edule* (EtAc-LME) in MKN-74, NUGC gastric cancer cells and non cancerous gastric mucous cells (GES-1), and to explore the mechanism of EtAc-LME induced apoptosis. **Methods:** The mechanism of EtAc-LME induced apoptosis was explored by analysing the activation of pro-caspases, PARP cleavage, expression of cytochrome-c (Cyt-c) was determined by western blotting, mRNA expression of Bcl-2, Bax by RT-PCR, loss of mitochondrial potential using DiOC6 dye, annexin binding assay and its influence on cell cycle arrest by flow cytometry. **Results:** The results indicated that EtAc-LME inhibited the gastric cancer cell growth in dose-dependent manner and cytotoxicity was more towards the gastric cancer cells (NUGC and MKN-74) compared to normal gastric cells (GES-1), suggesting more specific cytotoxicity to the malignant cells. Over expression of Cyt-c and subsequent activation of caspases-3 and down regulation of Bcl-2 and loss in mitochondrial potential in EtAc-LME treated MKN-74 and NUGC cells suggested that EtAc-LME induced apoptosis by mitochondrial dependent pathway. **Conclusions:** The present findings suggest that ethyl acetate extract of *Memecylon edule* induces apoptosis selectively in gastric cancer cells emphasizing the importance of this traditional medicine for its potential in the treatment of gastric cancer.

1. Introduction

Gastric cancer was the second most cancer causing death worldwide and the fourth most common cancer, with an estimated 737 000 deaths per year. Around one million cases of stomach cancer were recorded in 2008, accounting for around 8 percent of all new cancer cases and it was reported that the number of new cases will rise to 1.7 million by 2030[1,2]. Incidence rates for stomach cancer were the highest in Eastern Asia (China, Japan and Korea) and the lowest in Africa, Australia, New Zealand and North America. About 6 in 10 new cases of stomach cancer were diagnosed in Eastern Asia (5 in 10 for China and 1 in 10 for Japan)[1,2]. Chemotherapy plays an important role in the treatment of advanced gastric cancer, but its clinical applications are limited because of severe toxic effects. Hence, there is a

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need to search for novel anticancer agents with fewer side effects. Natural plant based therapies gain importance in the treatment of cancer because of low side effects and less cost.

Memecylon edule (ME) is a small green tree grown in India, especially the Deccan Plateau, including most of Karnataka, Andhra Pradesh, and parts of Tamil Nadu. It belongs to the family Melastomataceae and commonly known as Iron wood tree, kaayam, delek bangas, delek air, miat, and nemaaru. Traditionally, leaves of the ME have been used to treat leucorrhea, gonorrhoea, wound and gastrointestinal ailments[3]. Till today, there is no scientific reports have been published for its potential application for the medicinal use except one group who claimed the anti-inflammatory, analgesic and wound healing activities[4]. In the present study, we investigated the effect of ethyl acetate extracts from leaves of *Memecylon edule* (EtAc-LME) on the growth of human gastric carcinoma cells and further examined the apoptotic molecular events triggered by the extracts to establish the anticancer mechanism. The results of this study demonstrated that EtAc-LME induced specific cytotoxicity towards gastric cancer cells rather than the normal cells and triggered apoptosis by mitochondria mediated intrinsic caspase dependent apoptotic pathway. The present investigation will be useful to identify novel bioactive anticancer molecules for the treatment of gastric cancer.

2. Materials and methods

2.1. Chemicals

RPMI 1640 medium, Dulbecco's modified Eagle's medium, trypsin/EDTA, fetal bovine serum, penicillin, and streptomycin were purchased from Invitrogen Life Technologies, Inc. (Grand Island, NY, USA). Lactate dehydrogenase (LDH) assay kit was purchased from Roche Molecular Diagnostics (Mannheim, Germany). Propidium iodide (PI), acridine orange, ethidium bromide, RNase A, and MTT were purchased from Sigma chemical Co. (St. Louis, MO, USA). Beta-actin antibody was purchased from Abcam (Cambridge science park, Cambridge, UK). Anti-rabbit IgG HRP linked, Cytochrome-C rabbit mAb (monoclonal antibody), anti-cleaved caspase-3, anti-cleaved PARP and prestained protein marker (broad range) were purchased from Cell Signaling Technology, Inc (3 Trask Lane Danvers, MA, USA). Annexin binding assay kit was procured from BD Biosciences (San Diego, CA, USA). Polyvinylidene fluoride (PVDF) membranes and protein assay kit were purchased from Biorad (Biorad Hellas, USA).

2.2. Plant extraction

Dried leaves of ME were collected in the month of November in the Tirumala Tirupathi hills. The plant material was authenticated by Assistant Professor K. Madhava chetty, Department of Botany, S.V.University, Tirupathi, Andhra Pradesh, India (Herbarium number: *Memecylon edule* 591). The leaves of ME were shade dried and ground to coarse powder with high speed mechanical blender. The powdered material (500 g) was extracted successively with *n*-hexane, ethyl acetate, ethanol and water. The hexane, ethyl acetate and alcohol extracts were filtered and concentrated in rota vapour (Rotavac, Heidolf, Germany) at reduced pressure below 40 ℃.

2.3. HPLC analysis

The HPLC system (Shimadzu Corporation, Kyoto, Japan) was equipped with two Shimadzu LC-10 ATVP reciprocating pumps, a variable Shimadzu SPD-10 AVP UV-VIS detector and a Rheodyne Model 7725 injector with a loop size of 20 μL. Reverse-phase chromatographic analysis was carried out in isocratic conditions using a C-18 reverse phase column (Fortis H_2O, 250.0 mm×4.6 mm, particle size 5 μm). Running conditions are as follows: injection volume, 20 μL; mobile phase, 70% acetonitrile in water (20 mM ammonium acetate); flow rate, 1 mL/min; and detection at 290 nm.

2.4. Phytochemical analysis

The presence of phytochemicals, alkaloids (Draggendorff's reagent test), flavonoids (Shibata's reaction), saponins (Frothing test), tannins (5% ferric chloride), terpenoids (2,4-dinitrophenylhydrazine), glycosides (fehling's solution), steroids (Liebermann's Burchard test) and anthraquinones (Borntrager's test) were evaluated according to the methods described by Edeoga *et al*[5].

2.5. Cell viability studies

The effects of EtAc-LME on the viability of NUGC, MKN-74 and GES-1 cell lines were determined by using the MTT assay. Briefly, 96-well plates were plated in triplicate with 6 000 cells per well. After 12 h incubation, cells were treated with various concentrations of the extract (0, 12.5, 25.0, 50.0, 100.0, and 200.0 μg/mL) for 24 and 48 h. MTT solution (10 μL of 5 mg/mL concentration) was added to each well, and the cells were incubated for 4 h at 37 ℃. The medium was then removed, and 200 μL of DMSO was added to each well. Absorbance was determined at 570 nm using a microplate reader (SpectraMax Molecular devices, USA). The effects of the extract on cell viability were assessed by comparing the cell viability of treated cells with that of DMSO treated control cells, which were arbitrarily assigned as 100% viable.

2.6. Lactate dehydrogenase enzyme assay

The LDH assay was performed according to the manufacturer's protocol. Briefly, gastric cancer cells (NUGC

& MKN-74) and normal gastric cells (GES-1) were seeded on 96 well plate and were incubated with EtAc-LME at concentrations of 0, 25, 50, 100 and 200 μg/mL for 24 h at 37 ℃. After treatment, 50 μL of cell culture supernatant from each well was transferred to the wells containing 50 μL of tetrazolium salt reaction mixture and incubated for 30 min at room temperature in the dark condition. The spectrophotometric absorbance of the colored formozan was determined using the automated microplate reader at 490 nm wavelength. As a control for maximum LDH release, cells were treated with 2% triton X-100 for 10 min before running the assay. Untreated cells served as controls for spontaneous LDH-release. The necrotic percentage was expressed using the formula: (sample value/maximal release) ×100%.

2.7. Acridine orange/ethidium bromide staining

The effect on morphological changes induced by EtAc-LME was detected on NUGC cells after 24 h treatment by means of fluorescence microscopy with acridine orange (AO) and ethidium bromide (EB) staining (100 μg/mL for both AO and EB in phosphate-buffered saline). After 5 min, the wells were washed with PBS and the cells were viewed with a Nikon Eclipse inverted microscope at 200× magnification.

2.8. Flow cytometric analysis

The cell cycle analysis, annexin V binding and mitochondrial membrane potential were studied by flow cytometric analysis (Beckman coulter, Inc, Fullerton, CA, USA). At the optimal setting of the instrument and proper filters, the samples were recorded as a FCS file and analyzed by Summit V4.3.02 software. Before analysing the samples, cell suspension was filtered through muslin cloth to remove the debris and cell clumps.

2.8.1. Cell cycle analysis

For the cell-cycle distribution analysis, NUGC and MKN-74 cells (1×10^5/well) were plated in 6-well plate and incubated for 12 h at 37 ℃. NUGC cells were treated with EtAc-LME at concentrations of 100 and 200 μg/mL and MKN-74 cells were treated with 50 and 100 μg/mL for 24 h. After treatment, the cells were collected, fixed in 70% ethanol, washed in PBS, resuspended in 1 mL PBS containing 1 mg/mL RNase and 50 mg/mL PI, and incubated in the dark for 30 min at 37 ℃. Fluorescence emitted from the propidium iodide-DNA complex was quantified by using flow cytometer (Beckman coulter, Inc, Fullerton, CA, USA).

2.8.2. Annexin V binding assay

NUGC and MKN-74 cells were seeded on a 6-well plate at a density of 1×10^5 cells/well. After 12 h incubation at 37 ℃, cells were treated with EtAc-LME at concentrations specified in section 2.8.1. After 24 h treatment, cells were harvested, washed twice with ice-cold PBS, and further evaluated for apoptosis by double staining with annexin V-FITC and propidium iodide according to the manufacturer's instructions (BD Pharmingen, San Diego, CA, USA). The apoptotic cells were measured by a fluorescence-activated cell sorter analysis in a flow cytometer (Beckman coulter, Inc, Fullerton, CA, USA).

2.8.3. Mitochondrial membrane potential analysis

NUGC cells treated with EtAc-LME at concentration of 100 and 200 μg/mL for 24 h were pooled together along with the trypsinized cells and centrifuged at 1 000 r/min for 5 min at 4 ℃. The cell pellets were washed once with PBS before resuspending in 500 μL of PBS containing 50 nM DiOC6. Fluorescence intensities of DioC6 were analyzed by flow cytometry (Beckman coulter, Inc, Fullerton, CA, USA) with excitation and emission settings of 484 and 500 nm, respectively.

2.9. Western blot analysis

NUGC (100 & 200 μg/mL) and MKN-74 (50 & 100 μg/mL) cells treated with EtAc-LME for 24 h were collected and washed twice with cold PBS. The cells were then lysed with 100 μL of M-PER Mammalian Protein Extraction Reagent (Pierce, USA) with protease inhibitor cocktail (Thermo, USA) for 10 min on ice and subsequently cells were centrifuged at 13 000 r/min for 15 min at 4 ℃. Cell lysate supernatants were collected and stored at −20 ℃ until further use. The protein concentration in the cell lysates was determined by using protein dye reagent method (Biorad, Hercules, CA). Aliquots of the lysates (40 μg of protein) were separated via 10% SDS-PAGE and transferred onto a PVDF membrane (Biorad, Hercules, CA) using a glycine transfer buffer (192 mM glycine, 25 mM Tris-HCl, pH 8.8, and 20% v/v methanol). After blocking with 5% non fat dried milk, the membranes were incubated for 2 h with primary antibodies, followed by an additional 30 min of incubation with secondary antibodies in milk containing Tris-buffered saline and 0.1% Tween 20. Human anti-cleaved caspase-3, anti-PARP antibody, Cyto-c antibodies were used at a 1:1 000 dilution as primary antibodies, and horseradish peroxidase-conjugated goat anti-rabbit (Cell signalling technology, USA) at a 1:5 000 dilution was used as secondary antibody. The membrane was then exposed to X-ray film (CL-Xposure Film, Thermo scientific, Rockford, IL, USA). Protein bands were detected using chemiluminescent reagent (Super Signal West Pico, Thermo Scientific, Rockford, IL, USA).

2.10. RT-PCR studies

Total RNA was isolated from the cells (NUGC and MKN-74) after treatment with EtAc-LME at concentration specified in section 2.8.1, using the RNA isolation kit according to the manufacturer's protocol (Qiagen sciences, Maryland, USA). Two microgram of total RNA derived from the cells

was reverse-transcribed into cDNA with random hexamers using superscript vilo cDNA synthesis kit (Invitrogen, Carlsbad, USA) and subjected to PCR using a RT-PCR kit (Qiagen sciences, Maryland, USA). Real-time PCR on the Light Cycler (ABi-7900HT fast light cycler, AB applied biosystems, CA, USA) was performed in the presence of 5 μL of 2× quantitect SYBR green master mix, 1 μL of primer mix (The primer sequence are as follow: Bcl-2 forward 5′-GAG GAT TGT GGC CTT CTT TG-3′, reverse 5′-ACA GTT CCA CAA AGG CAT CC-3′; Bax forward 5′-TTT GCT TCA GGG TTT CAT CC-3′, reverse 5′-GTG TTT GGC TTC CTC CAA AG-3′ and beat actin forward 5′-GGC ATC CTC ACC CTG AAG TA-3′, reverse 5′-GGG GTG TTG AAG GTC TCA AA-3′) for each forward and reverse primer (each 0.5 μM final concentration), 2 μL of cDNA and H_2O up to 10 μL were added. Real-time PCR was performed with an initial denaturation step for 5 min at 95 ℃, followed by 40 cycles of 15 s at 94 ℃, 25 s at 57 ℃ as annealing temperature and extension step for 25 sec at 72 ℃. Direct detection of PCR products was monitored in real time by measuring the increase in fluorescence caused by the binding of SYBR Green Ⅰ Dye to dsDNA. Subsequently, the threshold cycle (C_t), the cycle number at which the amount of amplified gene of interest reached a fixed threshold, was determined. The relative quantification value of the target, normalized to an endogenous control and relative to a calibrator, was expressed as $2^{-\Delta\Delta Ct}$ (fold), where $\Delta C_t = C_t$ of the target gene − C_t of the endogenous control gene (β Actin) and $-\Delta\Delta C_t = C_t$ of the samples for target gene − C_t of the calibrator for the target gene[6].

2.11. Data analysis

Results are expressed as the mean±standard error mean (SEM) of data obtained from three independent experiments. All data were analyzed using one way ANOVA, followed by Dunnett's test for pairwise comparison using Prism software. $P<0.05$ was considered statistically significant for all tests.

3. Results

3.1. Extraction yield, fingerprinting and phytochemical estimation

Leaves of ME yielded 0.40% of hexane extract, 2.40% of ethyl acetate extracts, 1.45% of ethanolic extract and 4.90% of water extract. In the HPLC fingerprinting, the ethyl acetate extract of ME gave 18 peaks at the following retention time values: 1.80, 2.20, 2.80, 3.03, 3.69, 4.26, 4.60, 4.65, 5.51, 6.79, 7.36, 7.60, 8.38, 9.74, 12.57, 15.38, 16.20, and 20.18 min. The corresponding HPLC chromatograms are presented in Figure 1. The phytochemical analysis from ethyl acetate extract of ME showed the presence of alkaloids, flavonoids, tannins, saponins, and terpenoids, as well as the absence of glycosides.

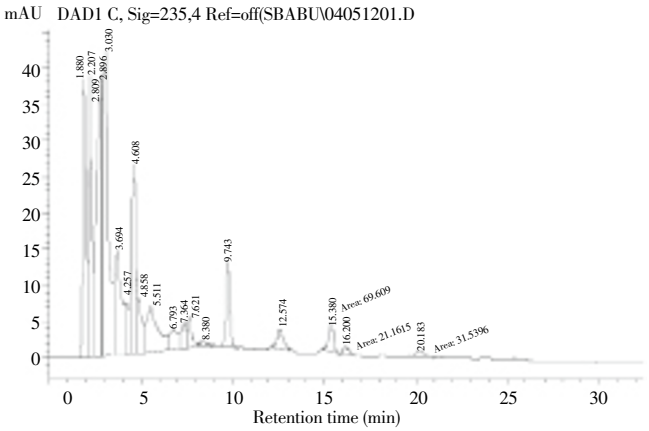

Figure 1. HPLC profile for ethyl acetate extract of Memecylon edule leaves.
Twenty microliter of extract was injected to the RP-HPLC, C18 column with diameter 250.0 mm×4.6 mm and particle size of 5 μm. Sample was eluted with the mobile phase of 70% ACN in water (20 mM ammonium acetate) at a flow rate of 1 mL/min.

3.2. Effect of ME on cell viability of gastric cancer cells

The effect of ME on cell viability of gastric cancer cells (NUGC & MKN-74) was assessed by using calorimetric MTT assay method. Data obtained after 24 and 48 h treatment showed that ME extracts had the potential to inhibit the growth of the gastric cancer cells, NUGC and MKN-74 cells in a time- and dose-dependent manner as shown in the Figure 2a and 2b. The IC_{50} values showed that the ME inhibit the growth of the gastric cancer cells more effectively than that of the normal gastric cells as depicted in Table 1.

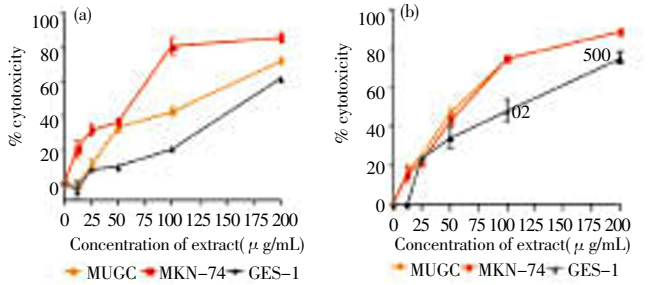

Figure 2. Cytotoxicity of EtAc-LME in human gastric cancer cells (NUGC and MKN-74) and normal gastric mucous cells (GES-1).
Cells were exposed to various concentrations of the extract (0-200 μg/mL) for (a) 24 h and (b) 48 h followed by analysis using an MTT assay. *n*= triplicate wells with triplicate experiments.

Table 1
IC_{50} values of EtAc-LME on gastric cancer cells (NUGC & MKN-74) and normal gastric mucous cells (GES-1) for 24 and 48 h treatment (mean±SD).

Cell line	24 h (μg/mL)	48 h (μg/mL)
GES-1	172.8±3.5	117.1±8.7
NUGC	104.1±3.5*	51.0±3.6*
MKN-74	52.2±1.0*	49.2±6.0*

n=triplicate wells, triplicate experiments. *$P<0.05$, statistically significant compared to normal gastric cells (GES-1).

3.3. Lactate dehydrogenase assay

In order to examine whether the observed cell death was due to necrosis or apoptosis, lactate LDH was measured as a necrotic marker in the cell culture medium after the cells were treated with EtAc-LME for 24 h. Necrotic cells, in contrast to apoptotic cells, disintegrate and release cytosolic enzymes such as lactate dehydrogenase into the cell culture medium which is associated with necrotic cell death. The extent of its activity in converting tetrazolium salt into red formazan product is directly proportional to the number of necrotic cells[7]. At concentration of 100 and 200 μg/mL, the % cytotoxicity of EtAc-LME on NUGC cells determined by MTT assay was found to be (42.3±4.7)% and (32.5±4.2)%, respectively. In case of MKN-74 cells, EtAc-LME showed (36.0±3.7)% and (31.1±6.0)% of cytotoxicity at concentration of 50 and 100 μg/mL. Even though the % cytotoxicity was high at the above mentioned concentrations, the % release of LDH was not significant when compared to the control cells. After treatment with EtAc-LME at higher concentrations *ie.* 200 μg/mL in NUGC cells and 100 μg/mL in MKN-74 cells, a significant release of LDH was observed as shown in Figure 3. These results indicated that at higher concentrations, the percentage of necrotic cell death was increased because of loss in cell membrane integrity.

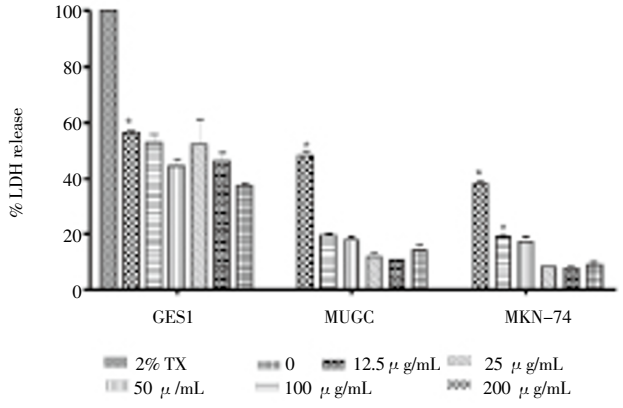

Figure 3. Effect of EtAc-LME on the release of LDH in human gastric cancer cells (NUGC, MKN-74) and normal gastric cells (GES-1). $n = 3$. *$P<0.05$ in comparison with control cells.

3.4. Cell morphological assessment

The morphological abnormalities induced by EtAc-LME in NUGC cells were studied under fluorescence microscopy using AO/EB staining technique. AO permeates the intact cell membrane and stains the nuclei green, whereas EB is taken up only when the membrane integrity is deteriorated, and stains the nucleus red. Intact cells therefore exhibit homogeneous green nuclei, whereas apoptotic cells show condensed or fragmented chromatin. Late apoptotic or necrotic cells have orange to red nuclei[8,9]. Compared with spontaneous apoptosis observed in control cells (early apoptotic 3.19%, 0% late apoptotic and 0% necrotic cells), NUGC cells treated with the EtAc-LME showed increase in percentage of early apoptotic cells (Figure 4). Cells treated with EtAc-LME at 200 μg/mL showed an increase in percentage of necrotic cells. Fluorescence microscopic images (Figure 4) of cells treated with 100 μg/mL of EtAc-LME clearly showed the morphological changes such as cell shrinkage, membrane blebbing (indicated by arrow), chromatin condensation with destructive fragmentation of the nucleus (inset ⅰ and ⅱ), suggesting that the ME induced cell death in gastric cancer cells by apoptosis.

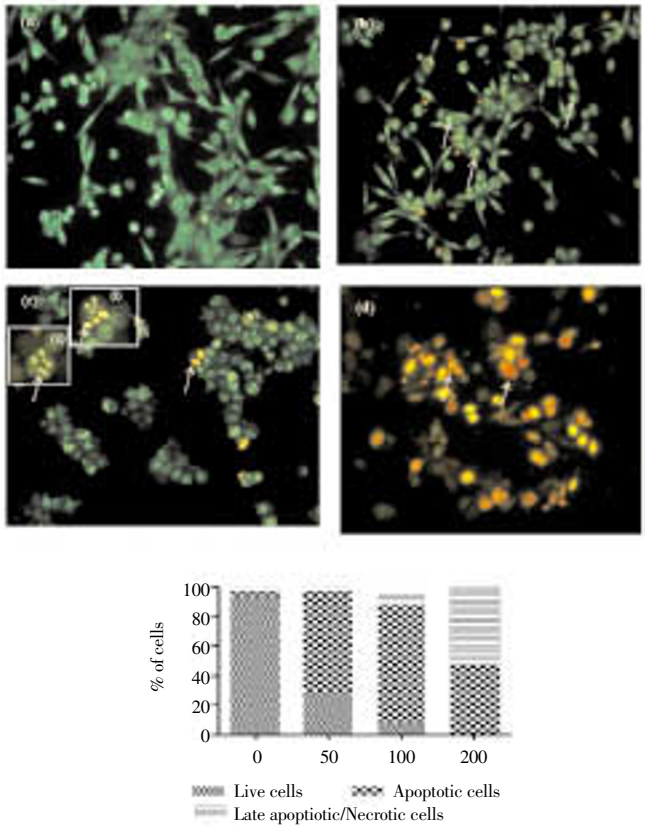

Figure 4. Morphological changes in NUGC cells treated with and without EtAc-LME for 24 h.
(a) Green live cells with intact membrane and nuclei; (b) cells treated with 50 μg/mL; (c) Early signs of apoptosis characterized by condensed chromatin, cell membrane blebbing, and destructive fragmentation of the nuclei (Inset ⅰ & ⅱ) at 100 μg/mL and (d) Late apoptotic cells/ necrotic cells characterised with orange-red staining of the nucleus after treatment with 200 μg/mL.

3.5. Effect on cell cycle progression

The effects of EtAc-LME on cell cycle progression were examined by propidium iodide staining method. The treatment of NUGC and MKN-74 cells for 24 h with extract resulted in a marked accumulation of cells in sub-G_1 phase (hypodiploid peak) and a slight reduction in G_1 and G_2/M and S phases as shown in Figure 5. When the cells were treated with 100 and 200 μg/mL (IC_{50} & IC_{80} values) of ME

extract, the percentage of NUGC cells undergoing apoptosis increased from 4.4% in control to 17.32% (3.9 folds) at 100 μg/mL and 23.17% (8 folds) at 200 μg/mL. Whereas, the percentage of MKN 74 cells undergoing apoptosis increased from 9.6% in control to 23.17% (2.4 folds) at 50 μg/mL (IC_{50} value) and 33.85% (3.5 folds) at 100 μg/mL (IC_{80} value). These results indicated that the ME extract inhibited the growth of the cancer cells by inhibiting the cell cycle.

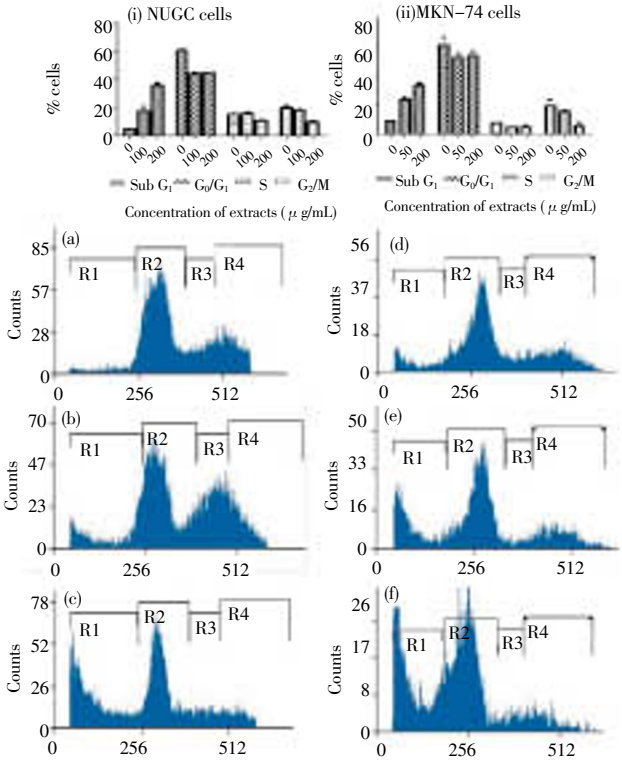

Figure 5. Effect of EtAc-LME on cell cycle progression of gastric cancer cells, (ⅰ) NUGC (ⅱ) MKN-74.
(a) NUGC-control cells; (b,c) NUGC cells treated with 100 and 200 μg/mL; (d) MKN-74-control cells; (e,f) MKN-74 cells treated with 50 and 100 μg/mL for 24 h followed by analysis of cell cycle distribution using propidium iodide cell staining method. All assays were done in duplicate (R1-Sub G_1, R2-G_0/G_1, R3-S phase, R4-G_2/M phase).

3.6. Annexin V binding assay

In normal cells, phosphatidylserine (PS) molecules are confined to the cytoplasmic surface of the plasma membrane. In apoptotic cells, PS is translocated from inner surface to outer surface of the plasma membrane. The human anticoagulant annexin V is a 35-36 phopholipid binding protein that has high affinity to the PS and is used to detect apoptotic cells. In case of necrotic cells, propidium iodide uptake will be enhanced because of disturbed plasma membrane. Hence, four different type populations of cells are easily distinguished during annexin V/PI staining[10]: cells that are unlabelled (viable cells), cells that have bound annexin V-FITC only (early apoptotic), cells that have een stained with propidium iodide only (necrotic) and those that have both bound with annexin V and been labelled with propidium iodide (late apoptotic/necrotic cells). Since, the morphological studies indicated that the EtAc-LME appeared to induce apoptosis; the annexin V-FITC/propidium iodide flow cytometric assay was performed to confirm ME induced apoptosis in gastric cancer cells. As shown in Figure 6, EtAc-LME treatment to NUGC cells for 24 h at concentration of 100 and 200 μg/mL increased annexin V-FITC binding by 8.1 fold and 12.8 folds, respectively as compared to the control cells. In case of MKN-74 cells, the annexin V-FITC binding was increased by 12.6 and 16.5 folds after treating the cells at concentration of 50 and 100 μg/mL, respectively. These results clearly showed that ME extract induced cell death by apoptosis.

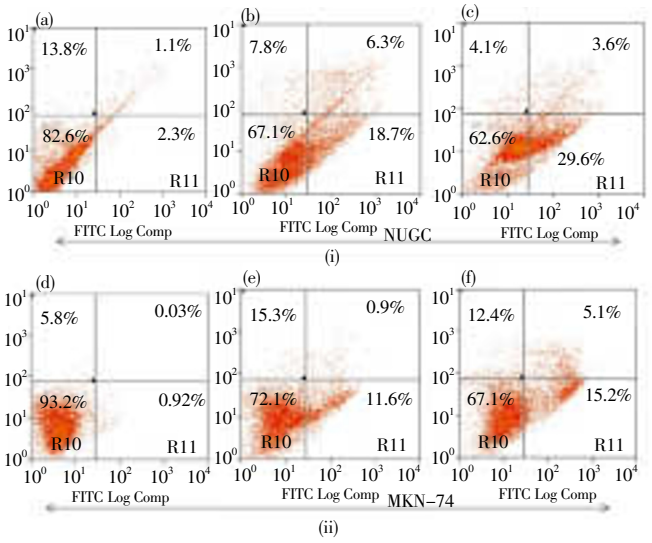

Figure 6. Effect on externalisation of phosphatidyl serine after the exposure of gastric cancer cells.
(ⅰ) NUGC (ⅱ) MKN-74 with EtAc-LME for 24 h (a) NUGC- control cells, (b,c) NUGC cells treated with 100 and 200 μg/mL, (d) MKN-74-control cells, (e,f) MKN-74 cells treated with 50 and 100 μg/mL for 24 h followed by analysis using the annexin V-FITC Apoptosis Detection Kit. All assays were done in duplicate (R8- Annex -ve, PI+ve ; R9- Annex +ve, PI+ve; R10- Annex -ve, PI-ve, R11- Annex +ve, PI-ve).

3.7. Loss of mitochondrial potential

The loss in the mitochondrial membrane integrity is one of the early events leading to apoptosis. Loss in mitochondrial membrane potential after treatment with EtAc-LME using ampholytic cationic fluorescent probe DiOC6[11,12] was observed as shown in Figure 7. The decrease in mean fluorescence intensity ($\Delta\Psi m$) about 15.5% and 27.9%, respectively was observed in NUGC treated cells when compared to the control cells.

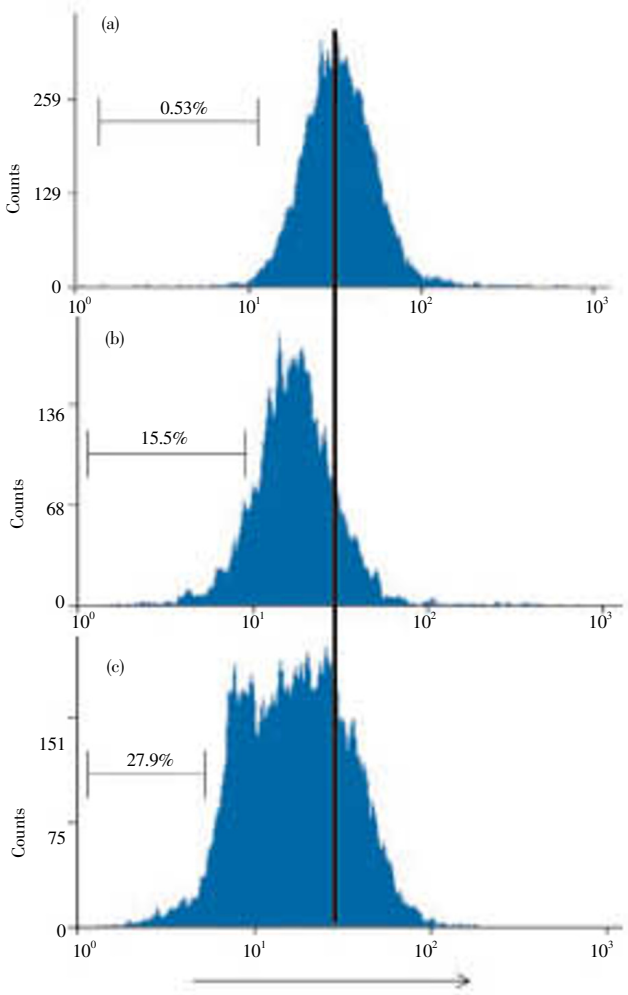

Figure 7. Effect of EtAc-LME on mitochondrial membrane potential of gastric cancer cells (NUGC).

(a) Control cells, (b) Cells treated with 100 μg/mL, (c) Cells treated with 200 μg/mL for 24 and stained with 50 nM of DiOC6.

3.8. Western blot and RT-PCR analysis for apoptosis-related protein expression

To elucidate the molecular mechanism of EtAc-LME induced apoptosis in gastric cancer cells, mRNA expression levels of Bax and Bcl-2 were examined by RT-PCR studies. The results of RT-PCR revealed that there was a significant down-regulation of mRNA expression of Bcl-2 in NUGC cells by 0.23 and 0.11 folds after treatment with 100 and 200 μg/mL respectively. Compared to control cells, the relative mRNA expression of Bcl-2 in MKN-74 cells was decreased by 0.66 and 0.68 folds, respectively after treating with 50 and 100 μg/mL for 24 h as shown in Figure 8. Expression of Bax was relatively constant in both NUGC and MKN-74 cell lines. The loss in mitochondrial membrane potential is usually accompanied by release of Cyt-C into the cytosol. As shown in Figure 9, Cyt-c levels were increased by 4.0 folds in NUGC and 3.7 folds in MKN-74 treated cells when compared to the control cells. As release of cytochrome c into the cytosol involved in the activation of the caspase-3 (main executioner caspase) with subsequent cleavage of cellular target proteins, such as nuclear PARP that triggers apoptosis[13]. Hence, the role of caspase-3 in response to the EtAc-LME treated gastric cancer cells was investiagted. Western blotting analysis revealed that EtAc-LME treatment to the gastric cancer cells resulted in proteolytic cleavage of procaspse-3 to active caspase-3 with subsequent cleavage of PARP into 89 kDa fragment.

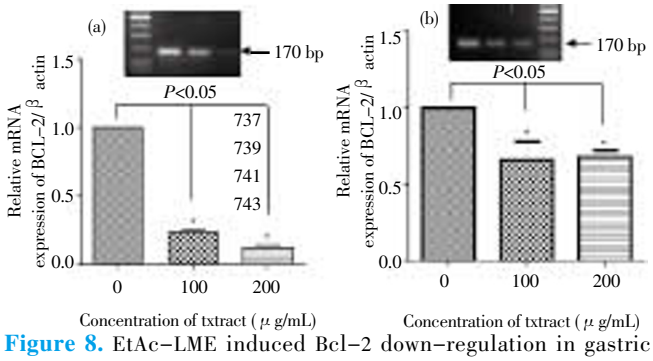

Figure 8. EtAc-LME induced Bcl-2 down-regulation in gastric cancer cells, (a) NUGC, (b) MKN-74.

Cells were treated with different concentrations of EtAc-LME as indicated in materials and method for 24 h and cells were harvested for RNA isolation and RT-PCR analysis. The amplified products were confirmed by running 2% agarose gel electrophoresis. Relative mRNA expression was calculated by $2^{-\Delta\Delta ct}$ method. *n*=triplicate samples, **P*<0.05, statistically significant when compared to the control cells.

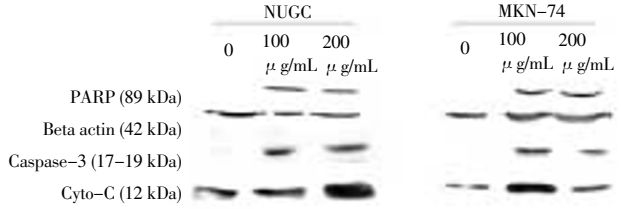

Figure 9. Western blot analysis of Cyto-c, caspase-3, and PARP protein levels in EtAc-LME treated gastric cancer cells (NUGC and MKN-74 cells) for 24 h.

Protein (40 μg) from each sample was resolved on 10 % SDS-PAGE and Western blot was performed.

4. Discussion

Natural products discovered from medicinal plants have played an important role in the treatment of cancer, having contributed considerably to approximately 60% of available cancer chemotherapeutic drugs[14,15]. The need to develop more effective and non toxic antitumor drugs has prompted investigators to explore new sources of pharmacologically-active compounds, especially from the natural products[16-20]. India is one of the sub continents having great biodiversity and wide use of traditional plants in ayurvedic medicine; however, only few medicinal plants have been scientifically proved for their applications in the

treatment of cancer. The present investigation was aimed to evaluate the antiproliferative and apoptogenic activity of Indian medicinal plant ME on human gastric cancer cells. Our preliminary screening for cytotoxicity studies of EtAc-LME has showed significant inhibition on the growth of gastric cancer cells. Based on the IC_{50} values, it was demonstrated that EtAc-ME inhibited the gastric cancer cell growth in a dose-dependent manner and cytotoxicity was more in the gastric cancer cells (NUGC and MKN-74) than in the normal gastric cells (GES-1). The high cytotoxicity towards malignant cells may in part reflect the difference in growth rates between malignant and non-malignant cells. Further studies in NUGC and MKN-74 cells were carried out by considering the IC_{50} (100 & 50 μg/mL, respectively) and IC_{80} values (200 & 100 μg/mL, respectively). Programmed cell death (apoptosis) is a physiological and crucial process that is regarded as the preferred way to eliminate cancer cells. Apoptosis is characterized by several morphological changes such as membrane blebbing, cell shrinkage, chromatin condensation, nuclear fragmentation and formation of apoptotic bodies[21]. The induction of apoptosis by EtAc-LME was evidenced from the morphological alteration such as severe membrane blebbing, DNA fragmentation, formation of apoptotic bodies that could be clearly visualized from fluorescence microscopic photographs. In order to confirm the apoptosis induced by EtAc-LME, other apoptotic markers like externalisation of phosphatidylserine and cell cycle arrest were studied using flow cytometric analysis. The translocation of phosphatidylserine towards the outer leaflet of apoptotic cells membrane was assessed using annexin V-FITC staining and propidium iodide staining techniques was used to check the cycle arrest. Percentage of early apoptotic cells was increased in a concentration-dependent manner in both NUGC and MKN-74 cells. Uncontrolled cell proliferation is associated with the loss of cell cycle checkpoints that regulate the passage through cell cycle. These check points monitor the integrity of DNA and ensure that the genes are expressed in a co-ordinate manner[22]. Cell cycle analysis showed that EtAc-LME induced a marked accumulation of the treated cells in a sub-G_1 phase and the DNA content was decreased in other three phases (G_0/G_1, S and G_2/M). These results indicated that the EtAc-LME triggered cell death by apoptosis in gastric cancer cells. To understand the molecular mechanism of EtAc-LME induced apoptosis in gastric cancer cells, expression of apoptotic related genes/proteins were further investigated. Regulation of apoptosis is a complex process and involves number of gene products such as Bcl-2 and Bax. Bcl-2 is a dominant negative inhibitor of Bax and inhibits apoptosis in cancer cells[23,24]. An important approach for successful anticancer therapy is to find new cytotoxic agent that are able to restore apoptosis through activation of caspases and/or inhibition of anti-apoptotic proteins like Bcl-2. From the results in our study, we observed that the mRNA expression of Bax levels in the EtAc-LME treated cells showed relatively constant expression in both NUGC and MKN-74 cells. Whereas, the mRNA expression of Bcl-2 was significantly decreased in EtAc-LME treated NUGC and MKN-74 cells, when compared to the control cells. Even though the Bax levels remained unchanged, the relative low expression of Bcl-2 sensitizes the gastric cancer cells to undergo apoptosis because of the homodimer formation of Bax protein[24]. The decrease in Bcl-2 expression and homodimer formation of Bax protein facilitates the permeabilization of the outer mitochondrial membrane and release of Cyt-c which further leads to activation of caspase-3. Hence in the current study, Cyt-c was analysed by western blot anaysis and we found that there was significant increase in Cyt-c level in EtAc-LME treated NUGC and MKN-74 cells. Furthermore, the activation of procaspase-3 which is main executioner caspase and subsequent cleavage of PARP were investigated. Treatment with EtAc-LME resulted in the cleavage of the procaspase-3 into the 17 kDa active-form and cleaved PARP (85 kDa) in both NUGC and MKN-74 cells. The active form of procaspase-3 and cleaved PARP was absent in control cells. The role of mitochondria for the induction of apoptosis by EtAc-LME was further confirmed by studying the change in mitochondrial potential by DiOC6 retained dye assay method. The decrease in mean fluorescence intensity ($\Delta\Psi$m) of DiOC6 in the treated gastric cancer cells suggested that the mitochondrial mediated cell death was involved[25]. These data clearly showed that EtAc-LME induced apoptosis by decreasing the expression of anti-apotic protein Bcl-2 leads to the collapse of mitochondrial potential which further causes the release of Cyt-c into the cytosol with subsequent activation of caspases and PARP cleavage.

In conclusion, this is the first study for evaluating the potential application of ME for the treatment of gastric cancer. This study showed that EtAc-LME significantly inhibited the growth of gastric cancer cells with minimal cytotoxicity towards the normal gastric cells. Treating gastric cancer cells with EtAc-LME led to accumulation of cells at sub-G_1 phase and induced apoptosis by mitochondria mediated intrinsic pathway. This study will helps us to establish the platform for generating more potent chemotherapeutic agents from natural resources for the treatment of gastric cancer.

Conflict of interest statement

We declare that we have no conflict of interest.

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