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XIAP inhibitor and antiestrogen embelin abrogates metastasis and augments apoptosis in estrogen receptor positive human breast adenocarcinoma cell line MCF-7

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Abstract Tamoxifen therapy for the treatment of hormone responsive breast cancer has limitations due to acquired resistance in the case of recurrences. Embelin, a known inhibitor of X-linked inhibitor of apoptosis protein (XIAP) was also reported to exhibit strong antiestrogenic effects in animal models. Dual role of embelin as a proapoptotic and antiestrogenic agent may have potential benefits in the therapy of breast cancer. In this study, the effects of embelin treatment on estrogen receptor positive Human breast adenocarcinoma (MCF-7) cells was investigated to primarily understand if embelin being an antiestrogen and XIAP inhibitor could be a potential alternative to tamoxifen therapy. Results revealed that, embelin at a concentration of 65 μg/ml attenuated proliferation, inhibited metastatic migration, modulated the expression of Bcl2, Caspases and induced apoptosis in MCF-7 cells which was found to be p53 mediated. Hence, chemotherapy with embelin could be a promising strategy to be experimented in hormone responsive breast cancers.

Keywords Antiestrogens · Embelin · Apoptosis · XIAP · MCF-7 · Breast cancer

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Introduction

Breast cancer is the most common malignancy in women and despite the advances in diagnosis and treatment, it presents the second leading cause of mortality due to cancer in women worldwide [1]. Approximately 70 % of breast cancers are estrogen receptor positive (ER+) and mitogenic estrogen signaling plays a pivotal role in the development and progression of ER+ breast cancer [2]. Endocrine therapies, especially treatment with antiestrogens like tamoxifen is the recommended first line of chemotherapy for ER+ breast cancer. Antiestrogens act by competitively binding to estrogen receptors on tumour cells and hence blocking the effects of estrogen [3]. The major setback in the treatment of breast cancer with antiestrogens like tamoxifen is the unresponsiveness to therapy due to acquired resistance or intrinsic insensitivity in case of recurrences. Thus, the search is on for alternative antiestrogenic compounds, which could be employed in the chemotherapy of breast cancer.

Dysfunction of the apoptotic machinery is a fundamental characteristic of cancer that allows the transformed cells to survive and proliferate. In part, this is due to defects in the execution phase of apoptosis. The inhibitor of apoptosis proteins (IAP) are one of the major protein families that regulates Caspase activation and programmed cell death. In particular, one of the IAP proteins, X-linked inhibitor of apoptosis protein (XIAP) has been identified as the most potent Caspase inhibitor to date [4]. XIAP is expressed in almost all tissues and cell types. However, it is often over expressed in tumours versus normal tissue [5, 6]. XIAP is capable of conferring resistance to cancer cells. The benzoquinone and the small molecular inhibitor of XIAP, embelin possess a wide spectrum of biological activities with strong inhibition of NF-KB and down

regulation of variety of gene products involved in tumour cell survival, proliferation, invasion, angiogenesis and inflammation [7]. The chemical structure of embelin has resemblance with that of natural coenzyme Q10 (ubiquinones) and the role of this is well defined in various biochemical protective mechanisms [8]. Embelin is reported as a potent oral contraceptive having 85.7 % antiimplantation activity in female rats. It is also reported to inhibit pregnancy and possess antiestrogenic and weak progestational activity [9–11]. The proapototic effects of embelin coupled with its strong antiestrogenic effects suggest the possibility of its potential therapeutic use in the chemotherapy of ER+ breast cancer. Hence the objective of the present study is to investigate the ability of embelin to attenuate cell proliferation, inhibit metastasis and augment apoptotic cell death in ER+ human breast adenocarcinoma cell line MCF-7. This is to understand the correlation between the dual role of embelin as an apoptosis activator and antiestrogenic compound, which was not previously investigated. With increased resistance being reported to antiestrogens like tamoxifen in case of recurrences, the current study gains potential significance in addressing clinical drug resistance in ER+ breast cancer patients.

Materials and methods

Cell culture and reagents

In the study, a widely used ER+ and estrogen dependent MCF-7 cell line was employed as in vitro model system and was procured from National Center for Cell Science (NCCS), Pune, India. MCF-7 cells were cultured in sterile ready to use Eagle's Minimum Essential Medium (MEM, AL075A, Himedia, India) supplemented with 1× Antibiotic Antimycotic solution (A007, Himedia, India) and 10 % Fetal Bovine Serum (FBS, RM1112, Himedia, India). Cells were grown under standard growth conditions (temperature 37 °C, 5 % CO_2 and 95 % humidity) in a CO_2 incubator (Forma Scientific, USA). When a confluent monolayer was formed, cells were detached with 0.25 % Trypsin-0.2 % EDTA in Dulbecco's phosphate buffered saline (PBS) (T-001, Himedia, India) and then subcultured at a split ratio of 1:3 in 12.5 cm^2 volume tissue culture flask (TCG2, Himedia, India). The media was changed three times a week. The cells were grown in growth medium containing 10 % FBS or maintained in maintenance medium containing 5 % FBS. After arriving at confluency, the cells were seeded onto 96 well microtitre plates (TPP96, Himedia, India) and were utilized for various cell proliferation and cytotoxicity assays.

A stock solution of 5 mg/ml of embelin (Sigma, St. Louis, MO, USA) was prepared in DMSO and stored as small aliquots at −20 °C. From the stock, appropriate dilutions were carried out to prepare various concentrations of embelin ranging from 1 to 100 μg/ml and used for the following cytotoxicity and cell proliferation assays. In the study, reference drug tamoxifen (Sigma, St. Louis, MO, USA) was used at a concentration of 25 μg/ml [12].

Cell proliferation, cytotoxicity and antimetastatic assays

Bromodeoxyuridine (BrdU) assay

Cell proliferation was measured by BrdU (5-bromo-2′-deoxyuridine) incorporation assay using a commercially available kit (Calbiochem/Merck, Whitehouse Station, NJ, USA) following the manufacturer's recommendations. Briefly, 10,000 cells/well were plated into 96 well plates in the presence of embelin and BrdU label (1:2,000 dilution) for 24 h. Plates were then washed, fixed with anti-BrdU antibody, and peroxidase goat anti-mouse IgG conjugate. Immunocomplex formation was measured using tetramethylbenzidine solution, and the reaction terminated using 2.5 N sulfuric acid. The measured intensity is proportional to the amount of incorporated BrdU in the cells. Absorbance was measured at 450 nm.

MTT assay

MTT Assay was performed based on the protocol described previously [13]. Briefly, a confluent flask was selected, trypsinized and 5 × 10^6 cells/ml was cultured in 96 well plate overnight and pretreated with various concentrations of embelin and tamoxifen and allowed to proliferate for 72 h (temperature 37 °C, 5 % CO_2 and 95 % humidity). 25 μl of MTT (10 mg/ml in PBS) was added to the wells and plates were incubated for 5–6 h. The formazan crystals formed were solubilised by adding 75 μl of di-methyl sulphoxide and the plate was read at 570 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA). A dose response curve was constructed applying the five parameter logistics using the Masterplex Readerfit: Curve fitting software for ELISA analysis (Hitachi solutions America, Ltd.). The concentration of embelin that inhibited cell growth by 50 % (IC_{50}) was calculated.

Trypan blue dye exclusion test

Trypan blue dye exclusion test was performed following the method described previously [14]. After incubation with embelin and tamoxifen for 24 and 48 h, cells were harvested by trypsinization, washed with PBS, stained with trypan blue (0.1 %) and the number of viable cells present

was counted under an inverted phase contrast microscope (Olympus, Japan) and the cell count was determined.

Clonogenic cell survival assay

A cell suspension (3×10^4 cells) in 4 ml of 0.4 % agar with complete medium was overlaid on a 60 mm dish containing 0.6 % agar base as described previously [15]. The media was changed two times a week and plates were incubated at 37 °C in a humidified incubator for 15 days. The colonies formed in the untreated control and the treated group were fixed with 3.7 % paraformaldehyde, stained with crystal violet (0.1 %) and counted under a light microscope (Olympus, Japan).

In vitro wound healing assay

Briefly, cells were seeded in a six well plate at a density of 5×10^4 cells/well, grown to near confluent monolayers in 10 % serum supplemented medium, and then starved overnight in a low serum medium (0.5 % FBS). Wounds were scratched (induced) through the cell monolayer using a sterile 200 µl pipette tip as described previously [16]. The cells were then washed twice very gently using PBS. PBS was removed and replaced with 2 ml of media containing test compounds and DMSO (0.1 %, v/v). After incubation for 12–24 h in a humidified atmosphere at 37 °C with 5 % CO_2, cells were fixed with 3.7 % formaldehyde and stained with crystal violet (0.1 %). Each well was photographed and the pictures were analyzed.

Assays to determine apoptotic cell death

Giemsa/crystal violet staining

Light microscopic analysis (treated and untreated cells) was carried out after fixing the cells in Carnoy's fixative and subsequent staining with Giemsa/0.1 % crystal violet. The morphology change in the untreated control and the treated cells were observed (40× magnification) under a trinocular inverted phase contrast microscope (Olympus, Japan).

Hematoxylin–eosin staining

Cells after treatment were fixed in Carnoy's fixative and the morphology change in the untreated control and the treated cells were analyzed by staining the cells with hematoxylin and eosin. Cells were observed (100× magnification) under a light microscope (Olympus, Japan).

Acridine orange/ethidium bromide (AO/EB) staining

Dual staining with acridine orange and ethidium bromide was performed based on the protocol previously described [17]. Cells were seeded in six well plates for 24 h and subjected to treatment with embelin and tamoxifen. After incubation, cells were harvested by trypsinization and washed with PBS, then stained with 0.1 mg/ml acridine orange and 0.1 mg/ml ethidium bromide. Stained cell suspension (10 µl) was placed on a clean glass slide and covered with a cover slip. The cells were then observed under a fluorescence microscope in both red channel (590 nm) and green channel (520–550 nm) and the percentage of apoptosis was calculated.

Hoechst 33342 staining

Chromatin condensation was assessed by nuclear staining with Hoechst 33342. After treatment with embelin and tamoxifen for 24 h, cells were harvested, washed with PBS thrice and then stained with 1 µl of Hoechst 33342 (5 mg/ml). The suspension was incubated at 37 °C in dark for 15 min. Stained cells were viewed and the images captured

Table 1 Sequence of primers used in RT PCR

Sl. no.	Gene	Primer sequence	Size of the product (bp)
1.	XIAP	F: 5′AAGAGAAGATGACTTTTAACAG 3′	575
		R: 5′ TGCTGAGTCTCCATATTGCC 3′	
2.	p53	F: 5′ CAGCCAAGTCTGTGACTTGCACGTAC3′	293
		R: 5′CTATGTCGAAAAGTGTTTCTGTCATC 3′	
3.	Bcl 2	F: 5′ ACGATAACCGGGAGATAGTGATG 3′	645
		R: 5′ CTGAGCAGAGTCTTCAGAGACA 3′	
4.	Caspase 3	F: 5′ CTTGGTAGATCGGCCATCTGAAAC 3′	405
		R: 5′ GGTCCCGTACAGGTGTGCTTCGAC 3′	
5.	Caspase 9	F: 5′CAA AGG AGC AGA AAG TAG TGA AG 3′	128
		R: 5′GAG GAA GGG CAG AAG TTC AC 3′	
6.	β-Actin	F: 5′ AGATGACCCAGATCATGTTTGAGAC 3′	720
		R: 5 CTGATCCACATCTGCTGGAAGGT 3′	

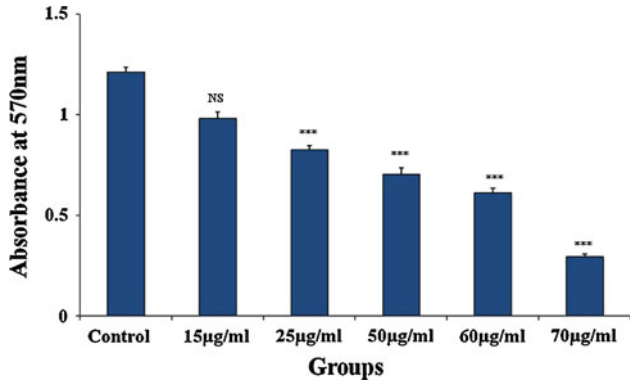

Fig. 1 Effect of embelin on proliferation of MCF-7 cells (MTT assay)—pilot studies. It shows the effect of different concentrations of embelin (15–70 µg/ml) on viability and proliferation of MCF-7 cells as determined by MTT assay. Treatment of MCF-7 cells with 25–70 µg/ml showed statistically significant ($P < 0.001$) decrease in the proliferation of MCF-7 cells. Data represent mean ± SD of six replicates. Intergroup comparisons were made between the cell control and the treated groups. Student's *t* test; ***$P < 0.001$ and *NS* non significant versus control

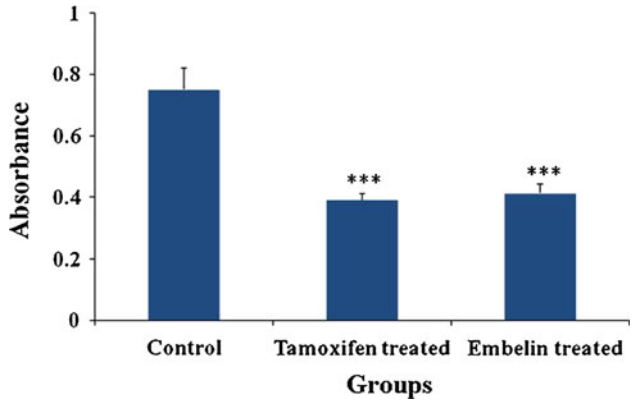

Fig. 2 Effect of tamoxifen and embelin on proliferation of MCF-7 cells (BrdU assay). It shows the effect of tamoxifen and embelin on MCF-7 cells as determined by BrdU assay. Treatment with embelin and tamoxifen resulted in a significant decrease in cell proliferation as compared with the control. Data represent mean ± SD of six replicates. Intergroup comparisons were made between the cell control and the treated groups. Student's *t* test; ***$P < 0.001$

under a fluorescent microscope (Leica DM LB2, Japan) using 350 nm extinction/stimulation and 460 nm emission. Chromatin integrity in the untreated control and the treated cells were analyzed.

Qualitative analysis of fragmented DNA

Cells (2×10^6/ml) were seeded in 25 cm^2 tissue culture flasks and allowed to attach overnight. Subsequently, cells were treated with embelin and tamoxifen. After incubation, cells were detached by trypsinisation, washed with PBS and total DNA was extracted following the protocol

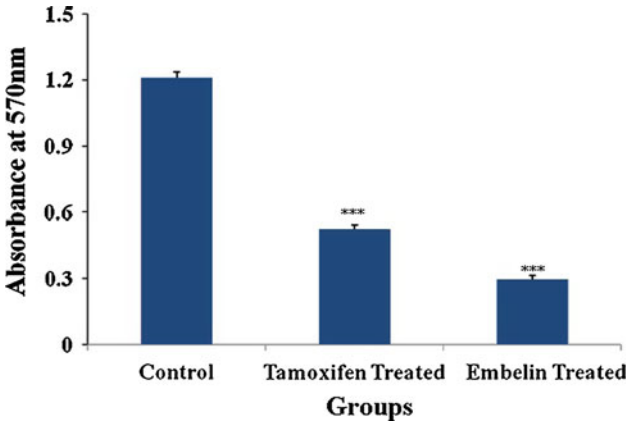

Fig. 3 Effect of tamoxifen and embelin on proliferation of MCF-7 cells (MTT assay). It shows the effect of tamoxifen and embelin on MCF-7 cells as determined by MTT assay. Treatment with embelin and tamoxifen resulted in a significant decrease in cell proliferation as compared with the control. Data represent mean ± SD of six replicates. Intergroup comparisons were made between the cell control and the treated groups. Student's *t* test; ***$P < 0.001$

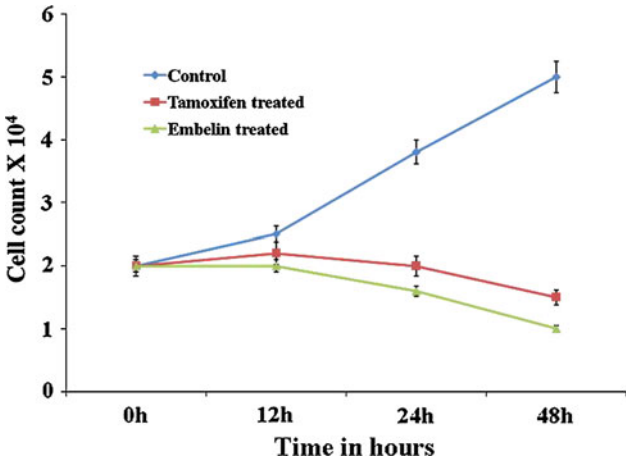

Fig. 4 Effect of tamoxifen and embelin on the growth of MCF-7 cells (Trypan Blue Dye Exclusion Test). It shows the effect of tamoxifen and embelin on the growth of MCF-7 cells as assessed by trypan blue dye exclusion test. Embelin and tamoxifen inhibited the growth of MCF-7 cells in a time dependent manner. Cells treated with embelin and tamoxifen for 24 and 48 h showed an appreciable decrease in cell count as compared to control cells

described previously [18] and loaded onto a 2 % agarose gel in TAE buffer and run at 100 V for 30 min.

Quantitative determination of fragmented DNA

Quantitative determination of fragmented DNA was performed using diphenylamine (DPA) as described previously [19]. Briefly, the procedure includes the lysis of cells and release of nuclear DNA, a centrifugation step with the generation of two fractions (corresponding to intact and fragmented DNA, respectively), precipitation of DNA,

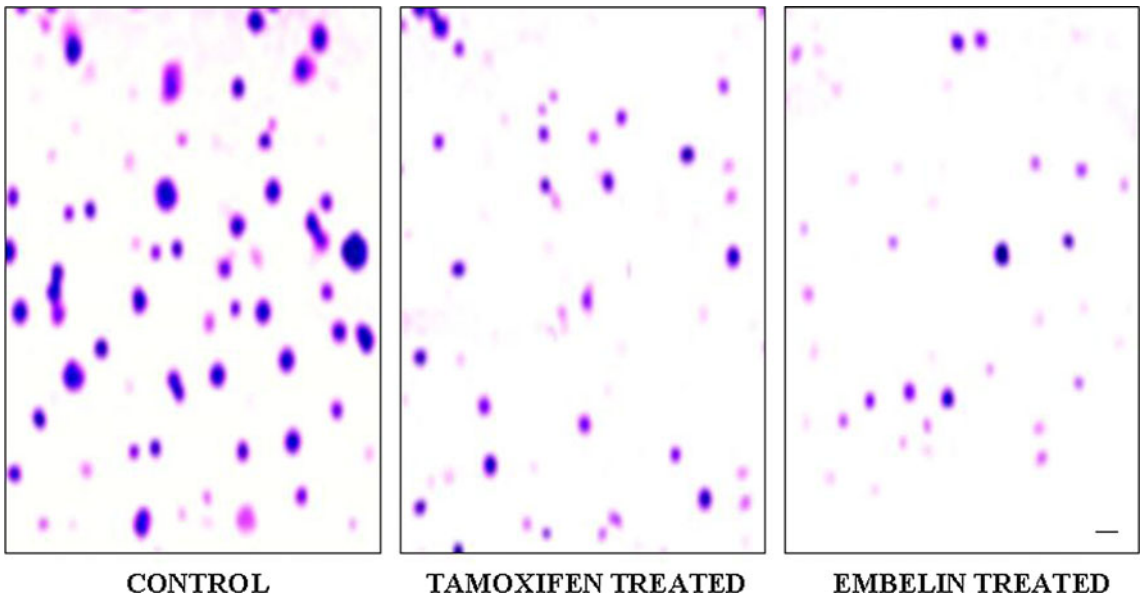

Fig. 5 Effect of tamoxifen and embelin on cloning efficiency and surviving fraction. Colonies formed in soft agar after 15 days of incubation were stained with 0.1 % crystal violet and photographed at ×10 magnification. Data are representative of three independent experiments. Magnification—×10. *Bar* represents 10 μm

hydrolysis and colorimetric quantitation upon staining with DPA at 600 nm using a microplate reader (Bio-Rad Laboratories, Hercules, CA, USA).

TUNEL assay

DNA Strand breakage and fragmentation of the MCF-7 cells following treatment with embelin and tamoxifen was ascertained by using the Fluorescein-FragEL™DNA Fragmentation Detection Kit following the manufacturer's instructions. The assay is based on the principle that terminal deoxynucleotidyl transferase (TdT) binds to exposed 3-OH ends of the DNA fragments generated in response to apoptotic signals and catalyse the addition of fluorescein-labeled and unlabeled deoxynucleotides. When excited, fluorescein generates an intense signal that can be detected by fluorescence microscopy and analyzed.

Comet assay

Single-cell gel electrophoresis (Comet assay) was performed as described earlier [20]. It is a sensitive method to visualize single strand breaks in nuclear DNA of cells. Briefly, cells were cultured in 24-wells plates. After treatment, cells were washed with 300 μl of PBS (pH 7.4), with 10 min of incubation at 37 °C. Cells were detached by incubation with 50 μl of trypsin (0.25 %) per well for 5 min, at 37 °C. To decollate the cells, 300 μl of medium containing 15 % fetal calf serum was added and pipetted three to four times. Then, cells were lysed for 2 h and transferred to fully frosted microscope slides (Menzel, Bonn, Germany). After electrophoresis under alkaline conditions (pH 13), DNA was stained with ethidium bromide, and the slides were examined under a fluorescence microscope (Leica DM LB2, Japan) and the results analysed using TriTek CometScore™ 15 software. The extent of DNA damage was expressed in terms of the percentage of the tail DNA, head DNA and tail moment.

Reverse transcription PCR analysis

Total RNA from the treated and untreated control cells were isolated using Trizol reagent. Oligo (dT)-primed

Table 2 Effects of tamoxifen and embelin on cloning efficiency and surviving fraction—colony formation in soft agar

Sl. no.	Groups	No. of colonies	Cloning efficiency	Surviving fraction	% Survival
1.	Control	38	0.76	–	100
2.	Tamoxifen treated	18	0.35	0.324	36.8
3.	Embelin treated	09	0.18	0.236	23.6

This table indicates the cloning efficiency and surviving fraction of the cells treated with tamoxifen, embelin and untreated control. The control cells showed a cloning efficiency of 0.76 as compared to a cloning efficiency of 0.18 and 0.35 observed with the cells treated with embelin and tamoxifen respectively. Decrease in the percentage of survival was observed in the treated cells as compared to untreated control

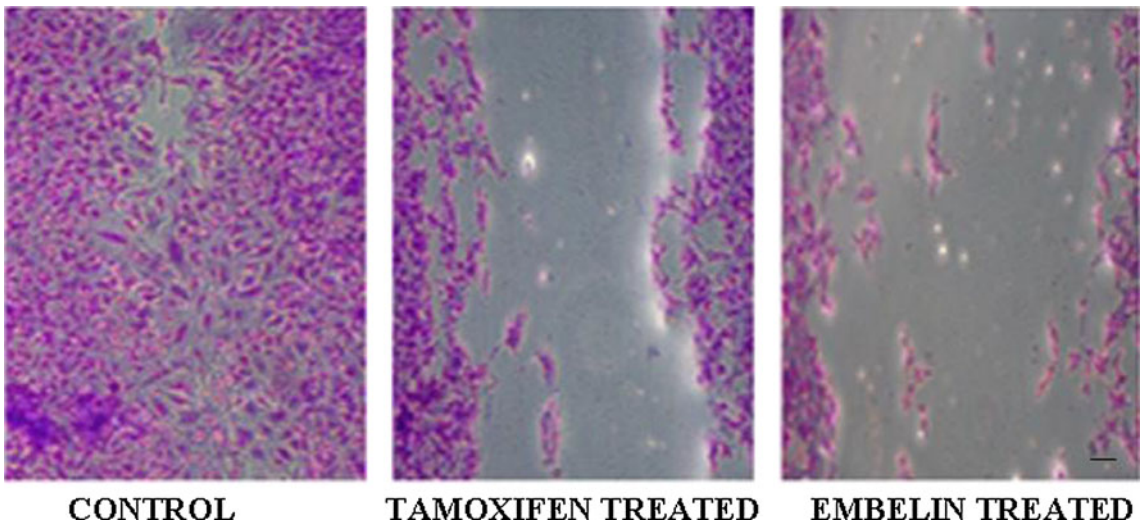

Fig. 6 Effect of tamoxifen and embelin on cell migration and invasion—antimetastatic activity. It shows the effect of tamoxifen and embelin on the migration of MCF-7 cells as assessed by in vitro wound healing assay. Wounds were induced by scratching and the effects of embelin and tamoxifen on cell migration after the desired incubation time was assessed by fixing the cells in formaldehyde and later staining with crystal violet. Embelin and tamoxifen almost completely clogged the migration of MCF-7 cells. *Bar* represents 10 μm

RNA (1 μg) was reverse transcribed with MulMV reverse transcriptase (Aristogene Biosciences Pvt Ltd., India). The obtained cDNA was used to determine the expression of XIAP, p53, Bcl 2, Caspase 3 and Caspase 9 using gene specific primers. Primer sequences for the genes analysed were listed in Table 1. β-Actin was used as the house keeping gene. The PCR products were analysed on 1 % agarose gels and densitometric analysis of the amplicons were carried out using the ImageJ software. The density values of the PCR products were normalized with the β-actin product levels. Each PCR reaction was repeated at least on three different occasions.

Statistical analysis

All the experiments were carried out in triplicate on at least three different occasions and the mean of the replicate values were taken. Values were expressed as mean ± SD. Statistical analysis of the data was determined by Student's *t* test and comparisons were made between the untreated control and treated groups.

Results

Pilot studies with embelin on MCF-7 cell line

Preliminary pilot studies (Fig. 1) were conducted with various concentrations of embelin ranging from 1 to 100 μg/ml. The concentration of embelin which showed maximum antiproliferative effect against the MCF-7 cell line was determined. Treatment of MCF-7 cells with 1–15 μg/ml of embelin showed no influence on the proliferation as compared to untreated control (data not shown), whereas concentration between 25 and 70 μg/ml showed statistically significant ($P < 0.001$) decrease in the proliferation of MCF-7cells. The concentration above 70 μg/ml that is 80–100 μg/ml (data not shown) showed no appreciable decrease in the proliferation of cells when compared to 70 μg/ml. Tamoxifen at a dose of 25 μg/ml was used as mentioned before.

Effect of tamoxifen and embelin on proliferation of MCF-7 cells

Results indicate that the cells treated with embelin showed marked decrease in proliferation, which was found to be statistically significant ($P < 0.001$) as compared to control (Figs. 2, 3). IC_{50} value of embelin was found to be 65 μg/ml whereas the IC_{50} value of tamoxifen was reported to be 25 μg/ml. Hence this particular concentration of embelin and tamoxifen which exhibited a marked influence on cell proliferation was selected for all subsequent proliferation and cytotoxicity studies. The IC_{50} values obtained for embelin were in agreement with the reported IC_{50} values of embelin on other carcinoma cell lines such as DLA (370 μg/ml) and K-562 (114.5 μg/ml) respectively [21].

Effect of tamoxifen and embelin on growth of MCF-7 cells

Effect of tamoxifen and embelin on the growth inhibition of MCF-7 cells was examined by trypan blue dye exclusion

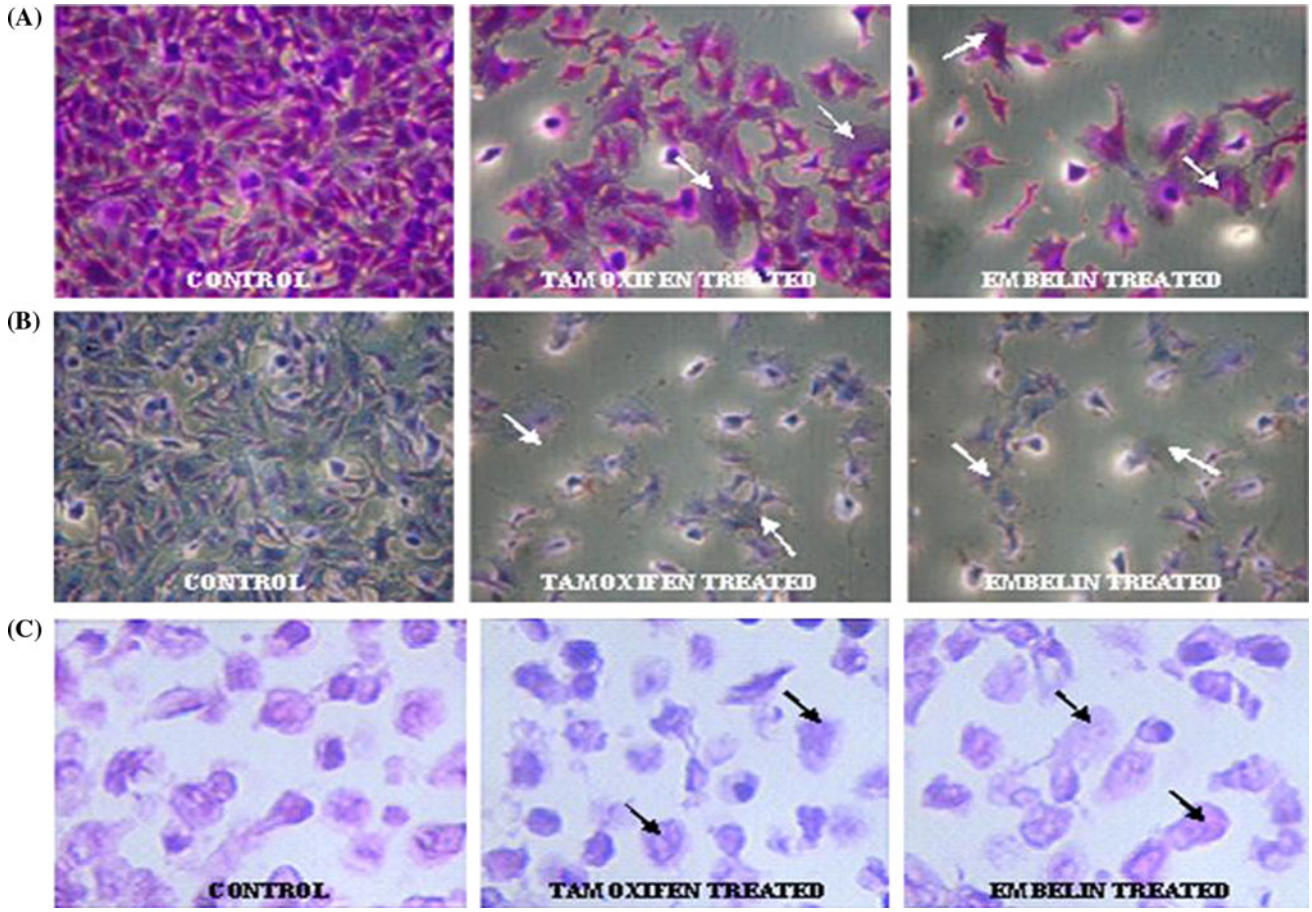

Fig. 7 Effect of tamoxifen and embelin cell morphology—light microscopy. It shows the effect of tamoxifen and embelin on the morphology of MCF-7 cells after suitable staining. **a** Crystal violet staining illustrate the appearance of apoptotic bodies, cytoplasmic vacuolization, cell shrinkage, chromatin condensation and appearance of micronuclei in embelin and tamoxifen treated cells and these were not evident in the control cells. **b** GIEMSA staining revealed cell shrinkage and nuclear fragmentations in embelin and tamoxifen treated cells whereas the changes were not observed in the control cells. **c** Hematoxylin–eosin staining revealed the appearance of heterochromatic nuclei and decreased mitotic activity in embelin and tamoxifen treated cells. Magnification: × 4× 10. *Bar* represents 40 μm

test. The results showed that the viability of MCF-7 cells was inhibited in a time dependent manner (Fig. 4). Cells treated with embelin and tamoxifen for 24 and 48 h showed a significant decrease in cell count as compared to untreated control cells.

Effect of tamoxifen and embelin on cloning efficiency and surviving fraction

Results revealed significant decrease in the number of colonies (Fig. 5) and decrease in the percentage of survival (Table 2) in the embelin treated group compared to that of untreated control. Control cells showed a cloning efficiency of 0.76 as compared to a cloning efficiency of 0.18 observed with the cells treated with embelin and 0.35 observed with the cells treated with tamoxifen.

Effect of tamoxifen and embelin on cell migration and invasion-antimetastatic activity

As shown in Fig. 6 tamoxifen and embelin treatment almost completely clogged the migration of MCF-7 cells. The inhibition of migration was found to be more evident in embelin treated cells, which demonstrated the antimetastatic potential of embelin.

Effect of tamoxifen and embelin on the morphology of MCF-7 cells

Crystal violet/Giemsa staining

The presence of apoptotic bodies, cytoplasmic vacuolization, cell shrinkage, formation of stretched nuclei,

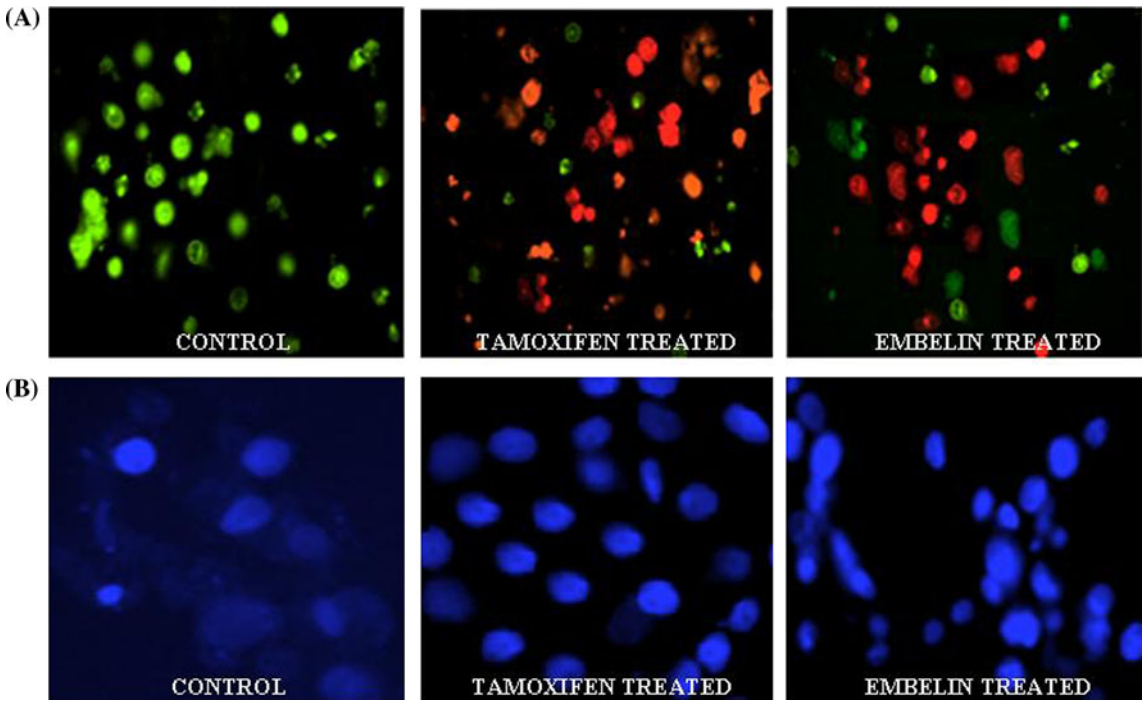

Fig. 8 Effect of tamoxifen and embelin on cell morphology—fluorescence microscopy. It shows the effect of tamoxifen and embelin on the morphology of MCF-7 cells as assessed by fluorescence microscopy. **a** AO/EB staining revealed that cells treated with tamoxifen and embelin showed a marked decrease in the number of live cells compared to the untreated control. **b** Hoechst 33324 staining revealed chromatin condensation and nuclear fragmentation in the tamoxifen and embelin treated cells which were not observed in untreated control. *Bar* represents 40 μm

Table 3 Percentage of apoptosis after AO/EB staining before and after treatment with tamoxifen and embelin

Sl. no.	Apoptosis	Control	Tamoxifen treated	Embelin treated
1.	EA	5 ± 0.57	12 ± 1.52***	17 ± 0.57***
2.	LA	7 ± 1.51	22 ± 2.51***	44 ± 2.51***
3.	Total apoptotic (TA)	13 ± 3.51	41 ± 2.51*	59 ± 5.77***

This table indicates the percentages of EA, LA and TA cells as assessed by AO/EB staining. EA cells were visualized as green areas with fragmented chromatin; LA dead cells were visualized as bright orange cells with condensed chromatin. The live cells were visualized as round and bright green cells. Data represent mean ± SD of six replicates. Student's *t* test; Intergroup comparisons were made between treated and untreated groups

*** $P < 0.001$; * $P < 0.05$

proapoptotic nuclei manifested as chromatin condensation at the periphery of the cell nucleus and appearance of micronuclei (apoptotic nuclei) were seen after staining the embelin and tamoxifen treated cells with crystal violet (Fig. 7a). Giemsa staining showed that the cells exhibited shrinkage and became smaller, the nuclei were broken, and cell debris with nuclear fragmentations varying in shape and size observed following embelin and tamoxifen

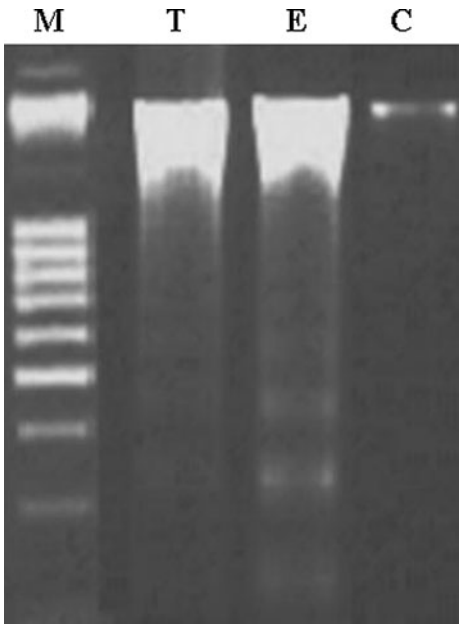

Fig. 9 Effect of tamoxifen and embelin on DNA fragmentation—agarose gel electrophoresis. It shows the qualitative assessment of the effect of tamoxifen and embelin on DNA fragmentation. A typical laddering pattern of DNA was observed in the treated cells which were not observed in the untreated control cells. (*M* marker, *C* control, *T* tamoxifen treated, *E* embelin treated)

Table 4 Quantitative estimation of fragmented DNA by diphenyl amine

Sl. no.	Groups	% of fragmented DNA
1.	Control	16.1
2.	Tamoxifen treated	52.6***
3.	Embelin treated	76.2***

Data represent mean ± SD of six replicates. Student's *t* test; inter-group comparisons were made between the treated and the untreated groups

*** $P < 0.001$

treatment (Fig. 7b). These features typical of apoptotic cell death were not observed in the untreated control cells.

Haematoxylin and eosin staining

Apoptotic hallmarks manifested as high levels of cytoplasmic vacuolization and cell shrinkage was observed after staining the cells with haematoxylin and eosin (Fig. 7c).The appearance of heterochromatic nuclei and decreased mitotic activity was observed in embelin and tamoxifen treated cells whereas they were not evident in the untreated control.

Effect of tamoxifen and embelin on chromatin integrity of MCF-7 cells

Hoechst 33324 staining revealed the appearance of fragmented apoptotic nuclei and chromatin condensation after treating with embelin and tamoxifen (Fig. 8a). Control cells displayed the nuclei with malignant morphology typical of MCF-7 cells while treated cells showed significantly reduced apoptotic nuclei.

Effect of tamoxifen and embelin on morphology of MCF-7 cells

Induction of apoptotic cell death was confirmed by morphological observation of the cells after AO/EB staining. As shown in Fig. 8b, the cells treated with embelin and tamoxifen showed a marked decrease in the number of live cells compared to the untreated control. Percentage of total apoptosis before and after treatment with embelin is represented in the Table 3. Compared with the spontaneous apoptosis observed in control MCF-7 cells [early apoptotic (EA) 5 %; late apoptotic (LA) 7 %], Cells treated with tamoxifen and embelin showed increased percentages of EA (12 and 17 %), and LA cells (22 and 44 %), with concomitant increase in the total number of apoptotic cells.

Effect of tamoxifen and embelin on DNA fragmentation in MCF-7 cells

Laddering pattern on agarose gel

In order to investigate whether morphological changes observed in the cells treated with tamoxifen and embelin were in tandem with chromatin degradation patterns, agarose gel electrophoresis of DNA extracted from cells

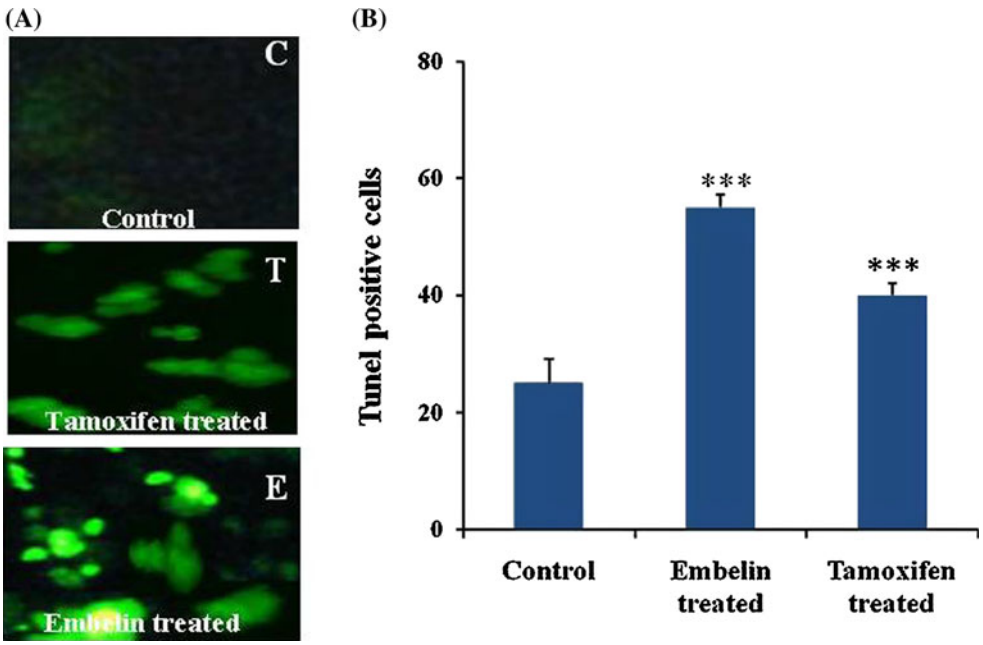

Fig. 10 Effect of tamoxifen and embelin on DNA damage—TUNEL assay. **a**—*C*, *T* and *E* indicate the control, tamoxifen treated and embelin treated MCF-7 cells. Fluorescent intensity being directly proportional to extent of DNA damage. A graph **b** showing the effect of embelin and tamoxifen on MCF 7 cell line. TUNEL positive cells were counted and data are expressed as mean ± SD. Apoptosis is significantly induced by embelin ($P < 0.001$)

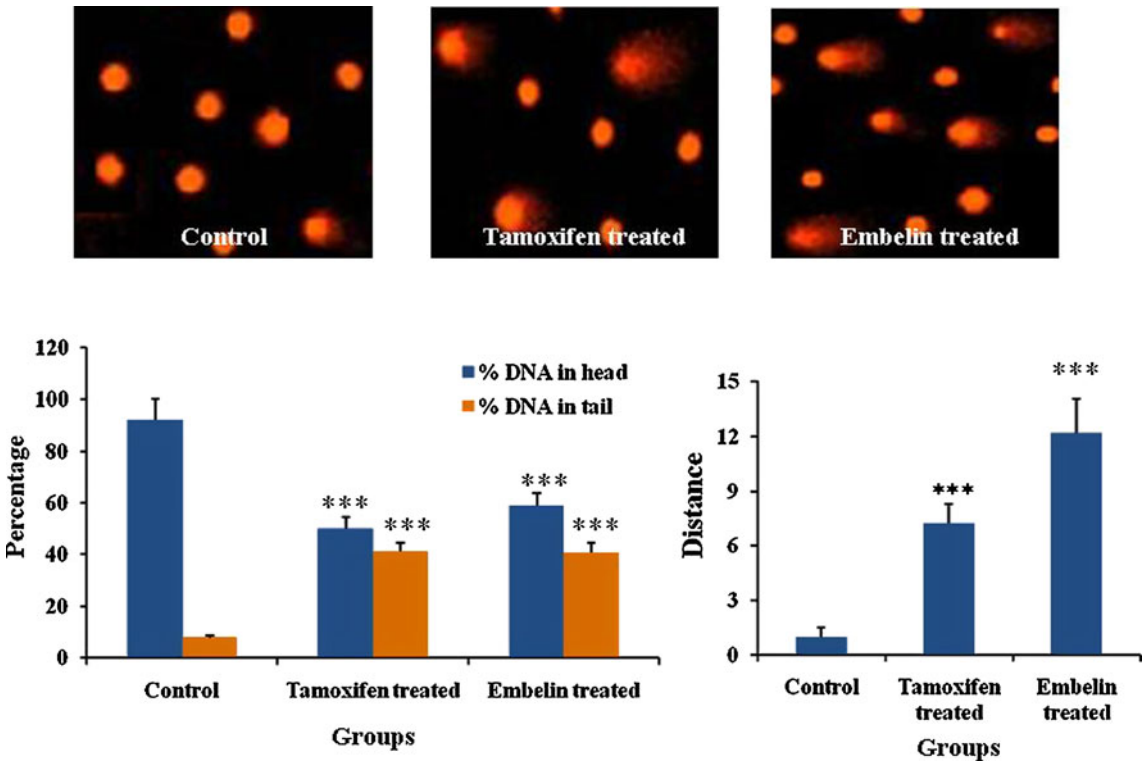

Fig. 11 Effect of tamoxifen and embelin on DNA damage—Comet assay. Appearance of Comet enabled the determination of major measurement parameters, including % DNA in head, % of DNA in the tail and Olive moment

treated with embelin were analyzed and a typical laddering pattern of DNA was observed (Fig. 9).

Quantitative estimation of fragmented DNA

Statistically significant increase in the percentage of fragmented DNA was observed on treatment with tamoxifen and embelin as compared to untreated control (Table 4).

Effect of tamoxifen and embelin on DNA damage—TUNEL assay

Figure 10a depicts the results of the TUNEL assay and subsequent fluorescence microscopic analysis of the control, embelin and tamoxifen treated MCF-7 cells. Figure 10b illustrates the number of TUNEL positive cells observed in the control, embelin treated and tamoxifen treated cells. Results indicate that embelin and tamoxifen treated cells showed increased fluorescence and statistically significant increase in the number of TUNEL positive cells ($P < 0.001$) as compared to the untreated control. This confirms the induction of DNA fragmentation and apoptotic cell death following embelin and tamoxifen treatment. More number of TUNEL positive cells were observed in the embelin treated cells as compared to the tamoxifen treated cells.

Effect of tamoxifen and embelin on DNA damage—Comet assay

The results of the Comet assay (Fig. 11) ascertained beyond doubt the induction of apoptotic cell death by embelin and tamoxifen. Results showed that embelin and tamoxifen treated cells exhibited DNA damage as evidenced by increase in the percentage of tail DNA, decrease in the percentage of head DNA and increased tail moment as compared to the untreated control which was found to be statistically significant ($P < 0.001$) .

Effect of tamoxifen and embelin on the expression of Caspase 3, Caspase 9, Bcl2, XIAP and p53

Decreased expression of XIAP, Bcl-2 and increased expression of p53, Caspase 3 and Caspase 9 was observed in the tamoxifen and embelin treated cells as compared to that of untreated control (Fig. 12a). Results of densitometric analysis and the difference in relative densities of the bands in different groups were illustrated in Fig. 12b.

Discussion

Cell proliferation is a culmination of a cascade of biochemical events which is mediated by several transcription

factors, cell signaling molecules and enzymes. The various assays performed to understand the effects of embelin on the cellular redox status, metabolic activity and clonogenic potential confirmed its cytotoxic and antiproliferative effects on human breast adenocarcinoma cell line MCF-7, which was found to be comparable to that of tamoxifen. In addition to this it was also observed that embelin and tamoxifen treatment clogged the migration of MCF-7 cells and exhibited marked inhibitory effects on cell migration. Cell migration is an important index of metastatic potential and a preliminary idea about the antimetastatic potential of test compounds could be revealed in vitro by the wound healing assay which involves complex and orderly sequence of events involving cell migration and proliferation [22]. In the current study, the antiinvasive potential as shown by the results of the scratch assay was found to be more evident in embelin treated cells compared to tamoxifen treated cells.

The fact that embelin exerted significant antiproliferative and antiinvasive effects, stimulated the interest to investigate whether the effect is mediated via apoptotic cascade, as induction of apoptosis is known to be a promising strategy for cancer prevention. A key regulator of apoptosis is the proapoptotic p53 tumour suppressor protein, which controls cell cycle progression and DNA repair [23]. p53 induces apoptosis by regulating the expression of proteins like Bcl2 and Bax. This concomitantly induces cytochrome C release and activates Caspase cascade (primarily Caspase 9 and Caspase 3) leading to apoptosis [24]. As any alteration in the expression of these proteins can be pivotal in the onset of oncogenesis, it was intended to study the modulation of XIAP, p53, Bcl2, Caspase 9 and Caspase 3 expressions under tamoxifen and embelin treatment on ER+ MCF-7 cell line. The results of RT-PCR analysis implicated that embelin induced an upregulation in the expression of p53, Caspases and down regulation in the expression of Bcl2, XIAP thereby inducing apoptotic cell death in MCF-7 cell line. This could be due to the strong inhibitory effects of embelin on the activation NF-KB [7, 25].

Interestingly, one of the mechanisms by which breast cancer patients develop resistance to tamoxifen therapy is through the activation of NF-KB. Hence, NF-KB inhibition coupled with potent antiestrogenic effects could enable embelin to antagonize E2 in an ER dependent manner to synergistically downregulate antiapoptotic genes and promote apoptotic cell death. Induction of apoptotic cell death upon embelin treatment was further confirmed by

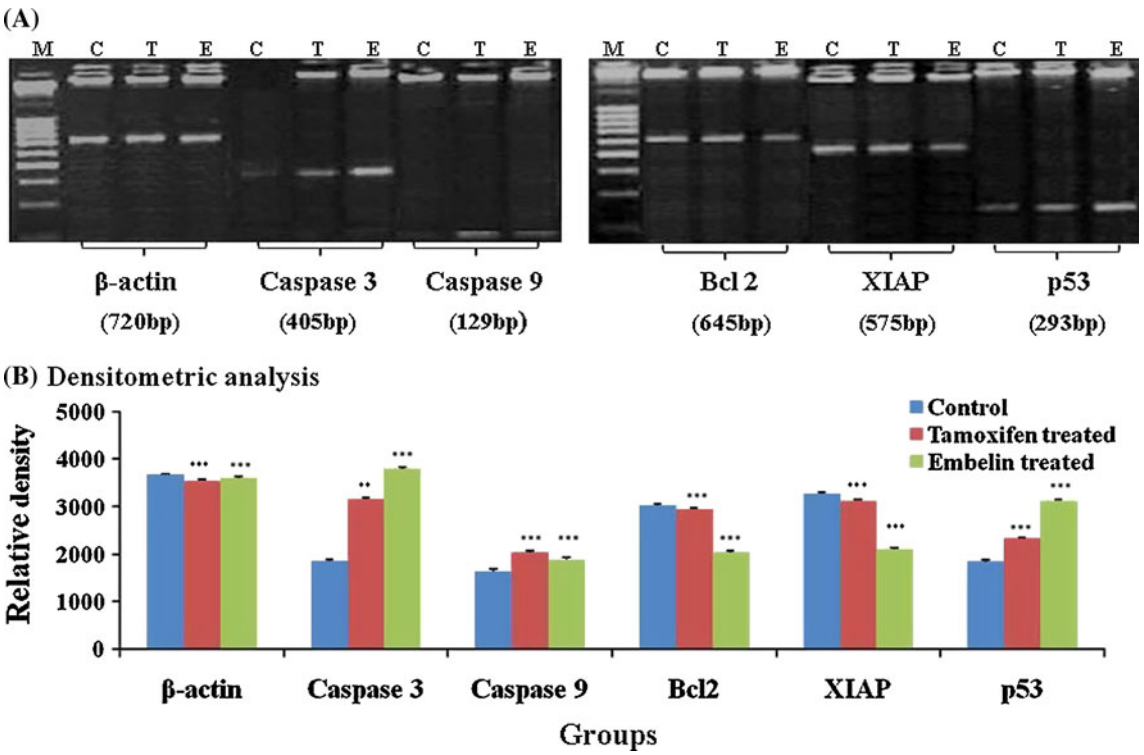

Fig. 12 Effect of tamoxifen and embelin on the expression of Caspase 3, Caspase 9, Bcl 2, XIAP, p53—reverse transcription PCR (*M* marker, *C* control, *T* tamoxifen treated, *E* embelin treated). Decreased expression of XIAP, Bcl-2 and increased expression of p53, Caspase-3 and Caspase-9 (**a**) was observed in tamoxifen and embelin treated cells as compared to that of untreated control. The relative densities as analysed from ImageJ software is as shown in **b**

morphological changes in the cell as observed by light microscopy and fluorescence microscopy analysis.

To conclude, we report for the first time the antiproliferative, antimetastatic and proapoptotic effects of embelin which is p53 mediated on ER+ breast cancer cell line MCF-7. With a sizable proportion of breast cancer patients becoming resistant to tamoxifen therapy, alternative therapeutic strategies to treat hormone responsive breast cancer by employing compounds like embelin which has a dual role as antiestrogen/XIAP inhibitor opens up interesting avenues for study.

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