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RESEARCH ARTICLE

ANTI-CANCER ACTIVITY OF (PHYLLANTHUS EMBLICA) PLANT EXTRACT INDUCED APOPTOSIS AND TARGETING SURVIVIN IN MCF-7 CARCINOMA CELLS

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ABSTRACT

Induce apoptosis in cancer cells has been a major study in the last decade. To study the effect of *Phyllanthus emblica* (*P. emblica*) has been investigated as an anti-cancer property. Nevertheless, their effect of growth inhibition of cancer cells and down regulation of survivin expression was studied in great detail. The MCF-7 cells were treated with various concentrations (0-100 µg/ml) of *P. emblica* extract. Apoptosis rate was measured, DNA damage was confirmed and the apoptotic proteins were evaluated by Western blot analysis. The *P. emblica* extract decreased the number of viable MCF-7 cells in a dose dependent manner with an IC_{50} of 54 µg/ml. Western blot analysis showed increased expression of pro-apoptotic protein caspase-3 in MCF-7 cells compared to untreated cells and decreased expression of the anti-apoptotic protein survivin. An induction of apoptosis was evidenced by (AO/EtBr). The *P. emblica* extract may have anti-cancer effects by inducing apoptosis signaling cascades *via* an upregulation of caspase-3 proteins and through the reduction of Survivin protein expression, which subsequently lead to increased DNA damage and apoptosis in breast cancer cells.

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INTRODUCTION

Programmed cell death (apoptosis) is an important process in the development of phytomedicine. This phenomenon occurs when the cells are exposed to certain toxic agents. Apoptosis is a crucial process for eliminating cancer cells. Therefore, induction of apoptosis is a key mechanism by which anticancer therapy works (Kundu *et al.,* 2005). In recent years, attention has been paid to potential anti-cancer components from traditional medicinal plants that can result in induction of cell apoptosis (Panichakul *et al.,* 2006). The plant genus *Phyllanthus* (*Euphorbiaceae*) is widely distributed in most tropical and subtropical countries. It is a very large genus consisting of approximately 550 to 750 species. Anti cancer effect of *P. emblica* in MCF-7 cells has never been reported earlier.

The development of new strategies for the treatment of cancer is immediate needful field. The mechanism of action of many anticancer compounds or drugs is based on their ability to promote apoptosis. There are many mechanisms by which apoptosis can be induced in cells (Sen and Incalci, 1992). Compounds suppressing the proliferation of malignant cells by inducing apoptosis may represent a useful mechanistic approach to both cancer chemoprevention and chemotherapy. However, the unfavorable side effects and the development of resistance to many currently used anticancer agents are serious problem (Khan and Mlungwana, 1999). Furthermore, the use of plant materials and extracts as alternative cancer therapies has attracted much recent attention due to the low toxicity and low cost of plant-derived materials (Hang *et al.,* 2005). Our earlier study reported that, Survivin was originally described as an anti-apoptotic protein (IAP), over expressed in a variety of human cancers and hence designated as a cancer-specific protein, rarely expressed in normal healthy adult tissues; however, up regulated in majority of cancers (Vivek *et al.,* 2011). It suppressed the apoptosis in cancer cells hence, this protein consider as therapeutic targeting of survivin in cancer therapy.

The present study, we concluded the following: MCF-7 cells are relatively sensitive to the cytotoxic effect of extract which induces apoptosis in MCF-7 cells by decreased the expression of anti-apoptotic survivin protein. Therefore, the present study was carried out to determine *P. emblica* extract is able to induce apoptosis in MCF-7 cells and to determine the mechanism responsible for its activity. Therefore, the target of extract inducing apoptosis in MCF-7 cells seems to be mediated by the inhibition in the expression of survivin protein.

MATERIALS AND METHODS

Materials

The leaves of *P. emblica* where collected from Coimbatore, Tamilnadu and identified by Botanical Survey of India (BSI, Coimbatore). 3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyl-terazolium bromide (MTT), dimethylsulfoxide (DMSO),

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purchased in Sigma chemical Pvt. Ltd were provided by Sigma Chemical Co. MEM medium, fetal bovine serum and penicillin/streptomycin were purchased from (Himedia, Coimbatore). All organic solvents and other chemicals were of analytical grade or complied with the standards needed for cell culture experiments, Survivin and Caspase-3 antibodies were purchased from Santa Cruz, CA.

Preparation of P. emblica extract

Plant material was pulverized using a milling machine and extracted with ethanol using a Soxhlet apparatus. The organic phase was evaporated under reduced pressure to obtain a residue. The residue was dried using a rotary evaporator to obtain the powder/paste. The required quantity of the dry powder/paste was dissolved in dimethyl sulfoxide (DMSO).

Cell culture

Breast cancer MCF-7 cells where purchased from the National Center for Cell Science (NCCS, Pune), cells were cultured in Modified Eagles Medium supplemented with 2 mM L-glutamine and Earle's BSS adjusted to contain 1.5 g/L Na bicarbonate, 0.1 mM nonessential amino acids, and 1.0 mM of sodium pyruate and maintained in carbon dioxide incubator at 37°C with 5% CO_2.These cells were plated at a concentration of $0.5 x 10^6$ and were grown in a 25 cm^2 flask in 5 ml of complete culture medium. Cell counts were made with a haemocytometer and viability of cells assessed by trypan blue exclusion.

Cell viability assay and LDH release assay

MTT assay was performed according to manufacturer's protocol. Cells seeded at a density of 0.5×10^6cells in 25cm^2 dish at 24 h are used and it is treated with ethanol extract of *P. emblica*. After 48 h, cells were incubated with MTT for 45 min at 37°C in dark. Formazan crystals were solubilized in dimethylsulfoxide and the absorbance was recorded at 560 nm. Untreated cells were used as control of viability (100%). The absorbance of each well was measured at 540 nm using an ELISA reader (BioRad, Model 680, USA), and the percentage of viability was calculated.

Cytotoxicity was determined by measuring the release of lactate dehydrogenase (LDH). The cells were pretreated with various concentrations (0, 25, 50 and 100 µg/ml) of *P. emblica* for 24 h, and the supernatant was used to assay LDH activity. A colorimetric assay was applied, according to which the amount of Formazan salt is proportional to LDH activity in the sample. The intensity of red color formed in the assay and measured at a wavelength of 540 nm was proportional to LDH activity and to the number of damaged cells. Data were normalized to the activity of LDH released from treated cells and expressed as a percentage of the control.

Morphological analysis

MCF-7 cells were seeded in 6-well plates (2.0×10^5cells/well) and incubated in a medium at 37°C under a 5% CO_2 for 24 h. The cells were pretreated with various concentrations of *P. emblica* extract. After incubation for 24 h, cellular morphology was observed using a phase-contrast microscope (Nikon, Japan). The photographs were taken at × 100 magnification.

Measurement of GSH and ROS

The measurement of GSH, cells was cultured in 75-cm^2 culture flask. After the treatment cell pellets from control and treated group were then lysed in cell lysis buffer [1×20 mM Tris-HCl (pH7.5), 150 mM NaCl, 1mM Na_2EDTA, 1%Triton, 2.5 mM sodium pyrophosphate]. Following centrifugation (15,000×g for 10 min at 4°C) the supernatant (cell extract) was maintained on ice until assayed for the cellular GSH. Briefly, a mixture of 0.1 ml of cell extract and 0.9 ml of 5% TCA was centrifuged (2300×g for 15 min at 4°C). Then 0.5 ml of supernatant added into 1.5 ml of 0.01% DTNB and the reaction was monitored at 412 nm. The amount of GSH was expressed in terms of mol/mg protein.

Levels of cellular oxidative stress were measured using the fluorescent probe, 2, 7-dichlorofluorescin diacetate (DCFH-DA). After treatment with these extract for 24 hours, cells were incubated for 30 minutes in the presence of 20 µM DCFH-DA followed by washing with phosphate-buffered saline. DCFH-DA is mainly trapped in the cytoplasm and is oxidized into highly fluorescent dichlorofluorescein (DCF) by intracellular ROS. The DCF fluorescence intensity of cells was measured in a fluorescence-activated cell sorting (FACS) Canto-II flow cytometer (BD Biosciences, San Jose, California).

(AO/EB) fluorescence staining

Characteristic apoptotic morphological changes were assessed by fluorescent microscopy using acridine orange/ethidium bromide (AO/EB) staining. Briefly, cells were seeded in 12-well plate at seeding densities of 1×10^5 cells and then treated with 75µg/ml of Pyrrolidine-1 for 24h. After washing once with phosphate buffered saline (PBS), the cells were washed with 100µl of 1:1 mixture of acridine orange/ethidium bromide (4µg/ml) solutions. The cells were immediately washed with DMEM, viewed, and photographed using Nikon inverted fluorescent microscope (TE-Eclipse 300). Viable cells stained only by AO were bright green with intact structure; apoptotic cells stained by AO_{LOW} and EB were red-orange with condensation of chromatin as dense orange areas and reduced cells. Data presented are representative of those obtained in at least three independent experiments done in triplicates.

Mitochondrial membrane potential (m)

Mitochondrial (m) was analyzed using JC-1, a lipophilic cationic fluorescence dye. JC-1 was added to each well and incubated for an additional 30 min at 37°C. After washing with PBS, the stained cells were assayed using a flow cytometer. For image analysis of mitochondrial m, the stained cells were mounted onto microscope slide in mounting medium. Microscopic images were collected using the confocal microscope Jc-1.

Measurement of apoptosis and cell staining

Apoptosis was investigated by staining the cells with Hoechst 33342 (Sigma). The cells were washed twice with phosphate-buffered saline (PBS) and then fixed in PBS containing 10% formaldehyde for 2 h at room temperature. The fixed cells were washed with PBS, and stained with Hoechst 33342 for 1 h at room temperature. The Hoechst-stained nuclei were excitation and emission wavelengths set at 358 and 461 nm, respectively, visualized using a fluorescence microscope.

DNA Fragmentation and Apoptosis Analysis

Cell death type was assessed by the detection of mono-oligo nucleosomes (histone-associated DNA fragments) using an ELISA kit (Cell Death Detection ELISA PLUS, Roche

Applied Science, IN, USA) following the manufacturer's instructions. In brief, the cytoplasmic lysates from untreated controls and extract treated cultures were transferred to a streptavidin-coated plate supplied by the manufacturer. A mixture of anti-histone biotin and anti DNA-POD were added to cell lysates and incubated for 2 h. The complex was conjugated and then the plate was read at a wavelength of 405 nm. The increase in mono-oligo nucleosomes production in cells lysates was calculated as the ratio of the absorbance of extract treated cells/absorbance of untreated control. Results were given as the mean ± SD of independent experiments.

Western blot analysis

Aliquots of samples with 70 µg of protein (determined by Bradford method) were fractionated in 12% SDS-PAGE and transferred to 0.45 µm nitrocellulose by wet transfer. The membranes were blocked with 5% fat free milk in PBS and then probed with primary antibody followed by secondary antibody. After washing 3 times in PBST, the membranes were visualized using enhanced chemiluminescence Western blotting detection system (Santa Cruz, CA).

Statistical analyses

Results are expressed as mean ± SD with the experiment repeated at least 3 times. Statistical evaluation was done using the Student's t-test. A p value of <0.05 was considered significant.

RESULTS

The growth inhibition property of *P. emblica* was evaluated in MCF-7 cells and showed dose depended cytotoxicity. Cells were treated with increasing concentration (0-100µg/ml) of *P. emblica* and cell viability was measured after 24 h. *P. emblica* treatment led to a significant decrease in cell viability and 50% cytotoxic concentration was observed at 54µg/ml. *P. emblica* treatments showed effect on MCF-7 cell proliferation and could significantly inhibit cell proliferation in a dose-dependent manner after 24 h (**Figure 1**).

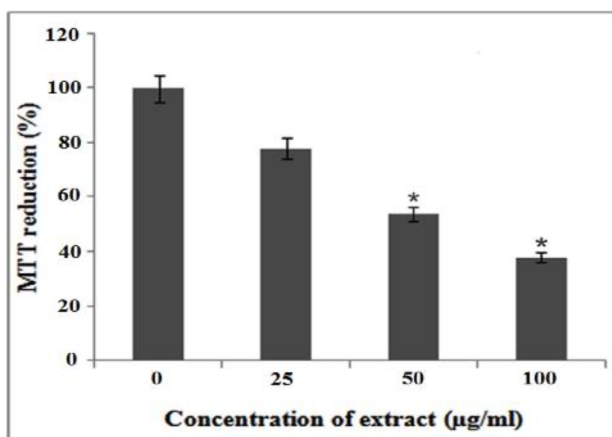

Figure 1 The effect of *P. emblica* extract on proliferation of MCF-7 cells treated with different concentrations (0, 25, 50 and 100µg/ml) of *P. emblica* extract. Data are represented as mean±SD.*Showed significant changes (p,0.05) after 24 hours of treatment with different concentrations of *P. emblica* extract using MTT assay with 50% reduction (IC_{50}) seen at 54µg/ml for MCF-7 cells.

Treatment for 24 h with 54 µg/ml of *P. emblica* extract led to a phenotypic conversion from cancer cells phenotype. Cells became more flattened in appearance, and showed an increase in cell–cell adhesion in comparison with untreated cells **(Figure 2a, b)**. After treatment with *P. emblica* for 24 h, the LDH release from MCF-7 cells were significantly

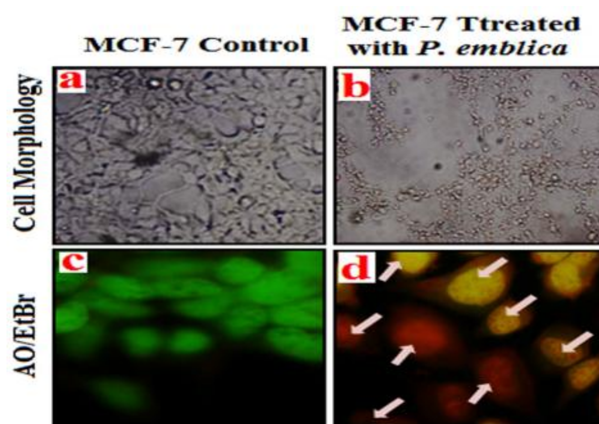

Figure 2: Bright field and fluorescence microscopy image of IC_{50} concentration of *P. emblica* treated on MCF-7 cells. Control cells of MCF-7 and cytotoxicity observed from bright field microscope (Top panel). Fluorescence microscopy study of AO/EtBr stained respective control cells appear in live cells in green color and orange colored apoptotic cells and necrotic cells appearing in red color indicated by arrows (Bottom penal).

increased as determined by the absorbance. A dose-dependent manner was observed. Compared with the control group, a more than 50% increase of absorbance at 100µg/ml *P. emblica* was observed **(Figure 3)**.

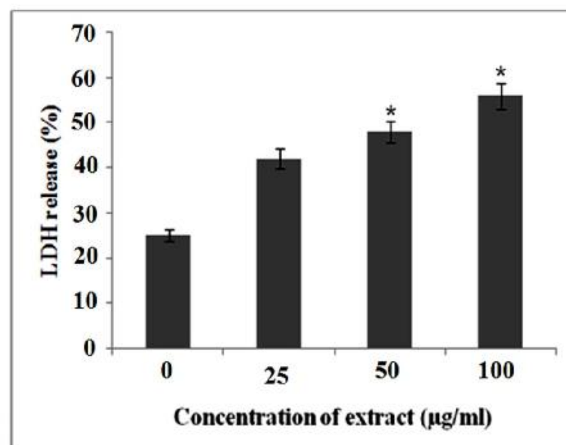

Figure 3 The LDH release in the culture medium is dramatically increased. Data were expressed as mean ± SD.*P < 0.05 vs. Control.

The augmented release of LDH into the media is reflective of cellular damage. Therefore, we conducted this experiment in order to estimate the release of LDH after treatment with various concentrations (0, 25, 50 and 100 µg/ml) of *P. emblica*. As expected, *P. emblica* caused cytotoxicity in a dose dependent manner increase in LDH-release, as compared to control cells.

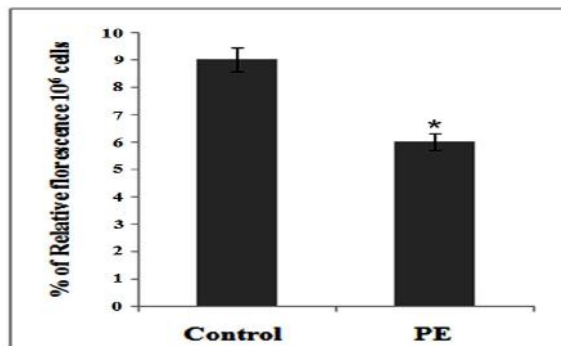

Figure 4(A) The GSH level was measured spectrofluorometrically. *significantly different from control cells (p < 0.05).

GSH is the major endogenous antioxidant scavenger that protects cells from oxidative stress through its ability to bind to and reduce ROS. Thus, preserving the GSH-mediated antioxidant defense is critical for cell survival. GSH is formed by -glutamate cysteine ligase (-GCL) and GSH synthetase (GSS). The protein expression of the catalytically active subunit of -GCL (GCLC) and GSS was decreased by *P. emblica* treatment (IC_{50}) when compared with control **(Figure 4A).** ROS is proposed as "redox messengers" in intracellular signaling at physiological levels while uncontrolled ROS production involved in apoptosis. Our results found that *P. emblica* extract induced the ROS formation shown in **(Figure 4B)**.

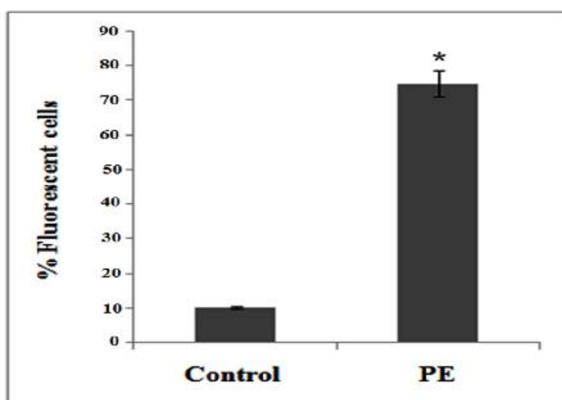

Figure 4B The level of ROS in *P. emblica* extract treated MCF-7 cells was measured. ROS level was quantified using DCF pre-treated cells after treatment with *P. emblica* extract.

Our findings demonstrated that the *P. emblica* induced apoptosis in MCF-7 cells. Treatment of MCF-7 cells with *P. emblica* "for 24h" resulted in cell shrinkage, chromosomal condensation, nuclear and plasma membrane convulsion and nuclear fragmentation indicating that the cytotoxic action of *P. emblica* was due to its ability to induce apoptosis by AO/EtBr dual staining **(Figure 2c, d).**

JC-1 staining showed results, in control group, there was intensive red fluorescence suggesting the high m. While after *P. emblica* (54µg/ml) treatment, the green fluorescence was greatly increased, suggesting the significant decreased of m. The green fluorescence was increased in 54µg/ml *P. emblica* treated cells **(Figure 5a, b)**. Furthermore, the JC-1 fluorescence ratio (red/green) was dramatically decreased after *P. emblica* "treatmen". To evaluate the cytotoxic effects of *P. emblica* in terms of apoptosis, the cellular nuclei were stained with Hoechst 33342 and assessed by microscopy in revealed that the control cells had intact nuclei, while the *P. emblica* treated cells showed blue emission causing highly condensed nuclear fragmentation, nuclei shrinkage which is indicative of apoptosis with apoptotic bodies formation (**Figure 5c, d**).

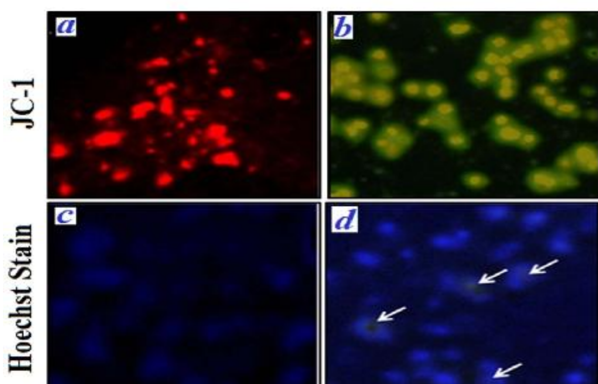

Figure 5 The m was analyzed with fluorescent microscopy after staining of MCF-7 cells was treated *P. emblica* with JC-1. Apoptotic cells were observed under a fluorescent microscope after Hoechst 33342 staining.

Apoptosis is characterized by the activation of endogenous endonucleases with subsequent cleavage of chromatin DNA into fragments **(Figure 6)**. After *P. emblica* treatment for 24 hr, DNA fragmentation events were clearly observed and cells suggesting the presence of apoptosis. To explore the potential mechanisms of *P. emblica*, the expression of several proteins related to apoptosis were determined in MCF-7 cells. Compared with the untreated cells, *P. emblica* significantly increased protein expression of caspase-3 and decreased survivin which was confirmed by the western blot analysis **(Figure 7)**; these results suggest that the apoptotic effects of *P. emblica* extract on MCF-7 cells are associated with an increase in caspase-3 activation.

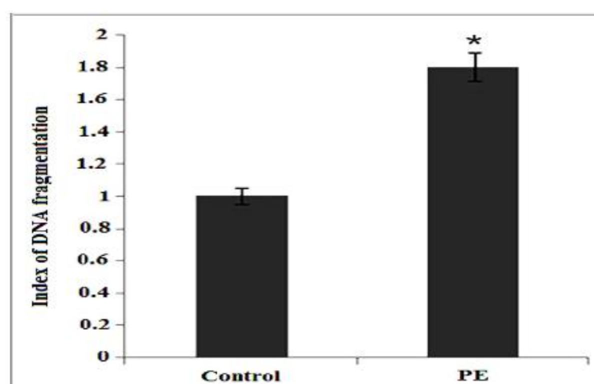

Figure 6 DNA fragmentation was quantified using an ELISA kit. The values are expressed as mean ± SEM. *significantly different from control cells ($p < 0.05$).

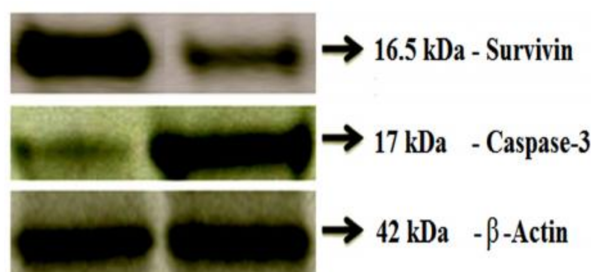

Figure 7: Effect of *P. emblica* extract on protein expressions. MCF-7 cells were treated with *P. emblica* for 24 h, apoptotic related protein expression were determined. Western Blot of Anti-apoptotic Protein Survivin and caspase-3 Expression in MCF-7 Cancer Cell Lines and - actin used as an Internal Control.

DISCUSSION

Survivin is essential for tumour cell proliferation as well as survival because of its regulation of cell cycle function. Hence, survivin is target for development of cancer therapy. Since survivin was known to regulate the cell cycle and appeared as an essential protein for the completion of Cytokines is our articles published in earlier (Vivek *et al.,* 2011). Results from our study demonstrated the down-regulation of survivin also a target for anticancer therapy.

Both the MTT and LDH assays confirmed that *P. emblica* extract significantly suppressed MCF-7 cells proliferation in a dose-dependent manner. The cytotoxic effect of *P. emblica* extract was tested *via* the lactate dehydrogenase

(LDH) release assay, based on the extent of LDH leakage into the medium. The augmented release of LDH into the media is reflective of cellular damage. Therefore, we conducted this experiment in order to estimate the release of LDH after treatment with various concentrations of *P. emblica* extract. As expected, *P. emblica* extract caused cytotoxicity in a dose dependent manner and increase in LDH-release, as compared to control cells. The *P. emblica* extract may induce apoptosis through the down-regulation of Survivin and through the up-regulation of Caspase-3 expression. Apoptosis occurs through the extrinsic (cytoplasmic) pathway and/or the intrinsic (mitochondrial) pathway (Guessous *et al.,* 2007). The survivin family is one of the most important classes of regulators involved in the inhibition of apoptosis.

Several natural compounds with anticancer effects can induce apoptosis of tumor cells (Shafi *et al.,* 2009). The *P. emblica* extract also induced apoptosis in MCF-7 cells. To understand the potential antitumor mechanisms of the *P. emblica* extract, the relative expression levels of the survivin, caspase-3 activity induced by extract. To understand the molecular mechanism of *P. emblica* induced growth inhibition, we found that intracellular activation of caspase-3. There was a marked increase in the activation of caspase-3 as evident from western blotting suggesting that caspase dependent apoptotic death could be another mechanism for the beneficial effects of *P. emblica*. There was an increase in activation of caspase-3 and it's well established that activation of caspase lead to degradation of cellular proteins, cell shrinkage, DNA fragmentation, loss of plasma membrane potential and membrane blebbing (Nicholson, 1999). The activation of caspase-3 induced chromosomal DNA break and finally the occurrence of apoptosis (Salvesen and Dixit, 1999). We found that survivin expression was decreased, whereas the expression of caspase-3 was increased.

Apoptotic signals are generally believed to be mediated through a hierarchy of caspase activation controlled by one of two distinct pathways that are associated with either mitochondrial caspase-8 or -9. The initiating caspases then converge on the central effector caspases, caspases-3 and -7 (Bursztajn *et al.,* 2000; Kuribayashi *et al.,* 2006). Although we did not find significant activation of down-stream caspase-8 there was, however, a significant increase of the active form of caspase-3 after 24 h of treatment with *P. emblica*, in a dose-dependent manner. These results demonstrated that the mitochondrial signaling pathway is involved in *P. emblica* induced apoptosis of MCF-7 cells.

In conclusion, the present study demonstrated that the *P. emblica* extract inhibited cancer cell proliferation *via* the induction of apoptosis. The *P. emblica* extract-induced MCF-7 cell death was shown to be due to apoptosis as demonstrated by the induction of caspase-3 activity and the observation of the cells containing fragmented nuclei and DNA. This apoptotic response is associated with the up-regulation of caspase-3 and the down-regulation of survivin. Therefore, we suggest that *P. emblica* extract is a promising molecule in cancer chemoprevention or chemotherapy. These results indicate that the *P. emblica* extract has anticancer activity *in vitro*. Further studies are needed to determine the molecular mechanisms of the active components and to evaluate the potential *in vivo* anticancer activity of the *P. emblica* extract.

"Conflict Of Interest Statement"

We declare that we have no conflict of interest.

Acknowledgements

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