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Apoptosis Induction of *Punica Granatum* Extract on Human Lung Cancer Cells

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ABSTRACT

The present study evaluated the ability of methanolic extract of *punica granatum* to induce apoptosis in lung cancer cell lines.A549 cells emerged as the most sensitive cell line for in-vitro growth inhibitory activity. A total of 50-60% confluent cells were treated with *Punica granatum* rind extract and their fraction for 48 hrs in complete growth medium. The ethyl acetate fraction induces uppermost cell death in the A549 cells which indicate the maximum growth inhibition. Ethyl acetate fraction of *punica granatum* extract induces apoptosis in A549 cells as indicated by nuclear condensation and increased annexin staining. It is possible that the use of *punica granatum* rind extract as a component in herbal medicines could be justifiable.

Keywords: Apoptosis, lung cancer, *punica granatum.*

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INTRODUCTION

Apoptosis is just one form of cell death. Cells may be eliminated by a number of alternative mechanisms including necrosis. In contrast, apoptosis is associated with the rapid engulfment and removal of cell corpses by phagocytic cells that recognize “eat-me” signals displayed on the outer surface of the apoptotic cell[1]. Understanding how apoptosis is regulated in cancer is therefore of major interest in the development of treatments for this disease. However, the role of the *Punica granatum* rind extract, in regulating the balance between cell proliferation and apoptosis via modulating the expression pattern of proliferation and apoptosis regulating genes in lung cancer cells, is not yet revealed. So the present investigation is targeted on the morphological changes in lung cancer induced by plant extract and the development of chemopreventive effect by the natural remedy on lung cancer. Hence it was planned to investigate on the apoptosis-inducing effects of methanolic extract of *Punica granatum* rind and the effect of other two fraction of *Punica granatum* rind i.e., alcohol fraction and ethyl acetate fraction on A549 cell line.

MATERIALS AND METHOD

Preparation of plant extract

Punica Granatum rind was collected from local market. It was authenticated by Dr.P.Jayaraman, Director of National Institute of Herbal Science, Plant Anatomy Research Centre, Chennai. A voucher specimen is maintained in plant anatomy research centre, Chennai (**PARC/2009/459)**. *Punica Granatum* rind were dried in shade and powdered mechanically. Then the powder is macerated with methanol and filtered. The residue settled was used for further analysis. Methanolic extract was subjected to column chromatography and fractionated. Further study was carried out with the crude methanolic extract and the two fractions obtained.

Maintaining and Storage of Cell Lines

A549 cells were procured from the National Centre for Cell Science, Pune, India. The cells were grown in monolayer culture in RPMI 1640 medium (sigma), supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin in a 5% CO_2 atmosphere at 37^0C.

Treatment with extracts

Punica granatum rind extract and their two fractions were dissolved in dimethyl sulfoxide (DMSO) (sigma, USA) and were used for the further treatment. A total of 50-60% confluent cells were treated with *Punica granatum* rind extract and their fraction for 48 hrs in complete growth medium.

Normal cell morphology of A549 cells

Normal Cell morphology was studied using haematoxylin – eosin stain[2].

Cell morphology assessment by inverted microscopy

For assessing morphological changes, confluent cells were treated with *Punica granatum* methanolic rind extract and their fractions. After treatment, photographs were taken using an inverted microscope.

Cell morphology assessment by acridine orange and ethidium bromide stain

The untreated and treated A549 Cells was washed with PBS, the cells were stained with 100µl of a mixture of acridine orange and ethidium bromide (1:1, 4µg/ml) solution. The cells were immediately washed once with PBS and viewed under a fluorescent microscope.

Nuclear stain by annexin and propidium iodide assay kit

The untreated and treated A549 Cells was washed with PBS, the cells was treated with 1x binding buffer, annexin-fluorescein isothiocyanate and propidium iodide as per the protocol described in the annexin V apoptosis detection kit from sigma. After 10-20 min, they are washed with PBS and the greenish apoptotic cells were viewed using a fluorescent microscope and photographed.

RESULTS AND DISCUSSION

Preliminary data showed that crude methanolic rind extract was predominantly effective among the other extract. So crude methanolic rind extract and two fractions was taken for further analysis. Normal A549 cell had flattened morphology in control medium as shown in figure 1a and A549 cells stained with haematoxylin – eosin stain was shown in figure 1b.

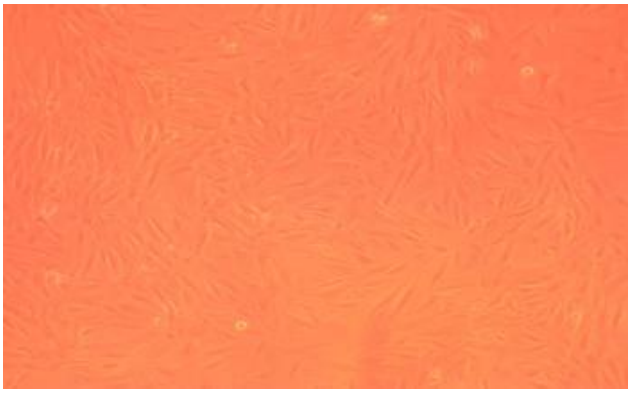

Figure 1a: Normal A549 cell morphology (with out stain)

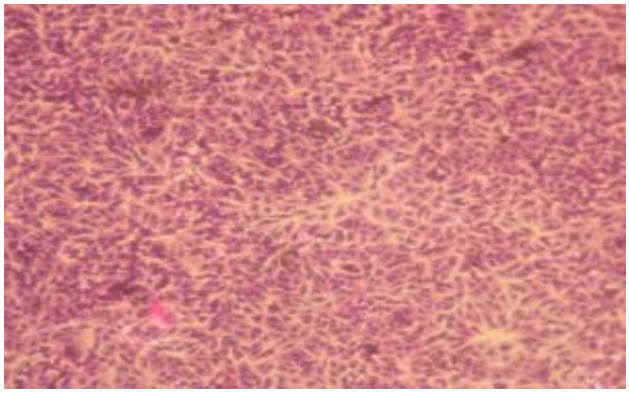

Figure 1b: Normal A549 cell morphology (Haematoxylin –eosin stain)

Apoptotic cell death - Morphological evidence

In order for a therapeutic agent to be truly effective, it should be toxic to tumor cells with out harming normal cells. In the present investigation of microscopic examination of normal A549 cell (Figure. 2a) appear healthy, high confluency of monolayer cells, translucent cytoplasm, cell shape

rules, long and slender polygonal, well-adherent, closely arranged with logarithmic growth. After 48 hrs treatment with methanolic rind extract no change in morphology was observed at low concentration (25µg/ml) but in higher concentration which is about >100 µg/ml changes in morphology occurs (Figure.2b) and on treatment with their two fractions, noticeable changes in the morphology and density of A549 cells were observed (Figure-2c, 2d).

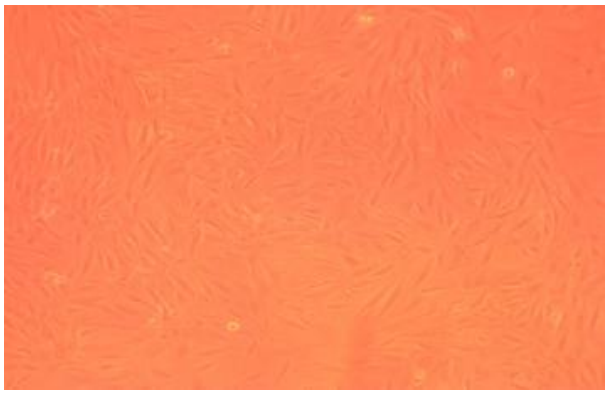

Figure 2a: Untreated A549 cell morphology

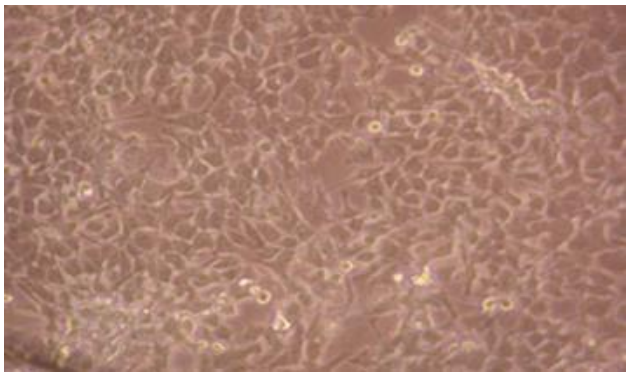

Figure 2b: Treated A549 cell morphology with crude methanolic extract

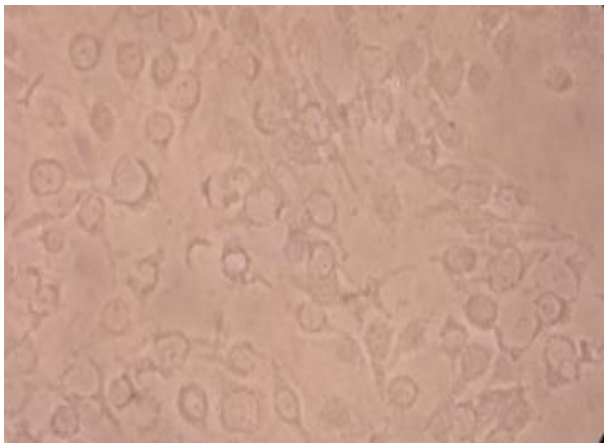

Figure 2c Treated A549 cell morphology with ethyl acetate fraction

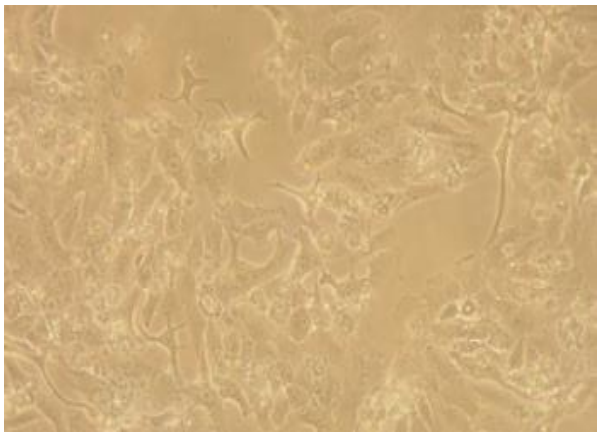

Figure 2d Treated A549 cell morphology with alcohol fraction

Morphological assessment of treated A549 with ethyl acetate fraction (25µg/ml) and alcohol fraction (50µg/ml) cells clearly indicated the play of apoptotic mechanisms leading to cell death. Morphological changes including cell shrinkage and loss of colony formation ability were observed. The treated cells appeared rounded off, shrunken and detached. 48 hrs after treatment extensive blebbing, presence of apoptotic bodies, dense cytoplasm, chromatin aggregates could also be seen in these cells. A cell undergoing apoptosis shows a characteristic morphology such as cell shrinkage and rounding because of the breakdown of the proteinaceous cytoskeleton by caspases, the cytoplasm appears dense, the organelles appear tightly packed, Chromatin undergoes condensation into compact patches against the nuclear envelope (also known as the perinuclear envelope) in a process known as pyknosis, all these morphological changes occurs in the cell

which is undergoing apoptosis[3]. The nuclear membrane gets destructed and the DNA inside it is fragmented in a process referred to as karyorrhexis. As a result the nucleus breaks into several discrete chromatin bodies or nucleosomal units due to the fragmentation of DNA[4]. The cell membrane shows irregular buds known as blebs and the cell breaks apart into several vesicles called apoptotic bodies, which are then phagocytosed. Based on the observation obtained in the present investigation it confirms that cell death caused by ethyl acetate fraction may be due to a programmed cell death – Apoptosis.

Morphological observation by acridine orange and ethidium bromide (AO/EB) staining

Staining the cells with fluorescent dyes, including acridine orange and ethidium bromide was used in evaluating the nuclear morphology of apoptotic cells. It could be noticed that apoptosis has been induced by *P. granatum* methanolic rind extract and their fractions on A549 cells. This was analysed by the presence of acridine orange and ethidium bromide staining (AO/EB staining). In the dual stain, the acridine orange is a vital dye that will stain both live cells and dead cells, whereas ethidium bromide will stain only those dead cells that have lost their membrane integrity and binds to the nucleus of the cell[5]. Control cells showed bright nucleus with uniform intensity and had not taken up ethidium bromide and it appears green in colour (figure 3a) where as the apoptotic cells appeared orange in colour. In the study upon treatment with crude methanolic extract, the cells appear green because of less cytotoxicity over lung cancer cell line (figure 3b) but in case of ethyl acetate and alcohol fraction it appears orange in colour due to presence of apoptotic cells which takes up the ethidium bromide stain (figure 3c and 3d). Consequently, using the AO/EB staining procedure, the morphological features of an A549 cells in apoptosis were dose dependent, i.e., a stronger apoptosis signal was induced with higher concentrations of the respective extract. Apoptosis is the process of physiologically programmed cell death in which intrinsic pathway participates in the cell death[6]. The mechanism of apoptosis seems to depend on the stimuli (intrinsic and/or environmental such as drugs, cytotoxins, irradiation, infectious agents, etc). The known molecular mechanisms of apoptosis include activation of cysteine proteases as ICE (IL-l beta converting enzyme); calpain; Fas signaling; cell cycle interfaces; stress responses; Bcl-2 family; and the tumor suppressor gene p53[7]. The human lymphocytes is treated with dxm which induced apoptosis in uninfected human lymphocytes through nuclear condensation, fragmentation and apoptotic body formation[8], the report is identical to the present observations in the A549 cells; this therefore illustrates a similar mechanism of apoptosis induction by dxm and *Punica granatum* rind. The interpretation in the present study could therefore be of clinical and therapeutic significance, as well as pathophysiological research interest.

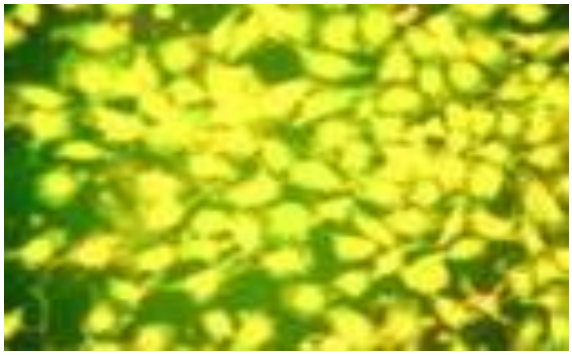

Figure 3a Untreated A549 cell morphology.

Figure 3b A549 cell morphology treated with crude methanolic extract.

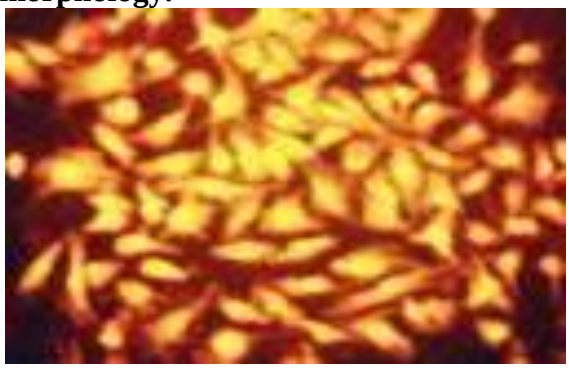

Figure 3c A549 cell morphology treated with ethyl acetate fraction.

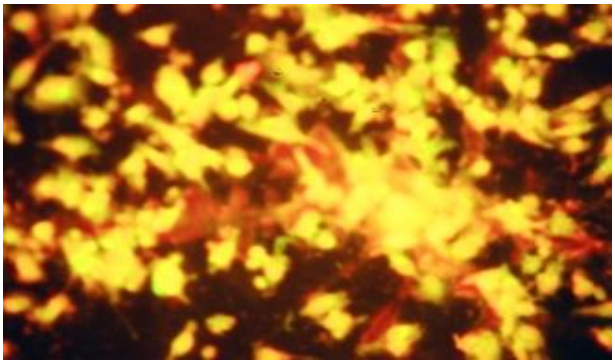

Figure 3d A549 cell morphology treated with alcohol fraction.

Annexin V FITC apoptotic cell staining

The Annexin V-FITC apoptosis detection kit was then employed to examine the influence of ethyl acetate fraction on the death of A549 cells. In the early stages of apoptosis, the cell membrane can expose phosphatidylserine which is annexin V-positive. Phosphatidylserine externalization not only takes place in apoptosis, but also occurs during cell necrosis. The difference between these two forms of cell death is that during the early stages of apoptosis, the cell membrane remains intact, whereas at the very moment that necrosis occurs; the cell membrane looses its integrity and becomes permeable. Therefore, the measurement of annexin V binding to the cell surface which is an indicator for apoptosis has to be performed in conjunction with a dye exclusion test to establish integrity of the cell membrane[9]. In combination with the membrane impermeable DNA stain PI, with a flow cytometer, one can distinguish at least three different cell types during apoptosis: viable cells (annexin V- and PInegative), early apoptotic cells (annexin V-positive but PI-negative), and necrotic or late apoptotic cells (annexin V- and PI-positive) [10]. In the present investigation observation was made on the binding of Annexin FITC to the phosphatidyl serine which is exposed in apoptotic cell which is indicated by positive green stain (figure 4a and 4b). The result confirms that the ethyl acetate fraction induces cell death by apoptotic process but not by necrotic pathway.

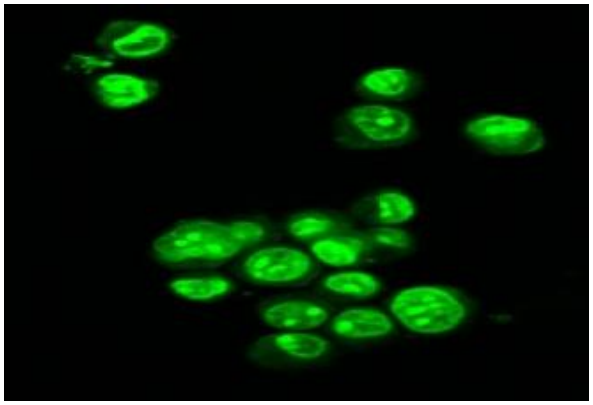

Figure 4a Treated A549 cell morphology with ethyl acetate fraction staining by annexin and PI kit.

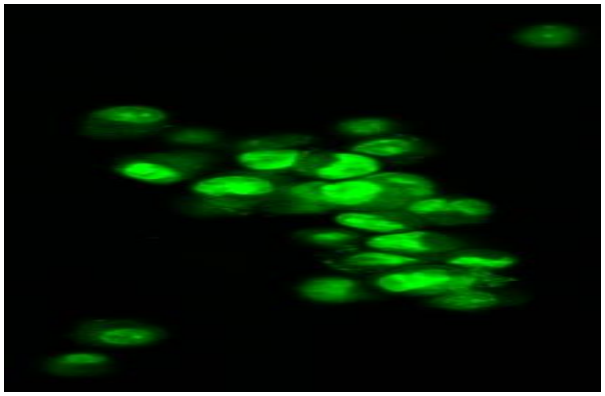

Figure 4b Treated A549 cell morphology with alcohol fraction - staining by annexin and PI kit.

CONCLUSION

The *punica granatum* extract induces apoptosis in A549 cells by induction of nuclear condenation, fragmentation, apoptotic body formation, flip flop movement and increased annexin staining.

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