

ANTICANCER ACTIVITY OF *PUNICA GRANATUM* RIND EXTRACTS AGAINST HUMAN LUNG CANCER CELL LINE

SANGEETHA JAYAKUMAR, SUMATHY HARIDASS, VIJAYALAKSHMI KRISHNAMURTHY*

Department of Biochemistry, Bharathi Women's College, North Chennai, Tamil Nadu, Chennai. Email: sangeetha_joe@rediffmail.com

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ABSTRACT

Natural products isolated as biologically active compounds with great therapeutic potential providing the molecular basis for most of drugs are currently in use, especially for cancer and infectious diseases. *Punica granatum* rind (PGR) extracts contains significant amount of polyphenolic compounds and exhibited powerful antioxidant potential *in vitro* system. The present study was undertaken to confirm the anticancer potential of PGR extracts on A549 cell line and to elucidate the mechanism of its action. PGR extract demonstrated a dose dependent inhibitory effect on cell growth (IC_{50} -80 μ g/ml), and it significantly inhibited the growth of A549 cells, FITC supported this result by showing increased cell death ethyl acetate treated cells. The results showed that ethyl acetate extract of PGR was found to be most potent when comparing to its counter parts.

Keywords: *Punica granatum*, cytotoxicity, MTT assay, DNA fragmentation, FITC.

INTRODUCTION

Lung cancer is the leading cause of cancer mortality in most countries^[1] and non-small cell lung carcinoma accounts for 70-80%. Tobacco smoking is the major risk factor for lung cancer^[2]. Benzo[a]pyrene (B(a)P), one of the important tobacco related carcinogen is significant for the formation of DNA adducts and ultimately cancer^[3]. Surgical resection and/or radiation ablation or systemic chemotherapy is the main lines of treatment for most cancers, but in case of lung cancer post-treatment recurrence is quite frequent. Although the cessation of smoking is important for lung cancer prevention, ex-smokers are still at a risk for developing lung cancer. Apoptosis, or programmed cell death (PCD), is a cellular homeostasis of multicellular organism. Cells die in response to a variety of stimuli and during apoptosis they do so in a controlled, regulated fashion. This makes apoptosis distinct from necrosis, which results in uncontrolled cell death ultimately leading to cells lysis, inflammatory responses and potentially, to serious health problems. Apoptosis, by contrast, is a process in which cells play an active role in their own death. Understanding how apoptosis is regulated in cancer is of major interest in the field of drug discovery for the management of cancer. Use of potential natural products as anticancer agents was recognized in the 1950s by the U.S. National Cancer Institute (NCI) and has since made major contributions to the discovery of naturally occurring anticancer agents^[4]. An alternative and novel approach for the management of lung cancer is chemoprevention through the recommended intake of health protective food especially those present in vegetables, fruits, beverages and spices in daily diet. *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 was targeted on the regulation of apoptotic pathway in lung cancer and the development of chemopreventive effect by the natural remedy on lung cancer.

MATERIALS AND METHODS

Chemicals

RPMI, MTT, DMSO, Annexin V and propidium iodide assay kit was purchased from Sigma, USA. All other reagents were of the analytical grade.

Preparation of plant extract

Punica Granatum was collected from local market and the rind was separated. They were dried in shade and powdered mechanically. Then the powder was macerated with methanol and filtered. The residue settled was used for further analysis. Methanolic extract (ME) was subjected to column chromatography and fractionated and stored in desiccator. Further study was carried out with the crude methanolic extract and the two fractions obtained.

Cell Line maintenance

A549 cells were procured from the National Centre for Cell Science, Pune, India. The cells were grown in monolayer culture in RPMI 1640 medium, supplemented with 10% fetal bovine serum, 1% penicillin/streptomycin in a 5% CO₂ atmosphere at 37°C.

Cell viability assay

Cell viability was assessed by MTT (3, 4, 5 dimethyl thiazol-2-yl)-2,5 diphenyl tetrazolium bromide) assay which is based on the reduction of MTT by the mitochondrial dehydrogenase of intact cells to a purple formazan product^[5]. 1×10^4 cells were seeded in a 96 well plate and incubated for 48 hours, cells were then treated with crude methanolic *punica granatum* rind extract and their two fractions with different concentration ranging from 12.5 to 100 μ g/ml for 48 hours. Then, MTT 50 μ g/ml was added in each well and cells were incubated for 4 hours. Supernatant in each well was removed carefully and 100 μ l of DMSO was added. Amount of formazan was determined by measuring the absorbance at 595nm using an ELISA plate reader (EL808 ultra micro plate reader biotek laboratories, USA). Data were calculated as percentage viability using the following formula.

$$\text{Cell death (\%)} = (\text{control OD} - \text{sample OD}) / \text{Control OD} \times 100$$

$$\text{Cell viability (\%)} = 100 - (\% \text{ cell death})$$

A graph was plotted against the % cell viability Vs dilution of the extract. The minimum concentration of extract that was toxic to lung cancer cells was recorded as the effective drug concentration.

DNA Fragmentation analysis

1×10^6 cells were incubated with 100 μ l of cell lysis buffer at room temperature for one hour and centrifuged for 15 min at 3000rpm at 4°C to sediment the cell debris. To the supernatant equal volume of phenol: chloroform: isoamylalcohol mixture was added and mixed well and centrifuged at 5000 rpm for 15min. Supernatant was transferred to new tube and the 3rd step was repeated again. To the final aqueous phase 40 μ l of 3.5M ammonium acetate was added, to this ice cold isopropanol was added to precipitate the DNA. This was incubated at -20°C for 1hour, followed by the centrifugation at 10000 rpm for 15min. Pellet was retained and washed with 70% ethanol and stored in 20-50 μ l of TE buffer. The samples were analyzed in 2% agarose gel stained with Ethidium bromide. Electrophoresis of DNA fragments at 50volts. Visualization of DNA fragments in the UV trans-illuminator.

Flow cytometry

Annexin V - FITC Apoptosis Detection Kit detects apoptotic cells by flow cytometry. Annexins are a group of homologous proteins which bind phospholipids in the presence of calcium^[6, 7]. Annexin V - FITC

is a fluorescent probe which binds to phosphatidylserine in the presence of calcium. Apoptosis or PCD, is a mechanism of cells used to negatively select cells that are deleterious to the host. The cellular changes involved in the process include loss of phospholipids asymmetry during the early stages of apoptosis. In living cells, phosphatidylserine is transported to the inside of the lipid bilayer by the Mg-ATP dependent enzyme, aminophospholipid translocase^[6]. At the onset of apoptosis, phosphatidylserine, which is normally found on the internal part of the plasma membrane, becomes translocated to the external portion of the membrane. The phosphatidyl serine becomes available to bind to the annexin V-FITC conjugate in the presence of calcium which is beginning of apoptotic process, and the binding of propidium iodide to the cellular DNA in cells where the cell membrane has been totally compromised. After 10 minute incubation period at room temperature the cells are analyzed by flow cytometry. Annexin V-FITC is detected as a green fluorescence and propidium iodide is detected as a red fluorescence.

1×10^6 cells / ml suspension of A549 cells were treated by the addition of $25 \mu\text{g/ml}$ of crude methanolic extract and two fractions namely ethyl acetate fraction and alcohol fraction. A control of non induced A549 cells was established at 1×10^6 cells / ml for a zero time data point. The cells treated with extracts were also maintained. Both A549 cell cultures were incubated for 1- 2 hours in a 37°C , at 5% CO_2 incubator. The cells were washed with PBS twice. The cells were resuspended in a 1x binding buffer at a concentration of $\sim 1 \times 10^6$ cells /ml. $500 \mu\text{l}$ of the cell suspension treated with extracts was added to a plastic $12 \times 75 \text{mm}$ test tube. $500 \mu\text{l}$ of the non-apoptotic cell suspension was added to a second plastic $12 \times 75 \text{mm}$ test tube. Then $5 \mu\text{l}$ of annexin V-FITC conjugate and $10 \mu\text{l}$ of propidium iodide solution was added to each cell suspension. The tubes were incubated at room temperature for exactly 10 minutes and protect them from light. The fluorescence of the cells were immediately determined with a flow cytometry. Cells which are early in the apoptotic process will stain with Annexin V-FITC conjugate alone. Necrotic cells will be stained by both the propidium iodide solution and Annexin V-FITC conjugate.

RESULT

Growth inhibitory effects of crude methanolic extract and two fractions of *Punica granatum* rind

Crude methanolic extract and their fractions inhibited the proliferation of human lung cancer cell line A549 in a concentration dependent manner as shown in figure 1. LD_{50} value of crude methanolic extract for A549 cells was also calculated and was found to be $> 100 \mu\text{g}$. The LD_{50} value of ethyl acetate and alcohol fraction obtained from crude extract for A549 cells was found to be $25 \mu\text{g}$ and $50 \mu\text{g}$ respectively. The highest concentration of the ethyl acetate fraction ($100 \mu\text{g}$) inhibited A549 cell growth to maximum level and exhibited higher cytotoxicity which may lead to programmed cell death.

DNA fragmentation analysis

DNA fragmentation which is a typical hallmark of the apoptotic cell death was analysed in the study (figure 2a and 2b). The alcohol and ethyl acetate fraction induces apoptosis in A549 cells resulting in the degradation of chromosomal DNA in to small oligonucleosomal fragments which results in fragmentation of DNA (Lane 2, 3, 4 and 5). But in case of crude methanolic extract there was no fragmentation of DNA (Lane 2, 3 and 4) seen but in the higher concentration which is around $150 \mu\text{g}$ we observed DNA fragmentation (Lane 5). In Case of untreated cells we observed the intact chromosomal DNA (Lane 6). These interpretations confirm cell death caused alcohol and by ethyl acetate fraction obtained from the crude methanolic extract through apoptosis.

Labelling with Annexin V FITC

Translocation of phosphatidyl serine (PS) from the inner face of plasma membrane to the outer cell surface occurs during the early stages of apoptosis. Apoptosis induced by CE, EAF and AF was quantified using Annexin V FITC kit (figure 3b, 3c and 3d) and the cells were scored by flow cytometry. Untreated cells labeling with Annexin V FITC was shown in figure 3a. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS. Annexin V FITC binds to the cell surface expressed PS of the apoptotic cells and not the necrotic cells. Therefore A549 cell line used in this study stain positive for annexin V binding after treatment with either ethyl acetate or alcohol fraction. But in case of ethyl acetate treatment the A549 cells exhibit higher percentage of apoptosis leading to cell death.

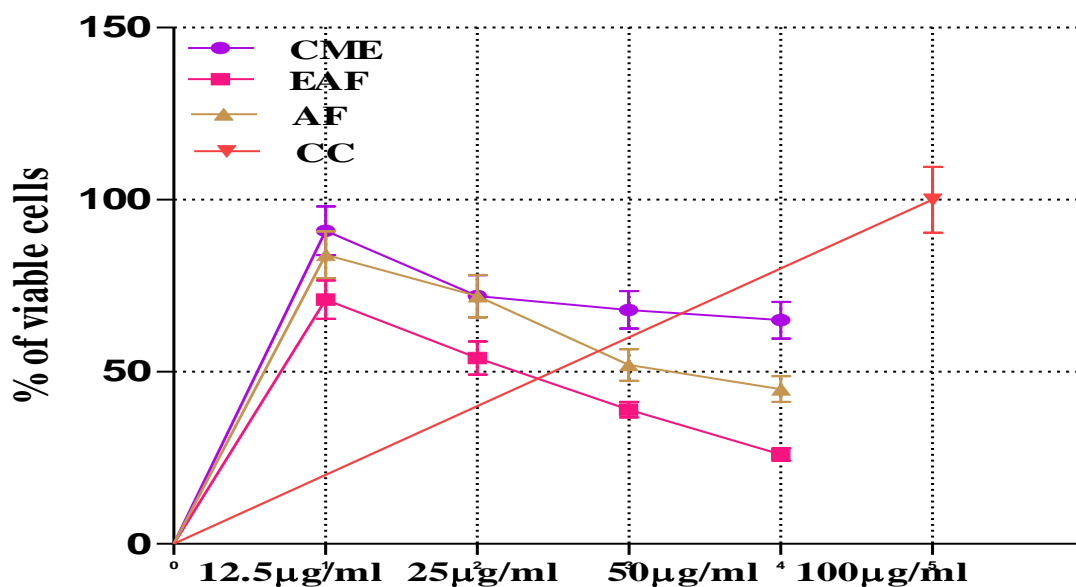


Figure 1: MTT Assay

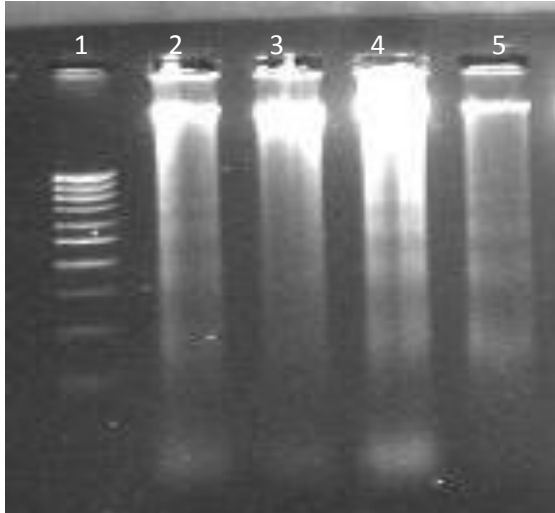


Figure 2a shows DNA fragmentation analysis using agarose gel electrophoresis.

- Lane 1: 1kb DNA ladder
- Lane 2: DNA fragmentation of A549 cells line treated with 50 µg of alcohol fraction
- Lane 3: DNA fragmentation of A549 cells line treated with 25 µg of alcohol fraction
- Lane 4: DNA fragmentation of A549 cells line treated with 50 µg of ethyl acetate fraction
- Lane 5: DNA fragmentation of A549 cells line treated with 25 µg of ethyl acetate fraction

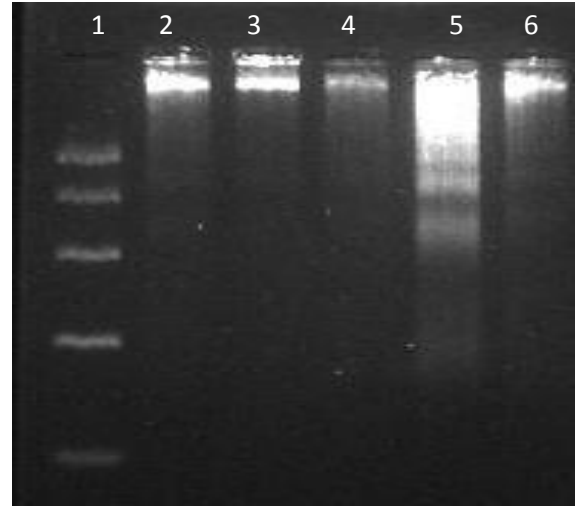


Figure 2b shows DNA fragmentation analysis using agarose gel electrophoresis for untreated and crude extract treated cells

- Lane 1: 1kb DNA ladder
- Lane 2: A549 cells line treated with 25 µg of crude methanolic extract
- Lane 3: A549 cells line treated with 50µg of crude methanolic extract
- Lane 4: A549 cells line treated with 100 µg of crude methanolic extract
- Lane 5: A549 cells line treated with 150 µg of crude methanolic extract. DNA fragmentation was observed.
- Lane 6: Untreated A549 cells lines with intact genome.

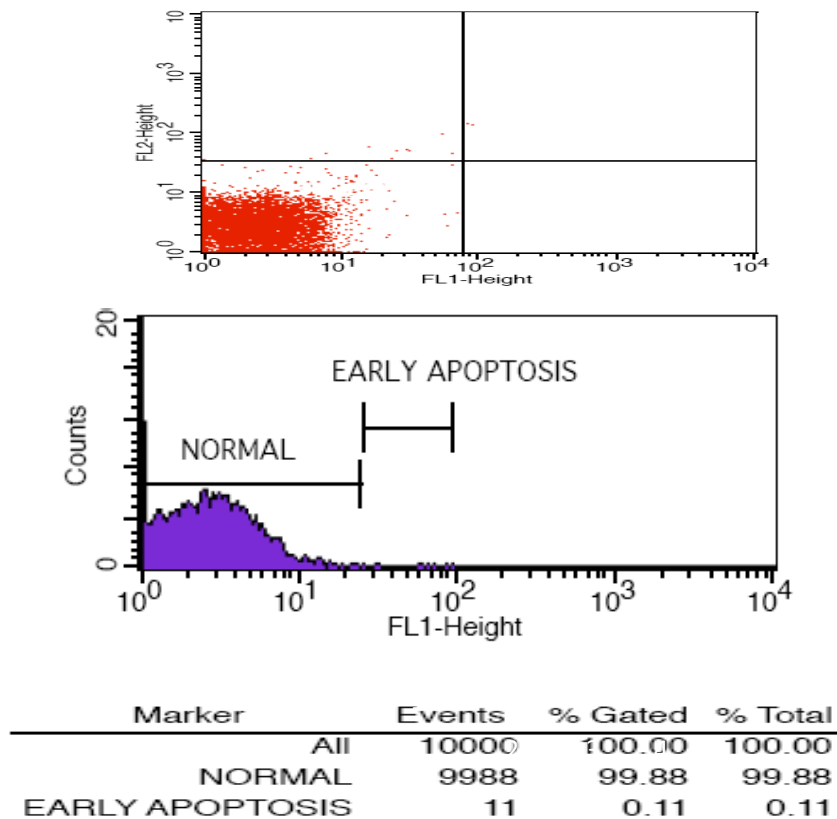


Figure 3a: Flow cytometry analysis for untreated cells with annexin V and PI

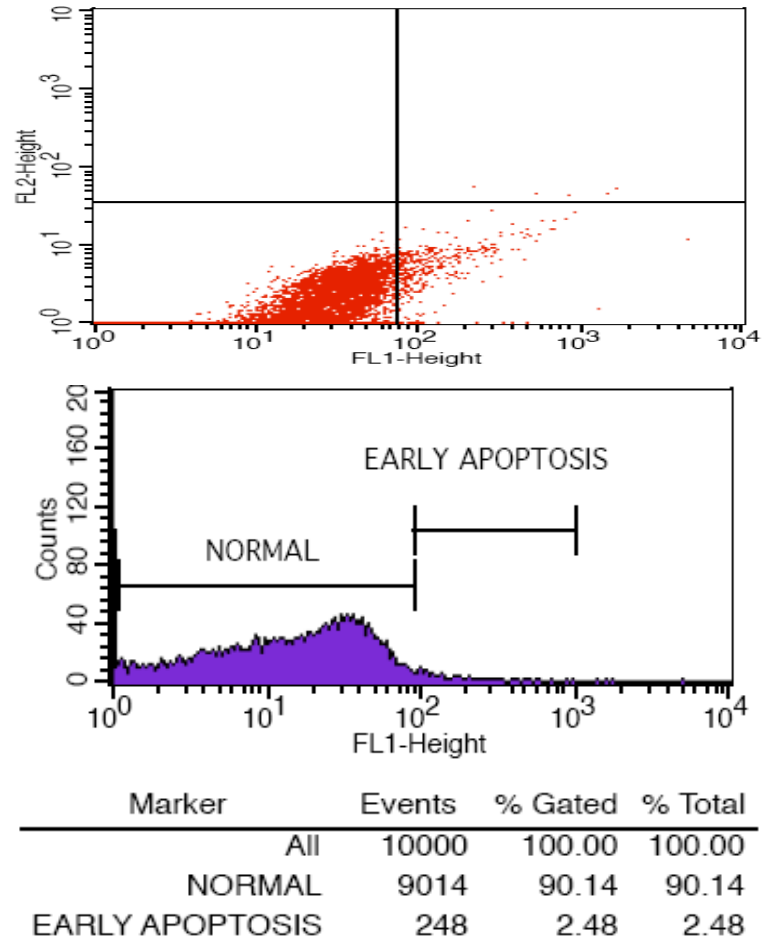
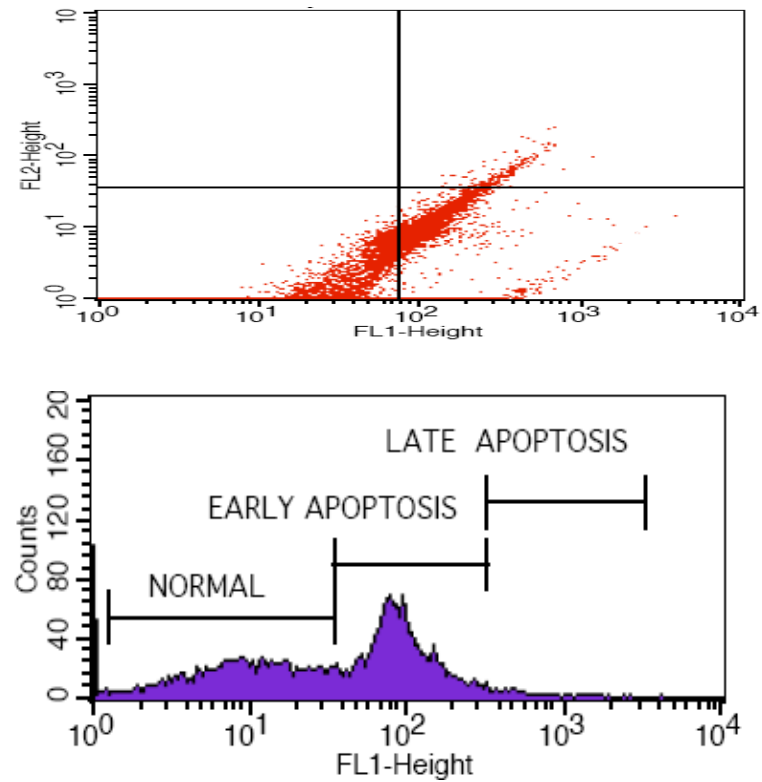
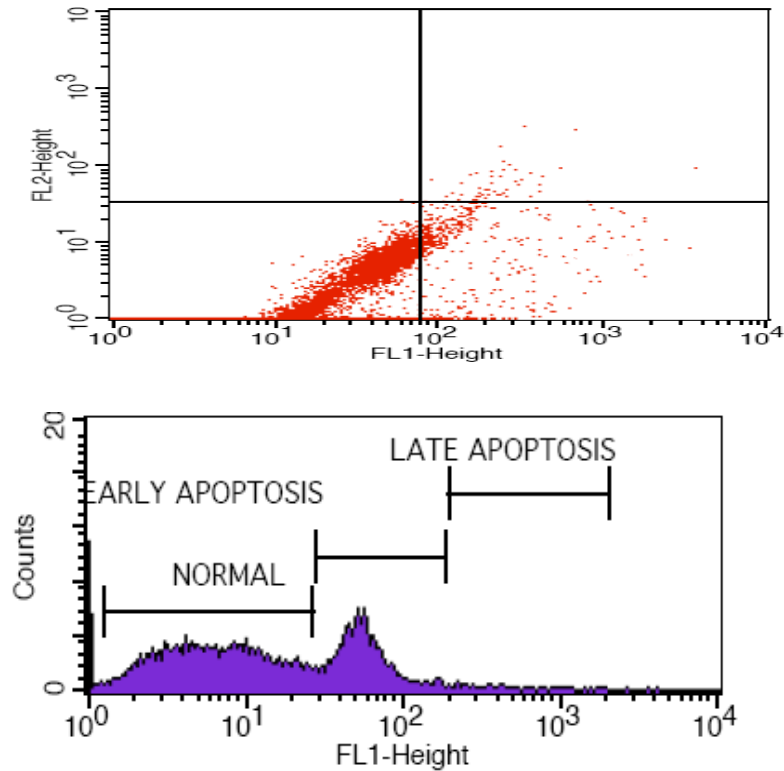


Figure 3b: Flow cytometry analysis for CE (25µg) treated cells with annexin V and PI



Marker	Events	% Gated	% Total
All	10000	100.00	100.00
NORMAL	4220	42.20	42.20
EARLY APOPTOSIS	5478	54.78	54.78
LATE APOPTOSIS	188	1.88	1.88

Figure 3c: Flow cytometry analysis for EAF (25µg) treated cells with annexin V and PI



Marker	Events	% Gated	% Total
All	10000	100.00	100.00
NORMAL	6272	62.72	62.72
EARLY APOPTOSIS	3371	33.71	33.71
LATE APOPTOSIS	145	1.45	1.45

Figure 3d: Flow cytometry analysis for AF (25µg) treated cells with annexin V and PI kit

DISCUSSION

Cancer continues to be a major health issue in terms of morbidity and mortality, in spite of advances in early detection and upgrading in treatment options. Cancer is a hostile disease, which if not detected at an early stage can metastasize to other organs of the body, potentially making even systemic chemotherapy ineffective. Carcinogenesis is a multistage development which is strongly based by a number of variables such as age, dietary habits and hormonal balance [9].

Lung cancer has the highest death rate in worldwide exceeding the mortality rates of colorectal, breast and prostate cancers [10]. Incidence and mortality from lung cancer in females is rising while it is declining in males in developed countries. This is the single most devastating cause of cancer related deaths. The majority of lung cancers occur in people who are either current or former smokers. Further approximately one out of every six people who develop lung

cancer never smoked. These statistics highlight that lung cancer development is a multifactorial process. More than 85% of all lung cancer cases occur among people who are either current or former tobacco smokers. The relationship between smoking and lung cancer is caused by the carcinogens present in tobacco smoke.

Cancer chemoprevention is rapidly emerging area of preventive oncology that focuses on cancer of the lung prevention by the administration of one or more synthetic or naturally occurring bioactive compounds to repress, reverse or extend the onset of lung tumor [11]. The chemoprevention compounds present in diet have higher capability in fighting against lung cancer proliferation by inhibiting the process of lung carcinogenesis through regulation of cell defensive and cell death machineries [12].

Pomegranate, used for centuries for its medicinal properties is now being recognized as a potential chemo preventive and anticancer agent. Increasing body of evidence has underscored the cancer

preventive efficacy of pomegranate both *in vitro* and *in vivo* animal models. The emerging data provide new insights into the molecular framework needed to establish novel mechanism-based chemo preventive strategies for various human cancers.

This study was designed to show the chemopreventive effect of *Punica granatum* rind extract of ganesh variety against lung cancer. Treatment of A549 cells with crude methanolic extract and their fractions resulted in decrease in cell viability.

Preliminary data showed that crude methanolic rind extract was predominantly effective among the other extract. So, crude methanolic rind extract and two fractions were taken for further analysis.

Antineoplastic effect on A549 Cells

Growth inhibition of pomegranate rind extracts on A549 cell line was studied by 3-(4, 5-Dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide assay. MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole), is reduced to purple formazan in mitochondria of living cells [5]. This reduction takes place only when mitochondrial reductase enzymes are active, and therefore conversion can be directly related to the number of viable cells. A solubilization solution (dimethyl sulfoxide, an acidified ethanol solution) is added to dissolve the insoluble purple formazan product into a colored solution. The absorbance of this colored solution can be quantified by measuring at wavelength between 500 and 600 nm by a spectrophotometer. In the present study A549 cells were treated with each herbal extract at a concentration ranging 12.5, 25, 50, 100 µg/ml for 48 hrs whereas cells incubated with DMSO were used as control. Ethyl acetate fraction had superior cytotoxic effects when compared to other fraction and crude rind extract in a concentration dependent manner. The ethyl acetate fraction induces uppermost cell death in the A549 cells which indicate the maximum growth inhibition.

The growth inhibition correlated considerably with the degree of treatment of the extract which induces cell death by inducing apoptosis in the target cells [13, 14]. Therefore ethyl acetate fraction from crude methanolic extract was more potent than the crude form which may be due to the action of purified bioactive polyphenols present in the ethylacetate fraction.

Apoptosis is the process of physiologically programmed cell death in which intrinsic pathway participates in the cell death [15]. 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-1 beta converting enzyme); calpain; Fas signaling; cell cycle interfaces; stress responses; Bcl-2 family; and the tumor suppressor gene p53 [16]. The human lymphocytes is treated with dxm which induced apoptosis in uninfected human lymphocytes through nuclear condensation, fragmentation and apoptotic body formation [17], 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. Apoptosis is identified as one of the most essential biological processes in eukaryotes which play a vital role in development and homeostasis, and also in several disease states [18]. The interpretation in the present study could therefore be of clinical and therapeutic significance, as well as pathophysiological research interest.

Determination of intrinsic apoptosis by DNA fragmentation

DNA fragmentation occurs in apoptotic cells that are caused by intrinsic activity which is induced by a variety of agents. This cleavage produces ladders of DNA fragments that are the size of integer multiples of a nucleosome length (180– 200 bp) [19]. The DNA fragmentation is due to the activation of caspase 3 of inactive CAD (caspase activated deoxyribonuclease) through removal of its inhibitors, i.e., ICAD [20]. As a biochemical hallmark of intrinsic apoptotic cell death, DNA fragmentation was used to determine whether the antiproliferative effect of *Punica granatum* methanolic rind extract and their fractions on A549 cells acts through the respective apoptosis pathway. As shown in Figure 2.9a, the

treatment of A549 cells with the ethyl acetate fraction resulted in the induction of intrinsic apoptosis activity at concentrations as low as 25 µg based on the IC₅₀ that was predetermined by MTT assay. A549 cells treated with 50 µg of alcohol fraction also induces apoptosis after 48 hrs showed typical features of DNA laddering on an agarose gel. In figure 2.9b, the untreated cells and 25 µg, 50 µg and 100 µg of methanolic crude extract treated cells produced intact genomes. Therefore, this study thus suggests that the ethyl acetate fraction at a concentration as low as 25 µg can induce nucleosomal DNA fragmentation of A549 cell due to intrinsic apoptosis processes.

Different stages of apoptotic cell determination by Annexin V FITC

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes will not uptake the PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are FITC Annexin V and PI negative; cells that are in early apoptosis are FITC Annexin V positive and PI negative so the cell appear green in color with fragmented nucleus inside; and cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive [21]. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in either case, the dead cells will stain with both Annexin-FITC and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death). In the present investigation the movement of cells through these three stages by ethyl acetate fraction and alcohol fraction suggests that apoptosis and the percentage of apoptotic cell is higher in case of ethyl acetate fraction treatment than alcohol treated cells.

The present study is the first report which suggests that the ethyl acetate fraction possess potent proapoptotic characteristics for human lung cancer cells (A549). These studies also suggest that ethyl acetate fraction may have significant potential in cancer therapy.

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 [22]. 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 loses 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 [23]. 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 PI-negative), early apoptotic cells (annexin V-positive but PI-negative), and necrotic or late apoptotic cells (annexin V- and PI-positive) [21]. 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. The result confirms that the ethyl acetate fraction induces cell death by apoptotic process but not by necrotic pathway.

CONCLUSION

The treatment of A549 cells with extracts confirm the highest percentage of apoptosis with the ethyl acetate fraction of *Punica granatum* rind extract which was confirmed by morphological changes, DNA fragmentation and loss of membrane integrity which lead to the translocation of phosphatidyl serine in the outer surface

of the plasma membrane were confirmed by binding with annexin V. The above result confirms the cell death was due to the apoptotic pathway and not by necrosis.

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