Punicaglin induces apoptosis in A549 cell line through mitochondria-mediated pathway

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Abstract. Lung cancer is the most common cause of cancer-related deaths worldwide. Punicaglin is an ellagitannin mostly found in pomegranate husk and shows very strong antitumoral activity. The purpose of this study was to investigate the mechanism in which punicaglin acts as an anti-proliferative agent on A549 cell line (adenocarcinomic human alveolar basal epithelial cells) and MRC-5 cell line (normal lung fibroblast cells). The cultured cells were treated with punicaglin at concentrations of 1–100 μM for 24 h. For this aim, cell growth inhibition, percentage of apoptotic cells, cell cycle distribution, morphological changes, cellular and mitochondrial reactive oxygen species (ROS) production, and expression of apoptotic proteins were evaluated. Cell viability test and morphological examinations showed that punicaglin at 50 and 75 μM concentrations exhibited toxic effect against lung cancer cells but not toxic against normal lung cells. Cytoplasmic ROS production decreased with the application of punicaglin, while the level of ROS released from mitochondria increased due to mitochondrial dysfunction. Studies of apoptosis indicated that both punicaglin concentrations induced apoptotic process in A549 cells. However, cell cycle was arrested in the G₁/S phase after punicaglin treatment. These findings suggest that punicaglin has antiproliferative and apoptotic properties in these concentrations.

Key words: A549 cell line — MRC-5 cell line — Punicaglin — Cell proliferation — Apoptosis

Introduction

Reactive oxygen species (ROS) are produced from several physiological processes in the body. Overproduction of these species can damage cellular components such as lipids, proteins and DNA. There are several diseases related to the high production of ROS like cardiovascular and inflammatory diseases and cancer (Vazhappilly et al. 2019). The carcinogenesis process is enhanced due to high ROS level in the organs (Lin et al. 2019). Inhaled toxins (e.g. smoke, chlorine, hydrogen chloride, hydrogen sulfide, sulfur dioxide, and nitrogen dioxide) are some sources for ROS production in the lung tissue (Jha et al. 2019).

Cancer is one of the diseases associated with ROS overproduction. Cancer is a major health concern in terms of morbidity and mortality. It is a fatal disease, which can be metastasised to another organ of the body, specially if undiagnosed at early stages (Monkkonen and Debmath 2018). Lung cancer is a cancer with high mortality rate, which is the most common cause of cancer-related deaths worldwide, and causes approximately 1.6 million deaths each year (Dela Cruz et al. 2011). Increasing incidence of lung cancer is associated with several risk factors including lifestyle choices, environmental factors, and genetic make-up, all of which contribute to the risk of developing lung cancer. Among many risk factors, tobacco smoke, radon, asbestos, and arsenic exposure have been linked with increasing rates of lung cancer (Schwartz and Cote 2016). Lung cancers are traditionally classified into two main histologic groups as small cell lung cancer and non-small cell lung cancer according to differences in the natural course of the disease and the treatment approaches. Non-small cell lung cancer constitutes approximately 85% of all lung cancers, and the most common subtypes are adenocarcinoma, squamous cell carcinoma, and large cell carcinoma (Zappa and Mousa 2016).
Due to concerns for general health, phytonutrients have attracted the consumers interest to prevent from chronic diseases and aging because of their potential curative, preventive and nutritive values (Wali et al. 2019). Phytonutrients have functionally been demonstrated as antioxidants, as well as modulators of enzyme activity, cell proliferation and apoptosis (Navaneetha Krishnan et al. 2019). Phytochemicals, like fruits and vegetable phenolics, may be responsible for the bioactivity in plant food diets that provide the health benefits. Numerous studies have shown that consumption of fruits and vegetables can reduce the risk of several cancer types due to their polyphenolic content. The suggested mechanisms of polyphenol action as anticancer agents include antioxidant, anti-inflammatory and antiproliferative activities as well as their effect on subcellular signaling pathways, stimulation of cell cycle arrests and apoptosis (Baby et al. 2018; Phan et al. 2018).

*Punica granatum* (pomegranate) belongs to family *Punicaceae* and is commonly known as pomegranate. Pomegranate has been used as traditional medicine in many countries for the treatment of dysentery, diarrhea, acidosis, hemorrhage, and respiratory pathologies (Shaygannia et al. 2016). Pomegranate leaf exhibits antihelmintic, antiparasitic, antidiarrhoeal, antioxidant, antitumoral and anti-inflammatory properties. Two types of polyphenols are found in pomegranate; anthocyanins (delphinidin, cyaniding and pelargonidin), responsible for the red color of the fruit, and hydrolysable tannins, mainly ellagittannins. These polyphenols are responsible for 90% of the antioxidant capacity of pomegranate and punicalagin alone undertakes more than 50% of this antioxidant property. Punicalagin is a hydrolysable tannin and it is considered to be the main compound in pomegranate leaf and husk (Berköz and Allahverdiyev 2017). Punicalagin is reported to have anticancer, antiatherosclerotic and antiobesity properties (Turrini et al. 2015). The purpose of this study was to investigate the mechanism in which punicalagin acts as an antiproliferative agent on A549 cell line which is an adenocarcinomic human alveolar basal epithelial cell culture model.

**Materials and Methods**

**Materials**

The adenocarcinomic human alveolar basal epithelial cell line (A549) and normal lung fibroblast cell line (MRC-5) was obtained from American Type Culture Collection (ATCC). Punicalagin, staurosporine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) dye, dimethyl sulphoxide (DMSO) and cell lytic buffer were obtained from Sigma-Aldrich Chemical Co, USA. The Annexin V- Fluorescein isothiocyanate (FITC) apoptosis detection kit was obtained from Merck Chemicals Ltd. Primary antibodies against β-actin, poly (adenosine diphosphate-ribose) polymerase (PARP), caspase-3, -8 and -9 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). A protein assay kit was obtained from Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, Massachusetts, USA). All cell culture chemicals were bought either from Sigma-Aldrich, Merck or other standard suppliers. All solvents used for the assays were high-performance liquid chromatography (HPLC) grade.

**Cytotoxicity of punicalagin on lung cells**

A549 cells was grown in Dulbecco’s modified eagle medium (DMEM) media with high glucose content without sodium pyruvate. Media was supplemented with 20% fetal bovine serum (FBS), 5% penicillin and 5% glucose. The cytotoxic effect of different concentrations ranging from 1-100 µM of punicalagin on A549 cells was investigated. In brief, a stock solution of 10 µM punicalagin was prepared in DMSO. A549 cells were seeded into 96 well tissue culture plates at 1×10⁴ cells/200 µl DMEM serum media. After 24 h, different concentrations of punicalagin were added to each well. The MTT determination was performed after 24 h incubation at 37°C and 5% CO₂. The plate was removed from the incubator and the sample (20 µl) from the MTT dye was added to each well. The plate was incubated for 2 h at 37°C. At the end of the incubation time, the culture media was aspirated and 50 µl of DMSO was added to each well. The produced formazan crystals were dissolved completely by shaking the plate for 30 s at room temperature. The colour intensity was measured at 492 nm using a plate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA) (Mosmann 1983). The MRC-5 cell line was seeded in DMEM with high glucose content without sodium pyruvate, supplemented with 10% FBS. 96 wells plates were coated for 15 min with a coating solution before seeding MRC-5 cells. The coating solution contained 50 ml serum-free media (without FBS), 65 µl bovine serum albumin (BSA) (100 mg BSA in 2 ml H₂O), 0.5 ml collagen (250 µl from stock + 9.75 ml H₂O) and 125 µl fibronectin. After 15 min, the coating solution was collected and reused for 5 times only. The MRC-5 seeding performance liquid chromatography (HPLC) grade.

**Morphological change in A549 and MRC-5 treated with punicalagin**

A549 cells and MRC-5 cell line were seeded at concentrations of 1×10⁶ cells/ml in 25 cm² flasks. After 24 h, two different concentrations of punicalagin (50 µM and 75 µM) were ap-
plied to each cell line. Cellular morphology was determined by microscopy after 24 and 48 h. Pictures of the cells were taken using an inverted microscope (Euromex, ED Arnhem, Netherlands) fitted with a camera (Nikon, Japan) at 10× magnification (Akter et al. 2012).

**Measurement of cellular ROS**

Dichlorofluorescein (DCF) dyes have the ability to diffuse across cell membranes. In the cytoplasm, these dyes are hydrolysed enzymatically by intracellular esterases and rapidly converted to DCF dye in the presence of ROS. The fluorescence intensity was proportional to the ROS content. The ROS levels in A549 cell line incubated with 50 µM and 75 µM punicalagin concentration were measured by flow cytometry using 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (CM-H$_2$DCFDA) dye. A549 cells were seeded in 25 cm$^2$ flasks at a concentration of 1×10$^5$ cells/ml. The treatments were applied once the cells reached 60–70% confluence. Following that, the cells were trypsinised and centrifuged for 3 min at 150 × g. The supernatant was discarded and the cells were washed and centrifuged with 3 ml phosphate buffered saline (PBS). Again, the supernatant was discarded and 1 ml PBS was added. The cells were incubated with 5 µM of 2',7'-dichlorofluorescein diacetate (DCFDA) (prepared in DMSO) for 30 min at 37°C and 5% CO$_2$. At the end of the incubation time, the cells were centrifuged for 3 min at 150 × g. The supernatant was removed and cells were re-centrifuged at the same speed. The cells were then incubated for 15 min in the dark at room temperature. At the end of this incubation, the cells were centrifuged for 3 min at 150 × g. Supernatant was removed and 500 µl cold binding buffer was added to each tube followed by 1.25 µl conjugated Annexin V-FITC. The cells were then incubated for 15 min in the dark at room temperature. All cells were then centrifuged for 3 min at 150 × g. Supernatant was removed and 500 µl cold binding buffer was added with 10 µl propidium iodide (PI) and mixed gently. The cells were kept on ice until analysed by flow cytometry (FACSCalibur, Becton Dickinson, USA). A minimum of 10,000 events was acquired in the gated regions using an emission wavelength of 520 nm. Experiments were carried out in triplicate to ensure reproducibility (Osseni et al. 1999).

**Measurement of mitochondrial reactive oxygen species**

Mitoxantrone red fluorogenic dye is a selective dye for the superoxide free radical in the mitochondria. The superoxide radical is generated as a by-product of oxidative phosphorylation which occurs in living cells. Only once this dye is oxidised by superoxide ion and it produces red fluorescence that can be determined by flow cytometry. The superoxide levels, in A549 cell line incubated with 50 µM and 75 µM punicalagin concentrations, were measured by flow cytometry using Mitoxantrone dye. A549 cells were seeded in 25 cm$^2$ flasks at a density of 1×10$^6$ cells/ml. Punicalagin at the concentrations of 50 µM and 75 µM were added to each cell line. Cellular morphology was determined by microscopy after 24 and 48 h. Pictures of the cells were taken using an inverted microscope (Euromex, ED Arnhem, Netherlands) fitted with a camera (Nikon, Japan) at 10× magnification (Akter et al. 2012).

**Cell cycle distribution**

Cells were seeded in 25 cm$^2$ flasks at a concentration of 1×10$^6$ cells/ml. Each punicalagin concentration were added once the cells reached 50–60% confluence using a 24 h exposure time. The cells were trypsinised and centrifuged for 3 min at 150 × g. The pellets were washed twice with PBS and re-centrifuged at the same speed. The cells were then...
re-suspended with 200 µl cold PBS. Cells were vortexed vigorously and fixed in 1 ml ice-cold fixing buffer (70% ethanol in PBS), then vortexed again for 2 min. The cells were incubated at 4°C for 24 h. On the following day, the cells were centrifuged to remove the fixation buffer. After this procedure, the cells were re-suspended in PBS with 10 µM of ribonuclease (RNAse) and incubated for 30 min at 37°C. At the end of this incubation, the cells were then stored on ice and 10 µl of 1 mg/ml PI was added to each sample and analysed by flow cytometry (FACSCalibur, Becton Dickinson, USA). At least 10,000 events were acquired in list mode, using an emission wavelength of 620 nm. The experiments were performed at least in triplicate for each treatment (Zhen et al. 2014).

**Determination of protein expressions by Western blot**

A 549 cells at a density of 1×10^6 cells/ml were seeded in 25 cm² tissue culture flasks. Once 50% confluence was achieved, cells were treated with 50 µM and 75 µM punicalagin and incubated for 24 h. After this incubation, the culture media was decanted and cells were washed with 5 ml PBS. The cells were then trypsinized and the resulting cell suspension centrifuged at 1500 × g for 3 min with 5 ml PBS to wash. Supernatants were removed and the cell pellets were lysed by adding 300 µl of lysis buffer. The last step for cell lysis was to keep the cells on ice for 20 min and then store them at −80°C until protein determination and Western blot experiment were carried out. Total protein concentrations of the supernatant were determined by Pierce™ BCA Protein Assay Kit (Thermo Scientific Pierce).

An equal amount of total protein (20–50 µg) was loaded into sodium dodecyl sulphate-polyacrylamide gel electrophoresis and separated by electrophoresis under 80–100 V. After electrophoresis, the protein bands were transferred to polyvinylidene difluoride (PVDF) membrane under 100 V for 1 h in cold water bath. Then, the PVDF membranes were blocked with PBS containing 5% non-fat milk or BSA for 1 h and incubated with the primary antibodies against PARP, caspase-3, -8, -9, and β-actin (1:1000 dilution) overnight at 4°C. The PVDF membrane was washed 3 times with PBS containing 0.05% Tween-20 (PBST) and incubated with the horseradish peroxidase (HRP)-conjugated secondary antibody (1:10000 dilution) at 37°C for 1 h. The PVDF membranes were washed 3 times with PBST again and labeled with the enhanced Pierce™ Fast Western Blot Kit (Thermo Scientific Pierce) for 1 min at room temperature in dark. Finally, the PVDF membranes were exposed to the imaging system (ImageJ software, National Institutes of Health, Bethesda, MD, USA) to capture the light signals of protein bands. The PARP and caspase-3, -8, and -9 protein levels were expressed as a relative value to that of β-actin.

**Statistical analyses**

All experiments were performed in triplicate. For the 96-well microtiter tissue culture plates, 3 replicate wells were used per category. The data were analyzed by Statistical Package for the Social Sciences (SPSS) statistics software (version 15.0, SPSS Inc, Chicago, IL, USA). For significant differences between untreated control and experimental values, the p value among groups was determined by one-way analysis of variance followed by Tukey’s test. The significance level was set at p < 0.05.

**Results**

**Cytotoxic effect of punicalagin on A549 and MRC-5 cells**

To measure the effect of punicalagin on the A549 and MRC-5 cell lines, cell viability was assessed by the MTT assay as shown in Figure 1. Punicalagin doses of 1–100 µM cause a significant dose-dependent inhibition of cell survival for A549 cells. The cell viability for A549 cells was 98.2 ± 1.6%, 95.2 ± 1.6%, 90.4 ± 1.2%, 82.7 ± 1.1%, 68.3 ± 0.8%, 51.4 ± 0.9%, and 31.8 ± 1.1% at punicalagin concentrations of 1, 5, 10, 25, 50, 75, and 100 µM compared to the untreated control group, respectively (Fig. 1). The IC_{50} value of punicalagin on A549 cells determined after a 24-h incubation time was found as 74.97 µM. Figure 1 revealed that 75 µM punicalagin concentration was almost the IC_{50} value of punicalagin on A549 cell line.
A549 cell line. Consequently, this concentration was used to induce an anticancer effect on A549 cells. However, a lower dose of 50 µM punicalagin was also tested in A549 cells to ascertain the efficacy of a lower concentration.

Different punicalagin concentrations (1–100 µM) were also tested on normal lung MRC-5 cells. In contrast to A549 cells, the cell viability for MRC-5 cells was 99.3 ± 1.4%, 97.3 ± 1.1%, 96.2 ± 1.3%, 95.8 ± 1.5%, 93.7 ± 0.8%, 90.7 ± 1.1%, and 81.2 ± 1.3% at 1, 5, 10, 25, 50, 75, and 100 µM punicalagin concentrations compared to the untreated control group, respectively. While the concentration of 1 µM punicalagin did not have a significant effect on the viability of the MRC-5 cell line (p > 0.05), 5–100 µM punicalagin concentrations significantly reduced the viability of this cells line as compared to the untreated control group (p < 0.001) (Fig. 1). It was observed that 1–75 µM punicalagin applications did not have a critical cytotoxic effect on MRC-5 cells (< 10%), but 100 µM punicalagin administration caused over 10% cytotoxicity on MRC-5 cells and exceeded the critical limit.

Morphological changes of A549 and MRC-5 cells induced by punicalagin

The antiproliferative effect of various punicalagin concentrations (50 µM and 75 µM) on A549 and MRC-5 cells was investigated by microscopy at 24 and 48 h (Fig. 2). Figure 2A showed a morphological change in the A549 cell line in response to punicalagin treatments compared with the untreated control. Conversely, the same concentrations of punicalagin had little effect on MRC-5 cells comparable to the untreated control, i.e. the cells appeared as healthy as the untreated control (Fig. 2B). This confirms the antiproliferative effect of punicalagin on the lung cancer cell line but not on normal lung cell line.

Cellular and mitochondria ROS measurement

Fifty µM and 75 µM punicalagin treatment significantly increased the mitochondrial ROS generation 1.84- and 2.18-fold compared to the untreated control group, respectively (p < 0.001). In contrast to mitochondrial ROS production, reduction in cytoplasmic ROS production was observed with 50 µM and 75 µM punicalagin compared with the untreated control (Fig. 3). The percentage of ROS inhibition was 12.39% at 50 µM and 25.03% for 75 µM compared to the untreated control group (p < 0.001).

Apoptosis in A549 cells

In the presence of 50 µM and 75 µM punicalagin concentrations for 24 h, apoptosis was significantly induced in the A549

![Figure 2. Effect of different punicalagin concentrations (50 µM and 75 µM) on the A549 (A) and MRC-5 (B) cell lines for 24 and 48 h. Images were captured using light microscopy at 100× magnification.](image-url)
Measurements of mitochondrial and cellular ROS in A549 cell treated with different punicalagin concentrations (50 µM and 75 µM) for 24 h. Cells were loaded with Mitosox stains and the fluorescence measured by flow cytometry to monitor mitochondrial and cellular ROS formation. Data represent the means ± SD of three independent experiments. * p < 0.001 and ** p < 0.0001 in comparison to untreated controls; significant difference was determined using one-way ANOVA followed by Tukey's test for multiple comparison.

A549 cell cycle phases after treatment with 50 µM and 75 µM punicalagin was shown in Figure 5. Arrest in the S-phase was observed by noting a significant decrease in the cell population, with an associated increase in the G1 population in comparison to untreated controls. Compared with the negative control, the percentage of apoptotic cells, in both the early apoptotic (staining with Annexin V) and the late apoptotic (staining with Annexin V+PI) stages were significantly increased (p < 0.0001). The proportion of cells in the early apoptotic stage was 1.2 ± 0.03%, 2.4 ± 0.02%, 1.5 ± 0.01%, and 2 ± 0.03% for untreated control, 50 µM, 75 µM punicalagin, and staurosporine (positive control), while the proportion in the late apoptotic stage was 7.7 ± 0.13%, 20.3 ± 0.16%, 12.4 ± 0.4%, and 19 ± 0.7%, respectively (Fig. 4). The proportion of cells undergoing necrosis (staining with PI) was 0.1 ± 0.01%, 0.2 ± 0.01%, 0.1 ± 0.01%, and 0.1 ± 0.01% for negative control, 50 µM and 75 µM punicalagin, and staurosporine respectively. The necrotic cells did not change significantly with punicalagin treatment compared with the negative control (p > 0.05) (Fig. 4).

Effect of punicalagin on cell cycle

A549 cell death treated with different punicalagin concentrations for 24 h by flow cytometric analysis. A. The flow cytometric analysis of Annexin V in A549 cells treated with 50 µM and 75 µM punicalagin for 24 h. Cells were harvested and stained with propidium iodide (PI) and Annexin V. Q1–Q4 quadrants indicate: Q1, cells stained with PI (necrotic cells); Q2, cell conjugated with Annexin V and stained with PI (late apoptotic/necrotic cells); Q3, unconjugated with Annexin V and unstained with PI cells (healthy cells); Q4, cells conjugated with Annexin V (early apoptotic cells). Staurosporine (1 µM) was assayed a positive control. B. The percentage of A549 cells in early apoptosis, late apoptotic/necrotic cell death and necrotic cell death pathways after treatment with 50 µM and 75 µM punicalagin concentrations for 24 h. Data represent the means ± SD of three independent experiments. * p < 0.001 and ** p < 0.0001 in comparison to untreated controls; significant difference was determined using one-way ANOVA followed by Tukey's test for multiple comparison.
cells treated with both concentrations of punicalagin. Consequently, the G2/M phase cell population was not affected by the treatment. The percentage of cells in the G1-phase was 48.7 ± 2.6%, 57.1 ± 1.6%, and 65.9 ± 1.9% for untreated control, 50 µM and 75 µM, (p < 0.01), respectively. The percentage of cells in the S-phase was 36.3 ± 1.8%, 30.9 ± 1.4% and 23.1 ± 1.1%, respectively (p < 0.05), while in the G2-phase, the percentage was 14.9 ± 0.5%, 12 ± 0.9% and 11 ± 0.6% for the untreated control, 50 and 75 µM punicalagin treatment (p < 0.01).

**Punicalagin induced apoptosis in the A549 cell line via the activation of caspases and PARP**

Induction of the apoptosis process by punicalagin through caspase activation and PARP cleavage has been also evaluated. Caspase-3, -8 and -9 protein expressions exhibited a dose-dependent increase after 50 and 75 µM punicalagin treatment (Fig. 6) (p < 0.05). Also, A549 cells treated with 50 and 75 µM concentrations of punicalagin showed a cleavage of PARP protein (p < 0.001) (Fig. 6).

**Discussion**

Several studies have shown that the polyphenolic compounds have the ability to inhibit cell proliferation in lung cancer (Amararathna et al. 2016). Li et al. (2016) demonstrated that pomegranate leaf extract showed anticancer activity in lung cancer in vitro by inhibiting proliferation, inducing apoptosis, cell cycle arrest and impairing cell migration and invasion but which of the phytochemicals in pomegranate leaf extract has not been shown to be responsible for this activity. It is thought that punicalagin, which is abundant in pomegranate leaf and peel, is responsible for this activity.

In this study, the antiproliferative effect of punicalagin was investigated in A549 cells as a model for lung cancer. The concentration of punicalagin that reduced cell viability to 50% was about 75 µM. A similar dose of 50 and 100 µM was also tested on a normal lung cell line (MRC-5) and showed no significant reduction of cell viability. Cell morphology for both A549 and MRC-5 cell lines treated with 50 µM and 75 µM punicalagin had a selective effect on lung cancer cells but not on normal lung cells. Seeram et al. (2005) observed a greater antiproliferative effect for pomegranate juice at 100 µg/ml, compared with ellagic acid, punicalagin and total pomegranate tannin when using different human cancer cell lines. In their research, the cell proliferation in all cancer cell lines was inhibited by 30% to 100% at 12.5 µg/ml to 100 µg/ml in a dose-dependent manner (Seeram et al. 2005). Li et al. (2016) showed that pomegranate leaf extract inhibited the proliferation of non-small cell lung cancer cells and had little cytotoxicity on normal cells. Similarly,
Larrosa et al. (2006) found that the cell viability of Caco-2 cells exposed to ellagic acid or punicalagin for 24 h was significantly reduced in a dose-dependent manner. Similarly, another study performed on acetone extract of pomegranate exhibited antiproliferative effect of the pomegranate on A549 lung cancer cell line at different concentrations (50–150 µg/ml) in a dose-dependent manner. However, this extract exhibited an insignificant effect on the cell viability of normal human bronchial epithelial cell at the same doses (Khan et al. 2007).

The ROS level in the A549 cell line was investigated to assess whether ROS played a significant role in the induction of apoptosis after exposure to punicalagin. According to several studies in which ROS levels in tumour cell lines after exposing them to polyphenol or flavonoids were measured, the induction or reduction of ROS levels lead to cell death (Renis et al. 2008; Singh et al. 2011; Yahloufi et al. 2018; Yang et al. 2018; Champ and Kundu-Champ 2019). Li et al. (2016) demonstrated that pomegranate leaf extract induced ROS level and loss of mitochondrial transmembrane potential in lung cancer cells. Mertens-Talcott et al. (2006) reported that ellagic acid and ellagitannine caused a significant increase in ROS production when applied to the Caco-2 cell line at different dilutions. However, the phenolic extract obtained from red muscadine grapes caused a significant reduction in ROS level at the lowest dilution, while the other dilutions from red muscadine grapes didn’t change the generation of ROS (Mertens-Talcott et al. 2006). In the current study, the ROS level in the A549 cell line was investigated to assess whether ROS played a significant role in the induction of apoptosis after exposure to punicalagin. Our study demonstrated that mitochondrial ROS formation was induced while cytoplasmic ROS was inhibited. This phenomenon was observed at all different punicalagin concentrations in this experiment. In agreement with this finding, the high antioxidant potential of punicalagin due to the presence of its phenol groups may explain the decrease in cytoplasmatic ROS (Mertens-Talcott et al. 2006). On the other hand, the increased amount of superoxide anion released from the mitochondria may be due to mitochondrial dysfunction caused by different punicalagin concentrations. When mitochondria loses their function, they release cytochrome c which activates caspase-9 (Larrosa et al. 2006). This result was confirmed by the activation of caspase-9, which supports our hypothesis that punicalagin causes mitochondrial dysfunction in A549 cancer cells.

Many studies conducted on cancer cell lines treated with polyphenols have found that different cancer cell lines died via the apoptosis pathway (Curti et al. 2017; Piccolella et al. 2019). In a study conducted by Seeram et al. (2005), significant induction of apoptosis in HT-29 colon cancer cell line was observed mostly by pomegranate juice followed by ellagic acid, punicalagin, total pomegranate tannins. However, pomegranate juice didn’t have any apoptotic effect on HCT 116 colon cancer cell line, although ellagic acid, punicalagin and total pomegranate tannins induced apoptosis at the same concentration (Seeram et al. 2005). Larrosa et al. (2006) found that both ellagic acid and punicalagin significantly induced apoptosis in Caco-2 colon cancer cells in a dose-dependent manner. In contrast, no apoptotic effect was observed in CCD 112 normal colon cell line at the tested concentrations (Larrosa et al. 2006). Hafeez et al. (2008) found the significant effect of delphinidin as a cell inhibitor in PC3 prostate cancer cell line. In the present study, A549 cells incubated with punicalagin, 50 and 75 µM, for 24 h showed apoptotic responses as measured by flow cytometry using an Annexin V kit. Annexin V dye
Apoptotic properties of punicalagin in A549 cell line

binds with phosphatidyl serine that has been externalized from the inner surface of the cell membrane to the outer surface during apoptosis (Crowley et al. 2016). As shown by Western blotting, the activation of apoptotic pathways was confirmed with caspase activation. The PARP cleavage was detected in A549 cells treated with punicalagin via a significant activation of caspase-3. Caspase-3 activation can occur via the intrinsic pathway (caspase-9) or extrinsic pathway (caspase-8). Caspase-9 is activated due to the release of cytochrome c from the mitochondria while caspase-8 is activated by transmembrane death receptors. In this study, Annexin V and morphological changes to the A549 cell line in the presence of 50 µM and 75 µM punicalagin concentrations confirmed the apoptosis cell death pathway.

Cell cycle arresting has been considered as a target for cancer therapy (Dickson and Schwartz 2009). Phenolic compounds showed different arresting phases in different cancer cell lines (Lazze et al. 2004). In our study, to investigate the possibility of cell growth arrest, cell cycle phases were explored when punicalagin was exposed to the A549 cell line for 24 h. Flow cytometry assays showed that A549 cells were arrested in the S-phase, and accumulated in the G1-phase caused by 50 µM and 75 µM punicalagin. This showed that punicalagin treatment has stimulated the apoptotic responses in A549 cells. Our findings are in agreement with the findings of another study conducted by Larrosa et al. (2006). In their research, punicalagin at 1, 10 and 100 µM and ellagic acid at 1, 10 and 30 µM caused arresting in the S-phase on Caco-2 cell line (Larrosa et al. 2006). Li et al. (2016) indicated that pomegranate leaf extract induced cell apoptosis and cell cycle abnormalities in lung cancer cells. In another study, ellagic acid and urolithins at 40 µM have individually been shown to cause accumulation of Caco-2 cells in the S-phase (Gonzalez-Sarrias et al. 2009). Khan et al. (2007) demonstrated that pomegranate extract has also been shown to have a chemotherapeutic potential against A549 lung cancer cells. Arresting of A549 cells was observed in the G1-phase of the cell cycle; the cell distributions were 65%, 70% and 72% for 50, 100 and 150 µg/ml extract concentrations, respectively (Khan et al. 2007).

According to the results obtained from all the previous experiments, punicalagin at 50 µM and 75 µM concentration was found to be an effective agent for apoptosis when used in the A549 cell line, but not in MRC-5 normal lung cell line, when compared to the untreated control. Cytoplasmic ROS production was decreased in cytoplasm and superoxide radical release from mitochondria was increased. Cell morphology changes, phosphatidylserine exposure and activation of caspases and PARP cleavage support the induction of the apoptosis pathway. The cell cycle was arrested by punicalagin in the G1/S-phase at the examined concentrations. All these findings showed that punicalagin may be a potential anticancer agent.

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