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Effect of Punicalagin on Human Colon Cancer Caco-2 Cells

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ABSTRACT

Introduction: There is increasing evidence of the presence of antioxidant and antimutagenic properties in some fruit species. Punicalagin extracted from pomegranate is reported to have anti-proliferative activities and able to induce toxicity in colon cancer cells. However, the molecular mechanisms involved are still poorly understood. In this study, *in vitro* apoptotic and anti-proliferative activities of punicalagin were investigated in human colon cancer Caco-2 cells. **Methods**: Cell viability was identified and a morphological change by punicalagin was taken. In addition, reactive oxygen species (ROS), annexin V and cell cycle were evaluated by flow cytometer at the tested concentrations of 50 and 75 µM. Moreover, the effects of punicalagin in Caco-2 cells and normal colon epithelial cell line (HCEC) were compared. The Caspases family was investigated as a marker of apoptosis by western blot. **Results**: Punicalagin (50 and 75 µM) showed toxic effects on Caco-2 cells but not on HCEC with both results being confirmed by morphological studies. In the presence of punicalagin, cytoplasmic ROS production decreased indicating antioxidant activity, whereas superoxide radicals released from mitochondria increased due to mitochondrial dysfunction. Annexin V and caspase family (9, 8 and 3) activation confirmed that cell death occurred via the apoptosis pathway in both concentrations of punicalagin. The cell cycle was arrested in the gap 1 (G1)/synthesis (S) transition phase at the concentrations of punicalagin tested. **Conclusion**: The *in vitro* results indicate that further studies are warranted to elucidate the potential role of punicalgin as an anti-cancer agent.

Key words: Annexine V, caspases, cell cycle, MTT & ROS

INTRODUCTION

Due to increasing concerns about general health, chronic disease prevention and ageing, consumers' interest in phytonutrients because of their potential curative, preventive and nutritive values has also increased (Guhr & Lachance, 1997). Phytonutrients have functionally been demonstrated as being antioxidants, as well as modulators of enzyme activity, cell proliferation and apoptosis (Balentine, Albano & Nair, 1999). Phytochemicals, such as fruit and vegetable phenolics, may be responsible for the bioactivity in plant food diets that provide health benefits. Numerous studies have shown that consumption of fruits and vegetables can reduce the risk of several cancer types due to their polyphenols content. The suggested mechanisms of polyphenol action as anti-cancer agents include antioxidant, anti-inflammatory and anti-

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proliferative activities, as well as their effect on subcellular signaling pathways, stimulation of cell cycle arrests, and apoptosis (Middleton, Kandaswami & Theoharides, 2000; Yang *et al.*, 2001). Pomegranate is widely consumed as fruit, juice, and as wine (Gil *et al.*, 2000). Pomegranate contains pectin, sugars, fibre and several tannins. It is also a rich source of anthocyanins such as delphinidin, cyanidin, and pelargonidin, and hydrolysable tannins such as punicalin, gallic acid, ellagic acid (EA), and punicalagin. Around 92% of pomegranate antioxidant activity is due to the presence of hydrolysable tannins (Khan, Adhami & Mukhtar, 2010).

One of the anti-cancer properties of polyphenols is their ability to induce cell cycle arrest and apoptosis (type of cell death). Cell death is a normal feature in our body; in healthy cases there is a balance between cell division and cell death (Khan *et al.*, 2010). Apoptosis is a programmed cell death process that normally eliminates damaged cells through the activation of caspase enzymes. Apoptosis is characterised by cell shrinkage (the nuclear chromatin condenses and cytoskeleton collapses) and engulfment by macrophage. In contrast to apoptosis, necrosis is uncontrolled cell death and characterised by cell swellings and bursting. It is caused by external factors that affect cells such as toxins, infection and trauma. In necrosis, cellular contents are spilled into the intracellular cell space and elicit an inflammatory response (Edinger & Thompson, 2004). Some dietary polyphenols such as epigallocatechin gallate (EGCG) and genistein have the ability to induce apoptosis in various cancer cell lines, but not to normal cell lines (Ahmad *et al.*, 1997).

From previous studies it is clear that pomegranate may have cancer-chemo-preventive effect, as well as cancer chemotherapeutic effect against colon cancer. It has also been found that pomegranate has an effect against prostate (Malik *et al.*, 2005), breast (Kim *et al.*, 2002) and lung (Khan *et al.*, 2007) cancer. An *in vitro* study done by Seeram *et al.* (2005) evaluated the anti-proliferative activities of punicalagin, EA, total pomegranate tannin (TPT) and pomegranate juice (PJ). The effects of these components were studied on human oral (KB, and CAL27), colon (HT-29, HCT116, SW480, and SW620) and prostate (RWPE-1, and 22Rv1) tumor cells. PJ showed the greatest anti-proliferative effect against all cell lines (Seeram *et al.*, 2005). Also, it depicted alteration in the regulatory molecules arresting in the G1 phase of the cell cycle (Malik *et al.*, 2005). Wang *et al.*, (2013) observed a dose-dependent anti-proliferative effect for 1-30 μg/ml punicalagin when using human U87MG glioma cell line through both apoptotic and autophagic pathways. Punicalagin and EA showed a protective effect against benzopyrene-induced deoxyribonucleic acid (DNA) adducts and anti-proliferative activity against lung cancer cells A549 (Zahin *et al.*, 2014). Furthermore, it has been speculated that EA, caffeic acid, luteolin and punicic acid are important components of pomegranate that significantly inhibit *in vitro* invasion of human PC3 when employed individually (Lansky *et al.*, 2005).

According to a number of studies on the anti-cancer properties of polyphenols, increasing the consumption of fruits, vegetables and dietary fibre may reduce the risk of colon cancer (Steinmetz & Potter, 1996; Briviba, Pan & Rechkemmer, 2002; Larrosa, Tomas-Barberan & Espin, 2006). This study therefore aimed to investigate the mechanism with which pomegranate's active component (punicalagin) acts as an anti-cancer agent. To achieve this, the colon carcinoma cell culture model (Caco-2) was used and the results were compared with HCEC.

METHODS

Materials

The human colorectal carcinoma cell line Caco-2 cells were obtained from the European Collection of Cell Cultures (ECACC), Salisbury, United Kingdom (UK). HCEC was gratefully received from Dr Karen Brown, University of Leicester, UK. Phosphate buffered saline tablets (PBS) were obtained from Oxoid, Hampshire, UK. Punicalagin, 2 amino-7-dimethylamino-2-methylphenazine hydrochloride (MTT dye) dimethyl sulphoxide (DMSO) and cell lysis buffer were obtained from Sigma-Aldrich Chemical Company, Poole, UK. The annexin V-FITC apoptosis detection kit was obtained from Merck Chemicals Ltd. Staurosporine was purchased from Calbiochem, Nottingham, UK. Foetal bovine serum (FBS), trypsin-EDTA solution, L-glutamine (200 mM), non-essential amino acids (NEAAs), Dulbecco's modified essential medium (DMEM), collagen and fibronectin, CM-H_2DCFDA, Mitosox WesternBreeze Antibody were obtained from Invitrogen, Paisley, UK. Poly adenosine diphosphate ribose polymerase (PARP), caspase 9, 8 and 3 were purchased from Cell Signaling Technology, UK.

Cytotoxicity and morphological analysis of punicalagin on Caco-2 and HCEC cells

Caco-2 was grown in DMEM media with high glucose content without sodium pyruvate. The media were supplemented with 10% FBS, 5% penicillin, and 5% glutamine. The cytotoxic effects of different concentrations ranging from 1-100 µM of punicalagin on Caco-2 cells were studied. In brief, Caco-2 cells were seeded into 96-well microtiterre tissue culture plates at 1×10^4 cells/200 µl DMEM serum medium. After 24h, punicalagin of a different concentration was added to each well. The MTT determination was performed after 24h of incubation at 37 °C and 5% CO_2. The plates were removed from the incubator and samples (20 µl) from the MTT dye were added to each well. The plates were incubated for 2h at 37 °C. At the end of the incubation period, the culture media were aspirated and 50 µl of DMSO was added to each well. The colour intensity was measured at 492 nm using a plate reader (Boehring CO, Marburg, Germany).

The HCEC cell line was seeded in DMEM with high glucose content without sodium pyruvate, but was supplemented with 10% of FBS. Tissue culture plates (96-wells) were coated for 15 min with a coating solution before being seeded with HCEC cells. The coating solution contained 50 ml serum-free media (without FBS), 65 µl Bovine Serum Albumin (100 mg BSA in 2 ml H_2O), 0.5 ml collagen (250 µl from stock + 9.75 ml H_2O) and 125 µl fibronectin. After 15 min, the coating solution was collected and reused a further five times only. The HCEC seeding density was 1×10^4 cells per well. The punicalagin concentrations of 50 and 100 µM were examined using HCEC and Caco-2 cells, and cytotoxicity was assessed by the MTT method as described above. Experiments were carried out in triplicate and repeated three times to ensure reproducibility.

Caco-2 cells and HCEC cell line were seeded at concentrations of 1×10^6 cells/ml in 25 cm^2 flasks. After 24h, two different concentrations of punicalagin (50 µM and 75 µM) were applied to each cell line. Cellular morphology was determined by microscopy after 24h and 48h. Pictures of the cells were taken using a phase contrast microscope (Zeiss Telaval inverted microscopy) fitted with a camera (Nikon, Japan) at 10 x magnification.

Measurement of cellular reactive oxygen species (ROS)

Dichlorofluorescein (DCF) dye or non-fluorescent dichlorodihydrofluorescein diacetate (H_2-DCFDA) has the ability to diffuse across cell membranes. The fluorescence intensity is proportional to the ROS content (Osseni *et al.*, 1999). The ROS levels in Caco-

2 cell line incubated with 50 μM and 75 μM punicalagin concentrations were measured by flow cytometry using H_2-DCFDA dye. Caco-2 cells were seeded in 25 cm^2 flasks at 1×10^6 cells/ml. The treatments were applied once the cells reached 60-70% confluence. The cells were incubated with 5 μM of dichlorofluorescein diacetate (DCFDA) for 30 min at 37 °C and 5% CO_2. At the end of the incubation period, the cells were kept on ice under low light conditions due to the high susceptibility of the dye to photo-oxidate until DCFDA florescence was measured using a BD FACSCanto flow cytometer (California, USA). At least 10,000 events were acquired in the gated regions using an emission wavelength of 520 nm.

Measurement of mitochondrial oxygen species

Mitosox 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 occurring in living cells. Only on oxidisation of this dye by a superoxide ion does it produce the red fluorescence that can be determined by flow cytometry (Liu, Liu & Dudley, 2010). The superoxide levels in Caco-2 cell line incubated with 50 μM and 75 μM punicalagin concentrations were measured by flow cytometry using Mitosox dye according to the manufacturer's instructions. The red fluorescence was measured using a BD FACSCanto flow cytometer (California, USA). At least 10,000 events were acquired in the gated regions using an emission wavelength of 620 nm.

Determination of apoptosis by annexin-V assay

Phosphatidylserine is a phospholipid located in the inner surface of the cell membrane. It is expressed on the cell surface during cell death by an apoptosis program and was measured by using annexin V–FITC apoptosis detection kit from Merck Millipore. The assay was performed according to the manufacturer's instructions to detect early and late apoptosis in Caco-2 cells incubated for 24h with different concentrations of punicalagin (50 μM and 75 μM) using a BD FACSCanto flow cytometry (California, USA). A minimum of 10,000 events were acquired in the gated regions. The emission was 520 nm for cells labelled with annexin V-FITC and 620 nm for cells labelled with propidium iodide (PI). The PI dye was used to distinguish the cells that had lost their membrane integrity. Staurosporine at 1 μM was used as a positive control for apoptosis.

Cell cycle and DNA fragmentation

Cells were seeded in 25 cm^2 flasks at a concentration of 1×10^6 cells/ml. Punicalagin concentrations were added once the cells reached 50-60% confluence after an exposure of 24h. The cells were trypsinised and the pellets were washed twice with PBS. The cells were then re-suspended with 200 μl cold PBS, vortexed vigorously, fixed in 1 ml of ice-cold fixing buffer (70% ethanol in PBS), and then incubated at 4 °C for 24 h. The fixation buffer was removed and 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 stored on ice, and 10 μl of 1 mg/ml PI was added to each sample and analysed by BD FACSCanto flow cytometry (California, USA). At least 10,000 events were acquired in list mode, using an emission wavelength of 620 nm.

Assessment of caspases family by western blot

Caco-2 cells treated with different concentrations of punicalagin (50 and 75 μM) for 24h were lysed as follows. After incubation, the cells were trypsinised and the resulting cell suspension was washed via centrifuging at 1500*g* for 3 min with 5 ml PBS. Supernatants were removed, and the cell pellets were lysed by adding 300

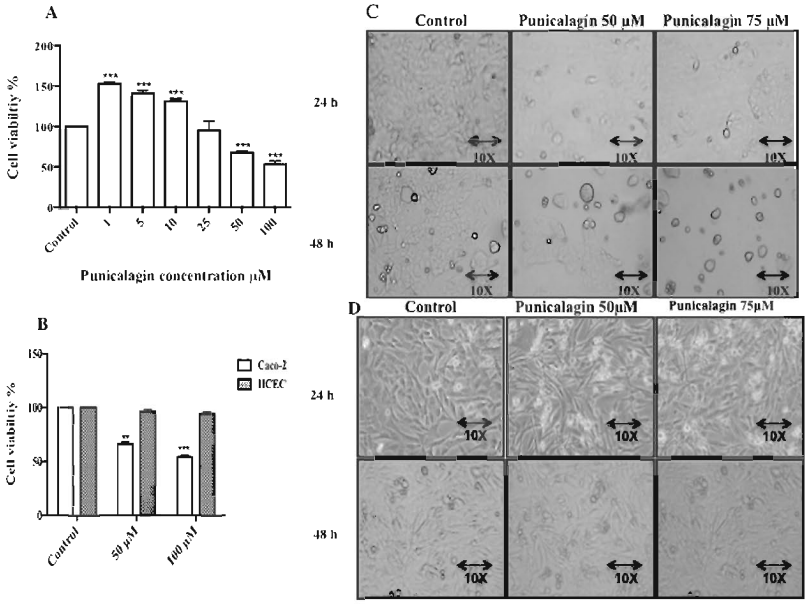

Figure 1. (A) Cell viability for different concentrations of punicalagin (1, 5, 10, 25, 50, 100, µM) incubated for 24 h in Caco-2 cell Lline. (B) The effect of punicalagin concenetrations (IC_{50}) on Caco-2 and HCEC cells for 24 h. The data represents the mean of three independent experiments (n=3 ± SD). Comparisons of means were made using a one-way ANOVA followed by Bonferroni corrections test (** = $p < 0.001$; *** = $p < 0.0001$. (C & D) Effect of punicalagin concentrations of 50 µM and 75 µM on the Caco-2 cell line and HCEC for 24 and 48h. Caco-2 images were captured using light microscopy at 10x magnification.

μl of lysis buffer (Sigma Aldrich Chemical Company). The lysis cell was kept on ice for 20 min and then stored at -80°C until protein determination was done by western blot experiment. The protein concentration for each sample was measured using a BioRad protein assay (Bradford, 1976) following the manufacturer's instructions. An Invitrogen NuPAGE 4-12% Bis-Tris gel was used for protein electrophoresis. The proteins were then transferred to a polyvinyl difluoride (PVDF). The results were visualised by chemiluminescence using Amersham film.

Statistical analyses

All experiments were performed in triplicate. For the 96-well microtitre tissue culture plates, four replicate wells were used per category. The data were analysed by Graphpad Prism 6 software. Significant differences between the control and the experimental values were evaluated using P-values determined by one way analysis of variance (ANOVA) followed by Bonferroni corrections. Significance was set at a cut-off level of ≤ 0.01.

RESULTS

Cytotoxicity and morphological analysis of punicalagin on Caco-2 and HCEC cells

To measure the effect of punicalagin on the Caco-2 cell lines, cell viability was assessed by the MTT assay as shown in Figure 1A. A concentration of 75 μM was the IC_{50} of punicalagin concentration on the Caco-2 cell line. Consequently, this concentration was used to induce an anti-cancer effect on Caco-2 cells. However, a lower dose of 50 μM punicalagin was also tested in Caco-2 cells to ascertain the efficacy of a lower concentration.

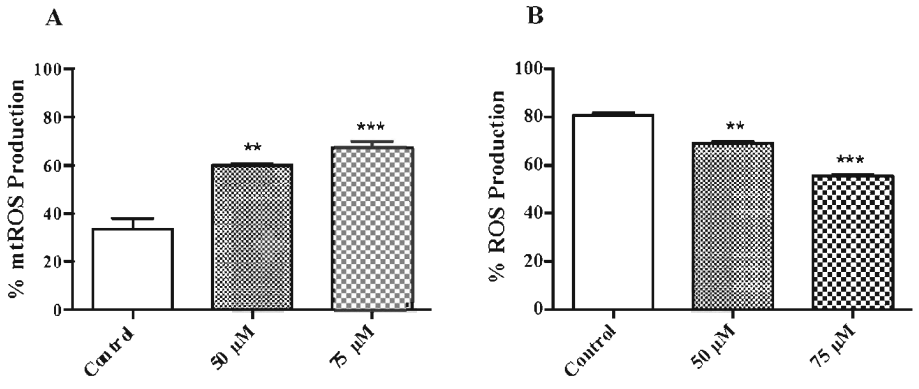

Figure 2. Measurements of mitochondria and cellular ROS in Caco-2 cells treated with different punicalagin concentrations for 24h. (A) Punicalagin-induced mtROS production in Caco-2 cells. Caco-2 cells were treated with 50 µM and 75 µM punicalagin for 24h. Cells were loaded with Mitosox stains and the fluorescence measured by flow cytometry to monitor mtROS formation. (B) Punicalagin cellular ROS inhibition in Caco-2 cells. Caco-2 cells were treated with 50 µM and 75 µM punicalagin for 24h. Cells were loaded with CM-H_2DCFDA and the DCF fluorescence was measured by flow cytometry to monitor ROS formation. The data represent the mean of three independent experiments (n=3 ± SD). Comparison of means was made using a one-way ANOVA followed by Bonferroni's corrections test (** = $p < 0.001$ *** = $p < 0.0001$).

The cell viability for Caco-2 cells were 152, 141, 131, 95, 67, 53, and 46% at punicalagin concentrations of 1, 5, 10, 25, 50, 100, and 150 μM, respectively. Different punicalagin concentrations (50-100 μM) were also tested on normal colon HCEC cells. The 50 μM and 75 μM concentrations showed no significant effect on the cell viability of the HCEC cell line as shown in Figure 1B. In contrast to Caco-2 cells, the cell viability for HCEC cells were 95% and 93%, at 50 and 100 μM punicalagin concentrations, respectively.

The anti-proliferative effect of various punicalagin concentrations (50 μM and 75 μM) on Caco-2 and HCEC cells was investigated by microscopy at 24 and 48h. The morphological change in the Caco-2 cell line in response to punicalagin treatments compared with the control is shown in Figure 1C. Conversely, the same concentrations of punicalagin had little effect on HCEC in comparison to the control (i.e., the cells appeared as healthy as the control) as shown in Figure 1D. This confirmed the anti-proliferative effect of punicalagin on the colon cancer cell line but not on the normal colon cell line.

Cellular and mitochondria ROS measurement

Mitochondrial ROS (superoxide anion) generation was significantly induced by (50 μM and 75 μM) concentrations of punicalagin (60%, 66%) compared with the control (30%) as shown in Figure 2A ($p \leq 0.001$). Mitochondrial dysfunction might have been caused by different punicalagin concentrations. This could be the reason for the increased production of superoxide anion. The activation of caspase 9 supported the hypothesis that punicalagin caused mitochondrial dysfunction. When mitochondria lose their function, they release cytochrome C, which activates caspase 9 (Larrosa *et al.*, 2006). In contrast to mitochondrial ROS production discussed above, the reduction in cytoplasmic ROS

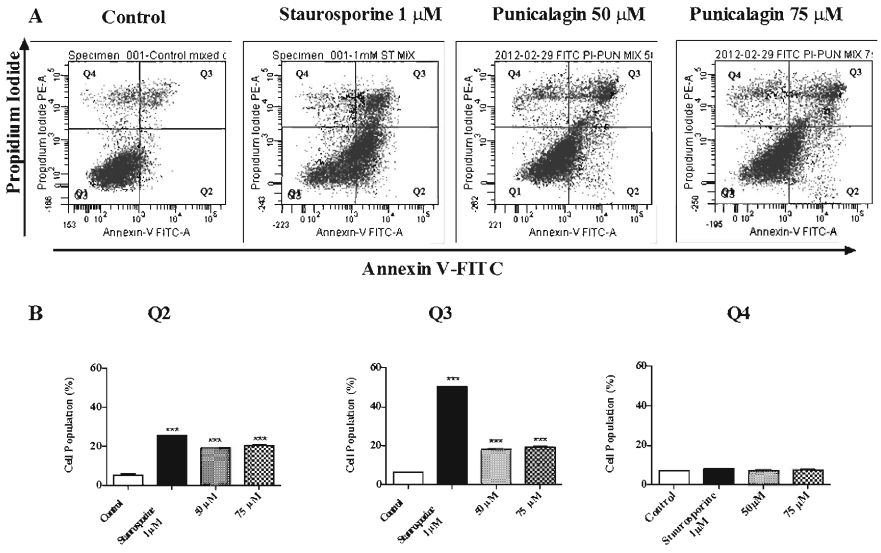

Figure 3. Detection of Caco-2 cell death treated with different punicalagin concentrations for 24h by flow cytometric analysis. (A) Flow cytometric analysis of Annexin V in Caco-2 cells treated with 50 μM and 75 μM punicalagin for 24h. Cells were harvested and stained with propidium iodide (PI) and Aannexin V (AV). Q1- Q4 quadrants indicate: Q1 = Healthy cells; Q2 = Cells conjugated with AV; Q3 = Cells conjugated with AV and stained with PI; Q4 = cells stained with PI. Staurosporine 1 μM was assayed as positive control. (B) Percentage of Caco-2 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 24h. Represented values are the mean of three independent experiments (n=3 ± SEM). Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (*** = $p < 0.0001$).

production was observed with 50 μM and 75 μM punicalagin compared with the control as shown in Figure 2B. The proportions of ROS inhibition were 69% at 50 μM and 54% at 75 μM, whilst the ROS production in the control was 81%.

Apoptosis in Caco-2 cells

In the presence of 50 μM and 75 μM punicalagin concentrations for 24h, apoptosis was significantly induced in the Caco-2 cell line. Compared with the negative control, the proportion of apoptotic cells, in both the early apoptotic (single annexin V) and the late apoptotic (annexin V plus PI) stages were significantly increased ($p \leq 0.0001$). The proportions of cells in the early apoptotic stage were 5%, 18%, 20%, and 28% for control, 50 μM, 75 μM punicalagin, and staurosporine (positive control), respectively, while the proportions in the late apoptotic stage were 6%, 17%, 19 %, and 50%, respectively. The cell population stained with PI only (necrotic cells) did not change significantly with treatment compared with the negative control ($p > 0.05$). The proportions of cells undergoing necrosis were 6.5, 7.5, and 8% for the negative control, 50 μM and 75 μM punicalagin, and staurosporine, respectively as shown in Figures 3A and B.

Effect of punicalagin on cell cycle

The Caco-2 cell cycle phases after treatment with 50 μM and 75 μM punicalagin are shown in Figure 4. Arrest in the S-phase was observed by noting a significant decrease in the cell population, with an

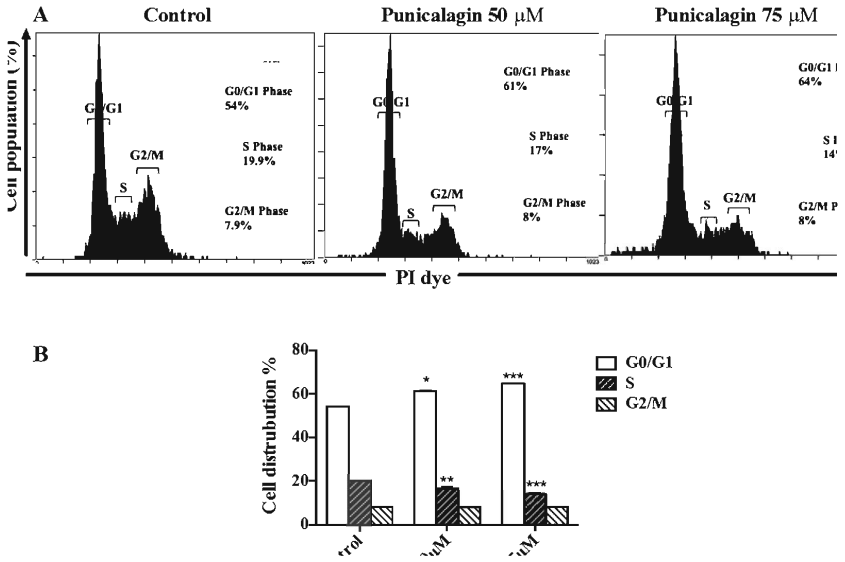

Figure 4. Cell cycle arrest in Caco-2 cells treated with different punicalagin concentrations for 24h by flow cytometry. (A) Caco-2 cells were treated with 50 μM and 75 μM punicalagin and fixed in 70% ethanol for 24h. Fixed cells were treated with RNAse (10 μM) and stained with 10 μl of 1 mg/ml propidium iodide stock. (B) Effect of 50 μM and 75 μM punicalagin concentrations at 24h on the Caco-2 cell cycle distribution. G1-phase (growth phase), S-phase (DNA synthesis phase), and G2-phase (growth 2 phase). In each phase, cells percentages were measured by flow cytometry. Represented values are the means of three independent experiments (n=3 ± SEM). Comparisons of means were made using one-way ANOVA followed by Bonferroni's test (*=$p < 0.01$, **= $p < 0.001$ and *** = $p < 0.0001$).

associated increase in the G1 population in cells treated with both concentrations of punicalagin. Consequently, the Gap 2 (G2)/mitosis (M) phase cell population was not affected by the treatment. The proportions of cells in the G1 phase were 45, 54, and 60% for the control, 50 μM and 75 μM ($p \leq 0.01$), respectively. The proportions of cells in the S phase were 26%, 19% and 14%, whilst in the G2 phase, the proportions were 7, 7.4 and 7.5 % for the control and 50 μM and 75 μM ($p \leq 0.001$), respectively.

Punicalagin induced apoptosis in the Caco-2 cell line via the activation of caspases and PARP

Induction of the apoptosis process by punicalagin through caspase activation has been studied. Western blotting of caspase 3, 8 and 9 as shown in Figure 5 illustrated a dose dependent increase in the levels of these proteins. Caspase 9 is activated due to the release of cytochrome C from the mitochondria and is involved in the intrinsic apoptosis pathway. On the other hand, caspase 8 is activated by transmembrane death receptors and is involved in the extrinsic apoptosis pathway. Activation of caspase 9 and caspase 8 led to the activation of caspase 3. Subsequently, the PARP is activated by caspase 3 during the process of apoptosis. Thus, PARP is an important downstream biomarker of apoptosis occurring by either the intrinsic or extrinsic pathways. Cells treated with different concentrations of punicalagin showed a cleavage of the PARP protein as represented by western

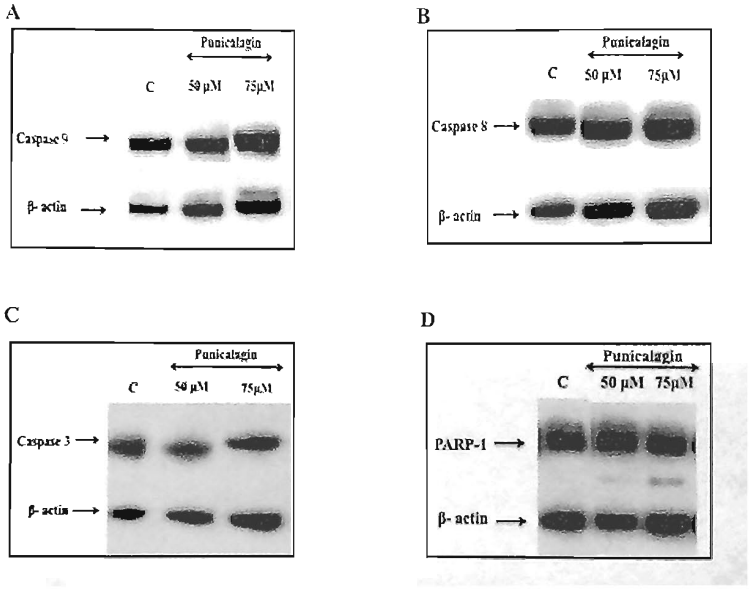

Figure 5. Western blot of caspases 3, 8, and 9 and PARP-1 expression in Caco-2 cells treated with different punicalagin concentrations. Protein was extracted from Caco-2 cells after treatment with 50 µM and 75 µM punicalagin for 24h. Cells were harvested and lysates were prepared. For each treatment, 20 µg protein was subjected to 4-12% SDS gel electrophoresis followed by western blot analysis and chemiluminescence detection. Loading of protein was confirmed using β-actin expression. Panel A: protein expression for caspase 9 (MW=47 kDa). Panel B: β protein expression for caspase 8 (MW= 57 kDa). Panel C: protein expression for caspase 3 (MW= 35 kDa). Panel D: protein expression for PARP-1 (MW= 116, 89 kDa.

blotting as shown in Figure 5. Therefore, the effect of punicalagin as an inhibitor of colon cancer cell growth is mediated by the activation of the caspase family.

DISCUSSION

Several studies have shown that the polyphenolic compounds have the ability to inhibit cell proliferation in different cancer cell lines (Seeram *et al.*, 2005; Khan *et al.*, 2007; Wang *et al.*, 2013; Zahin *et al.*, 2014). In this study, the anti-proliferative effect of punicalagin was investigated in Caco-2 cells as a model for colon cancer. The cytotoxicity of punicalagin was measured by the MTT assay. The concentration of punicalagin that reduced cell viability to 50% was 75 μM. Similar doses of 50 and 100 μM also tested on a normal colon cell line (HCEC) showed no significant reduction of cell viability. Cell morphology for both Caco-2 and HCEC cell lines treated with 50 μM and 75 μM punicalagin had a selective effect on colon cancer cells but not on normal colon cells.

The ROS level in the Caco-2 cell line was investigated to assess whether ROS played a significant role in the induction of apoptosis after exposure to punicalagin. The data demonstrated that mitochondrial ROS formation was induced while cytoplasmic ROS was inhibited. This phenomenon was observed at all different punicalagin concentrations used in this experiment. In agreement with this finding, Mertens-Talcott *et al.* (2006)

state that the high antioxidant potential of punicalagin due to the presence of phenol groups may explain the decrease in cytoplasmic ROS. On the other hand, the increased amount of superoxide anion released from the mitochondria might be due to mitochondrial dysfunction caused by different punicalagin concentrations. This result was confirmed by the activation of caspase 9, which supports this study's hypothesis that punicalagin causes mitochondrial dysfunction in Caco-2 cancer cells.

Many studies conducted on cancer cell lines treated with polyphenols have found that different cancer cell lines (from liver, colon, breast, and prostate tissue: HepG2, Caco-2, Mcf-7 and LNCap, respectively) died via the apoptosis pathway (Malik *et al.*, 2005; Zhang *et al.*, 2008). In the present study, Caco-2 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 binds with phosphatidyl serine that has been externalised from the inner surface of the cell membrane to the outer surface during apoptosis. As shown by western blotting, the activation of apoptotic pathways was confirmed with caspase activation. The PARP cleavage, which is an important apoptosis marker, was detected in treated Caco-2 cells via a significant activation of caspase 3. Caspase 3 activation can occur via the intrinsic (caspase 9) or extrinsic pathways (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, the significant increase in protein expression of caspase 9 and 8 were indicative that both pathways were involved in the mechanism of cell death by apoptosis.

To investigate the possibility of cell growth arrest, cell cycle phases were explored when punicalagin (50 and 75 μM) was exposed to the Caco-2 cell line for 24 h. Flow cytometry assays showed that cells were arrested in the S-phase, and accumulated in the G1-phase. The proportions of cells in the G1-phase were 45, 54, and 60% for the control, 50 and 75 μM punicalagin, respectively. The proportions of cells in the S-phase were 26, 19 and 14% for the control, 50 and 75 μM punicalagin, respectively (stated in the same order). It has been reported that arresting cells in the G1/S-phase is due to the down regulation of cyclin E (Lazze *et al.*, 2004). This cyclin is involved as a checkpoint for the transition from G1-to S-phase, the down-regulation of which leads to cell cycle arrest (Lazze *et al.*, 2004). Therefore, the arresting effect of different punicalagin concentrations could be due to cyclin E down regulation. Other polyphenols have shown different cell cycle arrest mechanisms in different cancer cell lines, arresting the cell cycle in the G1, S, S/G2 and G2 phases (Fresco *et al.*, 2006; Renis *et al.*, 2008; Hafeez *et al.*, 2008).

CONCLUSION

According to the results obtained from all the experiments, punicalagin, at both 50 μM and 75 μM, was found to be an effective agent for apoptosis when used in the Caco-2 cell line, but not in the normal colon cell line HCEC, when compared to the control. Cytoplasmic ROS production was decreased in cytoplasm, and superoxide radical release from mitochondria was increased. Cell morphology changes such as phosphatidylserine exposure, 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. Punicalgin may be a potential anti-cancer agent.

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