The Bioactive Properties of Pomegranate Polyphenol (Punicalagin)

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Abstract

Plant polyphenols are reported to have bioactive properties, which may be used for protection against diseases. Therefore, the aim of this research was to investigate the bioactive activities of a pomegranate tannin polyphenol compound, punicalagin. In particular, the antioxidant, antihypertensive and anticancer mechanisms were investigated.

Punicalagin was found in pomegranate husk but not in pomegranate juice when analysed by HPLC and LC-MS. Antioxidant mechanisms involved hydrogen peroxide scavenging, ferrous chelating and reducing ability. Higher hydrogen peroxide scavenging activity was achieved by 0.1 mg/ml from both punicalagin and pomegranate juice when compared with butylated hydroxytoluene (BHT) or trolox (p ≤0.05). Punicalagin and pomegranate juice exhibited ferrous chelating ability significantly lower than Ethylenediaminetetraacetic acid.

Cell toxicity induced by tert-butylhydroperoxide (3 mM) was significantly inhibited by punicalagin (5 and 10 μM) in Caco-2 cells; these results were confirmed by cell morphology. Punicalagin protection was achieved by inhibiting cellular reactive oxygen species (ROS) as well as malondialdehyde levels. Glutathione level was significantly increased in stressed cells pretreated with both concentration of punicalagin, indicating good antioxidant activity for punicalagin.

Punicalagin (1-60 μM) increased nitric oxide production in endothelial cells (EA.hy926) through decreased ROS levels and increased endothelial nitric oxide synthase enzyme (eNOS) activation. Activation of eNOS enzyme was achieved by an
increase of cellular calcium concentration. At the same examined concentration of punicalagin (1-60 μM), the activity of angiotensin converting enzyme (ACE) was significantly inhibited. The dual action of punicalagin as nitric oxide synthase inducer and ACE inhibitor showed antihypertensive effect.

Punicalagin (50 and 75 μM) showed toxic effects on the colon cancer cell line (Caco-2) but not on a normal colon cell line (HCEC); both results were 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 (caspase 9, 8 and 3) activation confirmed that cell death occurred via apoptosis pathway by both concentrations of punicalagin. The cell cycle was arrested by punicalagin in the G1/S-phase at the concentrations tested. The above findings indicating that punicalagin has antioxidant, antihypertensive and anticarcinogenic activity.
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<tbody>
<tr>
<td>ACE</td>
<td>Angiotensin Converting Enzyme</td>
</tr>
<tr>
<td>Ang</td>
<td>Angiotensin</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine Serum Albumin</td>
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<tr>
<td>Caco-2</td>
<td>Human Epithelial Colorectal Adenocarcinoma Cells</td>
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<tr>
<td>CVD</td>
<td>Cardiovascular Diseases</td>
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<tr>
<td>DHE</td>
<td>Dihydroethidium</td>
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<tr>
<td>DMEM</td>
<td>Dulbecco's Modify Eagle Medium</td>
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<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
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<tr>
<td>DPPH</td>
<td>2,2-Diphenyl-1-Picrylhydrazyl</td>
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<tr>
<td>EA</td>
<td>Ellagic Acid</td>
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<tr>
<td>Ea.hy926</td>
<td>Human Endothelial-Like Immortalized Cell Line</td>
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<td>EDTA</td>
<td>Ethylenediaminetetraacetic Acid</td>
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EGTA  Ethylene Glycol Tetraacetic Acid

eNOS  Endothelial Nitric Oxide Synthase

FBS  Foetal Bovine Serum

G1-phase  Growth 1 Phase

G2-phase  Growth 2 Phase

GSH  Glutathione

GSSG  Oxidized Glutathione

H-H-L  Hippuryl-Histidyl-Leucine

H-L  Histidyl-Leucine

H₂O₂  Hydrogen peroxide

HBSS  N-(2-hydroxyethyl) Piperazine-N-2-Ethanesulfonic Acid

HCEC  Human colonic epithelial cells

HCL  Hydrochloric Acid

HPLC  High Performance Liquid Chromatography

iNOS  Inducible Nitric Oxide Synthase
LC/MS  Liquid Chromatography Mass Spectrometre
MDA  Malondialdehyde
mtROS  Mitochondrial Reactive Oxygen Species
MTT  Tetrazolium Salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5 Diphenyltetrazolium Bromide
NADPH  Nicotine Amide Adenine Dinucleotide Phosphate Reduced
NaOH  Sodium Hydroxide
NEAA  Non-Essential Amino Acid
nNOS  Nuroal Nitiric Oxide Synthase
NO  Nitric Oxide
PBS  Phosphate Buffer Saline
PI  Propidium Iodide
PJ  Pomegranate Juice
PUNI  Punicalagin
ROS  Reactive Oxygen Species
<table>
<thead>
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<tr>
<td>S phase</td>
<td>DNA Synthesis S Phase</td>
</tr>
<tr>
<td>T-BOOH</td>
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Chapter 1
1 Introduction

1.1 Polyphenols

Plant foods like (fruits, vegetables and chocolate) and drinks like (tea, coffee and wine) contain several phenolic compounds which are secondary plant metabolites that exhibit nutritional quality (Ziccarelli and Basu, 2003; Crozier et al., 2009). Phenolic compounds are a large group of molecules with a variety of functions in plant growth, development and defence. Over the past decade, there has been a growing interest in identifying and developing phenolic compounds from plant extracts, as they are reported to have substantial benefits on human health (Gil et al., 2000; Zhang et al., 2005; Cvorovic et al., 2010). Polyphenol compounds have different biological activities like anti-carcinogen (Ahmad et al., 1997; Zhao et al., 2004; Kasimsetty et al., 2010b), anti-atherosclerosis (Aviram and Dornfeld, 2003; Adams et al., 2006), antioxidant (Kulkarni et al., 2004; Wolfe et al., 2008; Kunyanga et al., 2011), improvement of endothelial cell line and inhibition of cell proliferation (Leikert et al., 2002; Huang et al., 2004; Persson et al., 2009). Plant polyphenols can scavenge reactive oxygen species (ROS) and activate endogenous defence systems such as increasing glutathione level (Ziccarelli and Basu, 2003; Lima et al., 2006), activating antioxidant enzyme (Alia et al., 2006b; Lima et al., 2007), activating caspases (Li et al., 2003; Larrosa et al., 2006a) and increasing the activity of nitric oxide synthase enzyme (Martin et al., 2003; Appeldoorn et al., 2009).
1.1.1 Structure of Polyphenols

Phenolic compounds have one or more hydroxyl groups attached directly to an aromatic ring and their structures may range from simple phenolic molecules to high molecular mass complexes. Most phenolic compounds are present as esters or glycosides rather than free compounds (Balasundram et al., 2006).

1.1.2 Classification

Polyphenols are divided into several classes according to the phenol ring number they consist of, and to the structural elements that bind these rings to each other (see Figure 1.1). Flavonoids, phenolic acids, tannins, stilbenes and lignans are the main groups of polyphenols (D'Archivio et al., 2007). Different classes of polyphenols are present in the human diet like fruits (apple, raspberry, pomegranate and orange), vegetables (onion, tomatoes and broccoli), beverages (tea, coffee and red wine) and spices (turmeric, cinnamon and hot pepper) (Murcia et al., 2001; Bajpai et al., 2005). An example of a phenolic acid class is caffeic acid, which has antioxidant activity via scavenging ROS in endothelial cell line (EA.hy926) (Huang et al., 2004). Quercetin is another phenolic compound (subclass of flavonoids), which has a significant effect on increasing the activity of antioxidant enzymes such as catalase, superoxide dismutase, glutathione reductase and glutathione peroxidase in mouse liver (Shen et al., 2007) and liver cell line (HepG2) (Molina et al., 2003). Apoptosis in colon cancer cell line (HT29 and HCT 116) can be induced by tannic acid, a component in the tannin class (Seeram et al., 2005). Resveratrol is a type of stilbene that has the ability to inhibit the proliferation of cardiac fibroblasts in rat cardiac fibroblast cell line (Wang et al., 2007), as well as inhibiting the cell proliferation of human colon cell line (Fuggetta et al.,...
Moreover, it can increase nitric oxide and the nitric oxide synthase enzyme level in rat cardiac fibroblast cell line (Wang et al., 2007).

1.1.3 Flavonoids

Flavonoids are one of the phenolic compound classes that are found in the human diet. Flavanoids are low molecular weight and have 15 carbon atoms arranged in a C6-C3-C6 configuration; there are 2 aromatic rings A and B connected by a 3-carbon bridge into a heterocyclic ring (see Figure 1.2).
Flavonoids comprise anthocyanins, flavones, isoflavones, flavanones, flavonols and flavanols. The chemical structures of the main classes of flavonoids are depicted in Figure 1.3 (Merken and Beecher, 2000).

**Figure 1.2: General structure of flavonoid.** Source: Kar et al., 2006

**Figure 1.3: Subclasses of the flavonoids family.** Source: Gamet-Payrastre et al., 1999
1.1.4 Tannins

Tannins are one of the major polyphenol groups present in the human diet. Tannins are divided in two groups: hydrolysable tannin (ellagitannins and gallotannins) and condensed tannin (proanthocyanidins). Their chemical structures are illustrated in Figure 1.4.

Flavonoids and tannins are antioxidants as they have a high redox potential, permitting them to perform as reducing agents, hydrogen donors and single oxygen quenchers. They also have a metal chelating ability (Yen and Duh, 1994; Tsao and Yang, 2003; Gülçin et al., 2010). All these features are due to their phenolic structure (Passamonti et al., 2003; Crozier et al., 2009). Due to their presence in our diet and their protective effect from several diseases associated with oxidative stress, several studies on polyphenols activity were performed (Young and Woodside; 2001, Mertens-Talcott et
al., 2006; Bao and Lou, 2006; Kasimsetty et al., 2010b) (Figure 1.5). Epidemiological studies suggest that an intake of flavonoids and tannins is beneficial for prevention of cardiovascular, inflammatory and cancer diseases (Noda et al., 2001; Mertens-Talcott et al., 2006; Kunyanga et al., 2011). Pomegranates contain flavonoids (delphinidine, cyanindin, and peralgonidin) and tannin (punicalagin ellagic acid and gallic acid) (Malik et al., 2005). Therefore, the therapeutic effects of punicalagin as one of pomegranate phenolic compounds will be investigated in this research.

![Bioactive properties of polyphenol compounds. Source: Han et al., 2007](image)

Figure 1.5: Bioactive properties of polyphenol compounds. Source: Han et al., 2007
1.2 Pomegranate

Pomegranate is grown in the Mediterranean region, Afghanistan, Iran, India, China, Japan, Russia, Saudi Arabia and United States, particularly in Arizona and California. The history of pomegranates dates back to 800 years ago and it has been used worldwide as a folk medicine. In India (Nagaraju and Rao, 1990), Tunisia (Boukef et al., 1982) and Guatemala (Caceres et al., 1987), the pomegranate peels are dried and boiled in water and used both internally and externally as astringents and/or germicides especially for diarrhoea and ulcers. A mixture of pomegranate seed, juice and peel products paradoxically has been reported, not only to prevent abortion (Ramirez et al., 1988), but also conception (Gujral et al., 1960; Jochle, 1971; Zhan, 1995). Recent uses of pomegranate-derived products include hormone replacement therapy (Lansky, 2000) and cardiovascular protection (Shiraishi et al., 2002; Aviram and Dornfeld, 2003). People normally consume pomegranate as fresh fruit and drink or as food products e.g. (jams) and as dietary supplements (Lansky and Newman, 2007). The antioxidant capacity of different pomegranate cultivars reflected a significant concentration difference of some individual phenolic compounds (Arai et al., 1999).

1.2.1 Structure of Pomegranate

The pomegranate fruit can be divided anatomically into three compartments:

- Seeds: pomegranate seed oil contains punicic acid. It is an omega-5 long chain polyunsaturated fatty acid. It has been reported to have an anticancer effect because it contains a large number of potential bioactive substances (Lansky and Newman, 2007).
• **Juice**: Pomegranate is a rich source of anthocyanin. Delphinidin-3,5-diglucoside is a major anthocyanin in pomegranate juice. The juice also contains delphinidin-3-glucoside, cyanidin-3-glucoside, cyanidin-3,5-diglucoside, pelargonidin-3-glucoside and pelargonidin-3,5-diglucoside (Gil *et al.*, 2000). The juice also contains glucose, ascorbic acid, ellagic acid, gallic acid, caffeic acid, catechin, epigallocatechin gallate, quercetin, rutin, numerous minerals and amino acids.

• **Peels (pericarp or husk)**: pomegranate husk has been reported to have higher antioxidant activity than its seeds. Flavonoids and tannins are more abundant components in the peel (Ozcal and Dinc, 1993).

Pomegranate, as described previously, contains different classes of polyphenols that enables it to act as an antioxidant compound. Consequently, its bioactive compounds can scavenge reactive oxygen species (ROS) that are generated from normal metabolic functions and are responsible for several diseases.

### 1.3 Reactive Oxygen Species and Lipid Oxidation

#### 1.3.1 Reactive Oxygen Species (ROS)

Radicals are molecules with unpaired electrons that are highly reactive e.g. hydroxyl radical OH\(^{•}\) and superoxide radical O\(_2^{•−}\). Radicals are formed in all living organisms in normal metabolic pathways during the oxidation. The free radical level increases under certain circumstances e.g. environmental stress, wounding and pathogen attack. ROS can damage living organisms, such as cell membranes, protein, lipid and nucleic acids (Halliwell, 1991), when left unchecked Figure 1.6.
Reactive oxygen species are molecules that contain an oxygen atom and are highly reactive as a result of the presence of a free radical, or a configuration of the oxygen atom whereby there are more electrons than usual e.g. peroxide ions $O_2^{-2}$. Hydrogen peroxide ($H_2O_2$) is also considered as a ROS because of its reactivity. In fact, it is regarded as the most reactive radical known with an ability to react with a very wide range of bio-molecules (Halliwell, 1991).

![Illustration of diseases related to ROS production](Source: Brieger et al., 2012)

It has been demonstrated that the unsaturated lipids in cell membranes are susceptible to damage from free radical molecules that lead to uncontrolled chain reactions producing further oxidation products. Furthermore, other biological molecules like RNA, DNA and protein enzymes can also be damaged (Hanahan and Weinberg, 2000).
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1.3.2 Lipid Peroxidation

Lipid peroxidation can be defined as the oxidative damage to lipids comprising one or more carbon-carbon double bonds. The peroxidation of lipids produces free radicals which damage the cell membrane (Catala, 2006).

1.3.2.1 Types of Lipid Oxidation

Lipid oxidation occurs when free radicals attack the unsaturated lipids through different pathways: autoxidation, photoxidation and enzymatic oxidation.

a) Autoxidation: The process of autoxidation is triggered off when free radicals present in living cells interact with polyunsaturated and monounsaturated fatty acids, and result in the production of intermediate substances such as alkyl and peroxyl radicals (Kolakowska, 2002).

b) Photoxidation: In this process, light including ultraviolet light works as a source of energy which raises the oxygen from a ground state (O₂) to an excited energy state (¹O₂). The latter reacts with the unsaturated fatty acids to form free radicals (Kolakowska, 2002).

c) Enzymatic oxidation: The enzymatic oxidation reaction is determined by the presence of lipoygenase enzyme system that catalyses the reaction between oxygen and the unsaturated fatty acids in order to produce hydroperoxides (Wang et al., 1991).

1.3.2.2 Mechanism of Lipid Peroxidation in Cell Membrane

The process of lipid peroxidation is associated with several diseases linked with the undesirable effect produced in the cell. The mechanism of lipid peroxidation is
illustrated in Figure 1.7. The hydroxyl group removes a hydrogen atom from the carbon adjacent to the double bond, producing a fatty acid radical with a free electron on the carbon atom. The radical rearranges to give a stable conjugated structure. The free radicals can either crosslink with adjacent fatty acid radicals or react with oxygen to give a peroxyl radical. This subsequently starts a chain reaction, creating new peroxyl radicals. Lipid peroxidation can alter the fluidity of the membrane and transfer radicals onto the membrane proteins, especially receptors (Vermerris and Nicholson, 2006). The mechanism of lipid peroxidation comprises of three steps: initiation, propagation and termination.

**Initiation:** The initiation step of lipid peroxidation starts with the production of free radicals. This initiation process occurs slowly as the reaction between oxygen and the unsaturated fatty acids is difficult. A number of factors activate the initiation of lipid oxidation of PUFA like oxygen, metal ions, and ultraviolet light. Several enzymes have the ability to promote lipid oxidation such as lipoxygenase, and peroxidase. Lipoxygenase enzyme is the main cause of PUFA peroxidation as it is usually found in food (Halliwell and Gutteridge, 1984).

Through the initiation of lipid oxidation, a reactive species like the hydroxyl radical (OH·) extracts a hydrogen atom from PUFA (the methylene group). Therefore, a conjugated diene is produced as a result of the rearrangement of the double bonds of the carbon radical that is subsequently combined with an oxygen atom to form a peroxy radical (ROO·) (Niki *et al*., 2005). Photosensitisation is responsible for the production of the first few radicals in the reaction between a fatty acid double bond and an excited oxygen molecule. Pre-existing lipid hydroperoxide (ROOH) that has low reactivity
levels can be cleaved to produce alkoxy radicals (RO·). The new radical product is highly reactive and reacts with the allylic hydrogen atom of the unsaturated fatty acid to produce additional ROOH (Niki et al., 2005).

**Propagation:** Radicals such as alkoxyl and peroxyl abstract H atoms from the methylene group next to the double bond of the allylic position of the unsaturated fatty acids. Therefore, the autocatalytic chain reaction of lipid oxidation would proceed. The rate of propagation is based on the quantity of the double bonds present in the membrane (Niki et al., 2005).

Lipid hydroperoxides, which are products of ROS oxidation on polyunsaturated fatty acids, are heat labile and break down to produce secondary metabolite products like hydroxyl-fatty acids, epoxides and cleavage products like aldehydes including malondialdehyde (MDA). These radicals then bind and react with another unsaturated fatty acid to propagate the reaction. Consequently, the propagation step continues as long as the unsaturated fatty acid molecules are available (Niki et al., 2005).

**Termination:** Lipid oxidation can be terminated by the reaction of two free radicals to form non-radical products. The termination steps occur at low oxygen concentrations (Niki et al., 2005). For example, alkoxyl radicals via a radical recombination mechanism bind with another radical to form a stabilised ketone or a secondary alcohol through radical transfer. On the other hand, if no binding occurs, free radicals may react with protein molecules. This produces protein cross-linking and, therefore, severely damages the protein.
1.3.2.3 Defence System against Lipid Peroxidation

Since lipid peroxidation plays a key role in the pathogenesis of many diseases such as cancer, coronary heart disease and Alzheimer’s disease, several studies have suggested that free radical scavenging and antioxidant activity can help to prevent the above diseases (Noda et al., 2001). In fact, the living organism has developed various ways to deal with ROS by endogenous defence systems (antioxidant enzymes) and the exogenous antioxidants (provided from the diet) (Hayes and McLellan, 1999; Masella et al., 2005).
1.3.2.3.1 Enzymatic Defence System

The important antioxidant enzymes in the tissue are catalase, superoxide dismutase and glutathione peroxidase. H₂O₂, ROS, and superoxide radicals that are present in the cell environment can be scavenged by these defence system enzymes and prevent lipid oxidation in cells and cell membranes (Halliwell, 1991). The defence system enzymes are described below:

**Superoxide Dismutase (SOD)**

SOD is responsible for the elimination of superoxide (O₂⁻) by converting it to hydrogen peroxide (H₂O₂), which is less harmful to the tissue as compared to (O₂⁻).

\[ 2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2 \]

In humans, this enzyme is found in the cytoplasm, mitochondria and extracellular fluids. The location of SOD depends on the metal ion cofactors that are bound to the enzyme. Copper–zinc SOD is found in the cytoplasm and extracellular fluids, while manganese- SOD exists in the mitochondria (Halliwell and Gutteridge, 1990).

**Catalase (CAT)**

CAT can convert H₂O₂ to water and oxygen using a manganese or iron cofactor. This enzyme is found in all eukaryotic cells in peroxisomes organelle (del Rio et al., 1992). Catalase enzyme is activated at high concentrations of H₂O₂. Two molecules of H₂O₂ are needed in the active site of catalase to convert them to H₂O (Halliwell and Gutteridge, 1990).
Glutathione Peroxidase (GPx)

Glutathione peroxidase enzyme is responsible for the conversion of hydrogen peroxide to water using glutathione (GSH) in its reduced form as substrate. This reduced form of GSH converts to oxidized glutathione form (GSSG) (Halliwell and Gutteridge, 1990).

\[
H_2O_2 + 2 \text{GSH} \rightarrow 2 \text{H}_2\text{O} + \text{GSSG}
\]

The oxidized form (GSSG) is reduced by glutathione reductase to GSH. Therefore, the ratio GSH/GSSG should be maintained (Halliwell and Gutteridge, 1990).

\[
\text{GSSG} + \text{NADPH} + \text{H}^+ \rightarrow 2 \text{GSH} + \text{NADP}^+
\]

GPx enzyme is present in the cytoplasm and the matrix of mitochondria. Selenium is found in the active site of this enzyme (Brigelius-Flohe, 1999).

Both catalase and glutathione peroxidase take part in the breakdown of \(H_2O_2\) to \(H_2O\). The concentration of \(H_2O_2\) determines which enzyme will be activated. At higher levels of \(H_2O_2\), it is metabolised by catalase enzyme while, at lower concentrations, it is converted by GPx enzyme (Chance et al., 1979).

1.3.2.3.2 Antioxidant Defence System

Antioxidants are compounds that can scavenge free radicals and convert them to non-reactive molecules; these compounds are stable in the presence of free radical electrons (Young and Woodside, 2001). Antioxidants are classified into synthetic antioxidant like
butylated hydroxyltoluene (BHT) and natural antioxidant such as vitamin C and plant polyphenols. One of the most important subjects in human health and nutrition is the role of antioxidants to reduce lipid oxidation that form secondary oxidation products, which have harmful effects on the human body as they are linked with several diseases (Halliwell and Gutteridge, 1990; Niki et al., 2005; Lobo et al., 2010). Antioxidants such as vitamin E (tocopherols) and vitamin C (ascorbic acid) are commercially added to food products to retard lipid oxidation. Based on their structures, both components have the ability to prevent fat oxidation processes and therefore prevent ROS production (Flora, 2009).

A. Synthetic Antioxidants

Synthetic antioxidants are cheaper to produce and are more stable than natural antioxidants. Butylated hydroxytoluene (BHT) and butylated hydroxyanisole (BHA) are the most used synthetic antioxidants in the food industry (Iverson, 1999), although their use is restricted in some countries due to the possible effects on some enzymes in the human body (Nakatani, 1997). Therefore, research on natural antioxidants has witnessed an increase due to these safety concerns (Pokorny, 2007).

**Butylated hydroxy toluene (BHT)**

Butylated hydroxytoluene (BHT) (Figure 1.8) is a hydrophobic synthetic antioxidant that minimises lipid oxidation by donating on hydrogen atom to a free radical made during the initiation or the propagation step to produce a stable, unreactive molecule. Although, BHT is used as food an additive to prevent food spoilage, there are many safety concerns (Williams et al., 1999).
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Figure 1.8: The chemical structure of butylated hydroxy toluene (2,6- bis (1,1-dimethylethyl) – 4 methylphenol. Source: Gülçin et al., 2009

B. Natural Antioxidants

The concern about using natural antioxidants in food has increased over the past 20 years due to consumer concern of using synthetic antioxidants in food. Plant products like vegetables e.g. (broccoli, onion and tomato) (D’Archivio et al., 2007), fruits (e.g. strawberry, apple and pomegranate), herbs (oregano, thyme and rosemary) (Viuda-Martos et al., 2010) and spices (cinnamon, cloves and chilly pepper) contain natural antioxidants (Dai and Mumper, 2010). Consumption of these antioxidants (polyphenols and flavonoids) may have a role in the prevention of chronic diseases like hypertension, cardiovascular diseases (Aviram and Dornfeld, 2001) and cancer (Ames et al., 1993).

The phenolic compounds often exhibit similar or greater activity than synthetic antioxidants, for example, ethanolic extract from peanut hulls (1.2 mg/ml) showed significant antioxidant activities similar to BHA and catechin (Yen and Duh, 1994). Moreover, tannic acid showed the highest scavenging activity for H2O2 compared with α-tocopherol, BHT and trolox (Gülçin et al., 2010). The most common and effective antioxidant used in food products is ascorbate (Figure 1.9). Ascorbate is a very reactive
compound that acts as a reducing agent and, therefore, has the ability to scavenge oxygen (McGregor and Biesalski, 2006). It binds with an unpaired electron in the free radical and forms stable molecule; it also chelates metal ions (McGregor and Biesalski, 2006).

![Chemical structure of ascorbic acid](image1.9)

*Figure 1.9: Chemical structure of ascorbic acid. Source: Dragsted, 2008*

The other natural antioxidant that has been used as a food additive is trolox, (Figure 1.10). It is a water-soluble analogue of vitamin E as the presence of hydroxyl groups reduces the hydrophobicity of the compound. The hydroxyl group can stop lipid autooxidation by interfering with either the chain propagation or the decomposition processes. Due to its powerful antioxidant activity, it is usually used as a positive control standard during any experiment in order to investigate the antioxidant activity of other substances (Poljsak and Raspor, 2008).

![Illustration of the chemical structure of trolox](image1.10)

*Figure 1.10: Illustration of the chemical structure of trolox. Source: Menichetti et al., 2005*
Antioxidants can induce their effect via different mechanisms, which can be classified as:

1. Antioxidants having one or more hydroxyl groups have the ability to donate hydrogen atom or electron to the lipid oxidation species and produce stable molecules (Flora, 2009).

2. Antioxidants have the ability to chelate metals such as Fe\(^{2+}\) that can initiate free radical formation (Flora, 2009).

Antioxidants are normally added to food for two reasons:

1. To avoid food taste changes by delaying lipid oxidation caused by processing or long storage (Pokorny, 2007).

2. To reduce free radical concentration in the body after food ingestion and, therefore, controlling any cellular changes that may lead to diseases, such as strokes, atherosclerosis and cancer (Pokorny, 2007).

Polyphenols are antioxidants from plant foods that react in the body to enhance health and protect the human body from different diseases by different mechanisms. In fact, polyphenols are used as food additives because they are rich sources of natural antioxidants such as vitamin E, C and \(\beta\)-carotene. One of the important mechanisms of antioxidants as protective agents is their ability to inhibit lipid peroxidation. Several studies showed the ability of phenolic compounds to prevent lipid peroxidation (Dinis et al., 1994; Murcia and Martinez-Tome, 2001; Peng and Kuo, 2003). To this end, the focus of this research is to investigate these mechanisms further. Pomegranate is a fruit that has different phenolic compounds such as punicalagin as mentioned earlier. The cyto-protective effect of punicalagin against
lipid peroxidation and other mechanisms was examined in order to investigate the antioxidant properties of this compound.

1.4 Antioxidant Activity of Pomegranate

Antioxidants are compounds that have the ability to delay or inhibit the oxidation of oxidisable molecules through scavenging free radicals and reducing the oxidative stress (Ames et al., 1993).

Pomegranate contains high levels of flavonoids and polyphenols that have potent antioxidant protection against heart disease and cancer (Aviram et al., 2000). Anthocyanidins cyanidin, pelargonidin and delphinidin are three antioxidant types found in pomegranate, which have shown inhibitory effects on $H_2O_2$-induced lipid peroxidation in rat brain homogenate with delphinidin being the most potent and pelargonidin the least (Halvorsen et al., 2002).

The ability of dietary flavonoids to prevent lipid peroxidation in Caco-2 cell lines has been investigated by Peng and Kuo, (2003a). Quercetin, myricetin, luteolin and epigallocatechin gallate (EGCG) inhibited lipid peroxidation in Caco-2 cell lines whereas kaempferol, genistein and daidzein did not. Seeram et al., (2005) described the antioxidant activities of punicalagin, ellagic acid (EA), total pomegranate tannin (TPT) and pomegranate juice (PJ) on human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1,22Rv1) tumor cells. The trend of antioxidant activity was highest for PJ then TPT, followed by punicalagin and EA had the lowest activity (Seeram et al., 2005). Alia et al., (2006) studied the catalase and SOD levels in liver cells (HepG2) pretreated with 50 and 100 $\mu$M of quercetin and rutin and then
incubated for 3 hours with 3 mM T-BOOH; there were no significant changes in both enzyme levels. Similar results were found for oral cell line (NHEK, OSC-2 and OSC-4) treated with 100 μM epigallocatechin gallate (green tea polyphenol) (Yamamoto et al., 2004). Lima et al., (2006) indicated that polyphenol showed experimental protective effect against oxidative damage induced by T-BOOH. Luteolin (200 μM), 45 μM quercetin, 370 μM caffeic acid and 180 μM rosmarinic acid inhibited the glutathione depletion caused by 2 mM T-BOOH in liver cell line (HepG2) (Lima et al., 2006). Further, when oxidative stress was induced by 2 mM T-BOOH for 5 hours in liver cell line HepG2 (Lima et al., 2007), the addition of methanolic and water polyphenol extracts from Salvia plant significantly prevented glutathione depletion. Pomegranate fruit extract PFE showed higher antioxidant activity than tea polyphenol epigallocatechin-3-gallate, in human lung carcinoma A549. The PFE extract showed a dose-dependent decrease in A549 cancer cell viability and smaller effects on normal human bronchial cells (Khan et al., 2007).

In this thesis, human colon carcinoma cell line (Caco-2) was used to investigate the antioxidant activity of pomegranate compound (punicalagin) under oxidative stress caused by tert-butylhydroperoxide (T-BOOH). Caco-2 monolayer cell line exhibits features closely resembling intestinal epithelial cells such as tight junctions and brush border microvilli when grown under normal conditions (Pinto et al., 1983). Therefore, Caco-2 cell line has been used as a model to study the transportation of compounds like fatty acids, polyphenols and flavonoids through the intestinal epithelium (Meaney and O'Driscoll, 2000; Aherne and O'Brien, 2000; Tian et al., 2009). In addition, Caco-2 cells are used as an in-vitro model for many other purposes such as studying the potential absorption of drugs (van Breemen and Li, 2005), investigating the
biotransformation of compounds (Dai et al., 2008) and investigating the protective effects of natural antioxidants against oxidative stress (Aherne and O'Brien, 2000).

Tert-butyl hydroperoxide (T-BOOH) is an organic peroxide that can be converted to other alkoxyl and peroxyl radicals by cytochrome P450 enzymes and by free metal ions that can produce ROS. Consequently, these free radicals initiate lipid peroxidation chain reactions, produce cell toxicity by reducing glutathione level, damage the DNA and affect the membrane potential of mitochondria, and consequently cause cell death (apoptosis) (Kim et al., 1998; Aherne and O'Brien, 2000; Lima et al., 2006).

Several studies have shown that high intakes of polyphenols may protect the human body from cardiovascular diseases (Aviram and Dornfeld, 2003; Adams et al., 2006). The possible protective mechanism against heart diseases could be related to the polyphenols effect, namely anti-platelet aggregation, vasodilator properties and prevent LDL oxidation. In this study, the effect of punicalagin as an antihypertensive agent has been studied via different mechanistic pathways.

1.5 Cardiovascular Disease (CVD)

1.5.1 Cardiovascular Disease Epidemiology

Cardiovascular disease affects the circulatory system (heart, artery and blood vessels). This disease is a leading cause of death all over the world (Heidenreich et al., 2011). In the USA, according to the American Heart Association report in 2013, cardiovascular disease (CVD) deaths were 21.42% (Go et al., 2013). According to the World Health Organization (WHO, 2013), in the United Kingdom, CVD was one of the main causes
of death in 2011 reaching about 19.77%, while in Saudi Arabia the death rate from CVD was 23.98% in the same year (Finegold et al., 2012).

1.5.2 Risk Factors Related to CVD

There are several factors related to this disease such as age, gender (Jousilahti et al., 1999), high blood pressure (hypertension), family history, high level of serum cholesterol, smoking, extreme alcohol consumption, being overweight, lack of physical activity, and high blood glucose level (diabetes mellitus). In addition, the cardiovascular function in healthy people can be altered due to some physiological and morphological changes related to aging (Kurian and Cardarelli, 2007). Each factor has individual contributions in every country, based on people’s life styles.

Cardiovascular disease can be reduced by avoiding some risk factors like increasing physical activity, eating healthily, managing weight, avoiding smoking and stopping or decreasing alcohol consumption.

One important factor associated with increasing cardiovascular disease is high blood pressure (hypertension) (Lin et al., 2004) which has increased the number of deaths worldwide. According to WHO, in 2011 the mortality rate by this disease was 11, 3 and 109 per 100,000 of population in the United State, the United Kingdom, and Saudi Arabia, respectively. This rate ranks US, UK and Saudi Arabia number 152, 183 and 4 respectively in the world (WHO., 2013). Regulating blood pressure can be achieved by physical exercise, losing weight, and minimising salt consumption (Devi et al., 2013).
1.5.3 Cardiovascular Disease and ROS

Oxidative stress that initiates increased ROS level leads to heart disease (Paravicini and Touyz, 2006). One of the ROS production pathways in endothelial cells is through the stimulation of the redox signaling cascade like NADPH oxidase. This enzyme is stimulated by angiotensin II (Rey et al., 2001). High levels of superoxide radical \( \text{O}_2^– \) and low levels of endogenous antioxidant enzymes were observed in the blood of hypertension patients (Paravicini and Touyz, 2006).

1.5.4 Angiotensin Converting Enzyme (ACE)

In the human body, blood pressure is regulated by several mechanisms, one of which is the renin-angiotensin system (RAS) (Hall, 1991). Angiotensin converting enzyme (ACE) is one of the key elements of this system. This enzyme is found in many tissues including lung, endothelial cells, epithelia kidney cells and brain tissues. The ACE enzyme has two active sites; the N domain and the C domain. Both of these catalytic domains are zinc dependent (Hubert et al., 1991) as illustrated in Figure 1.11.

ACE enzyme converts the inactive biological molecule decapeptide (angiotensin I) to the potent octapeptide (angiotensin II) by removing the dipeptide His-Leu from the C-terminal in angiotensin I (Hubert et al., 1991).

\[
\text{Angiotensin-I} \quad \text{(Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu)} \\
\downarrow \\
\text{Angiotensin-II} \quad \text{(Asp-Arg-Val-Tyr-Ile-His-Pro-Phe)} - \text{His-Leu}
\]
Angiotensin II acts as a vasoconstrictor leading to blood pressure increase, as well as prothrombotic action via adhesion on the aggregation of platelets (Benigni et al., 2010) (Figure 1.12). Therefore, inhibiting the activity of ACE enzyme is a target for pharmacologists to treat hypertension patients. Several ACE inhibitor mechanisms on the market are based on the strong binding between the zinc atom present in the active site of the enzyme and the strong chelating group on the inhibitor (Acharya et al., 2003).
Several studies have concluded that the inhibition of ACE by synthetic drugs like captopril or natural inhibitors like polyphenols provide antihypertensive effect in hypertension patients (Aviram et al., 2000). The synthetic inhibitors of ACE enzyme such as captopril and lisinopril cause side effects like headaches, dizziness and cough in hypertension patients (Rossetto, 1987). In contrast, the natural ACE inhibitors like polyphenols do not exert any undesirable effects on the human body (Tabassum and Ahmad, 2011). Therefore, extensive research has been performed on natural products for use as alternative treatments for hypertension disease. The powerful effect of pomegranate juice on hypertension patients was studied by Aviram on ACE enzyme inhibitor, in 2001. A two-week consumption of pomegranate juice (50 ml) inhibits about 36% of the ACE enzyme activity (Aviram and Dornfeld, 2001).
Numerous studies were conducted on polyphenols to examine their effects as inhibitors of ACE activity, for example flavan-3-ols (epicatechin and catechin) inhibited ACE enzyme in rabbit lung (Actis-Goretta et al., 2003). A significant inhibition on ACE enzyme activity was observed in endothelial cell line treated with green tea and black tea and their flavanols (epicatechin, epigallocatechin, epicatechingallate and epigallocatechingallate) (Persson et al., 2006).

1.5.5 Effect of Polyphenols on Nitric Oxide (NO) Production

In cardiovascular systems, nitric oxide plays an important role in human health, such as vasodilation of the arteries and decrease in the stickiness of platelets (Dejam et al., 2004). The generation of NO in blood vessels is involved in the regulation of vessel functions. L-arginine (amino acid) is a source of NO due to the action of nitric oxide synthase in the presence of O$_2$ (Andrew and Mayer, 1999). Nitric oxide synthase enzyme has three isoforms: inducible (iNOS) isoform, which is independent of calcium ion; neuronal (nNOS) and endothelial (eNOS) are both calcium-dependent enzymes (Andrew and Mayer, 1999).

nNOS

Neuronal nitric oxide synthase (nNOS) generates NO in nervous tissues. It is located in the plasma membrane and has a role in cell communication. It is a calcium-dependent enzyme (Andrew and Mayer, 1999).

iNOS

Inducible nitric oxide synthase (iNOS) produces NO in an oxidative environment. The large amount of NO can react with superoxide and form peroxynitrite, which induces
cell toxicity. It is a calcium-independent enzyme (Andrew and Mayer, 1999).

**eNOS**

Endothelial nitric oxide synthase (eNOS) enzyme is localised in caveolae (small invagination of the plasma membrane). This enzyme is bound with fatty acids; one molecule of myristate and two molecules of palmitate. The enzyme remains inactive until calcium calmodulin replaces caveolin and binds to eNOS, thus activating the enzyme as illustrated in Figure 1.13 (Andrew and Mayer, 1999).

![Figure 1.13: Endothelial nitric oxide synthase cell location. Source: Michel and Feron, 1997](image)

For several years, researchers investigated the antioxidant activity of polyphenol compounds such as cyanidin, catechin and gallic acid for human health protection from cardiac disease (Morton *et al.*, 2000; Actis-Goretta *et al.*, 2003; Wang *et al.*, 2007). Red wine polyphenols have the ability to increase the activity and the expression of eNOS enzyme as well as increasing calcium concentration in cytoplasm leading to increased levels of NO (Leikert *et al.*, 2002). Additional research on pomegranate juice
found that NO level was increased without any significant effect on eNOS enzyme activity or expression. The researchers explained the significant increase of NO to be due to the antioxidant property of pomegranate juice that protects NO from ROS destruction (Ignarro et al., 2006). Resveratrol, epicatechingallate and epigallocatechin have significant effects on eNOS expression and, therefore, the level of NO was found to increase in EA.hy926 cell line (Appeldoorn et al., 2009).

In the present study, human endothelial immortalised cell line EA.hy926 has been used as a model for endothelial cell line to investigate the effect of pomegranate compound (punicalagin) as an angiotensin converting enzyme inhibitor and nitric oxide synthase enzyme activator. The EA.hy926 cell line is derived from the invasion of HUVEC (human umbilical vein endothelial cells) with lung carcinoma cell line A549 (Edgell et al., 1983). In the endothelial cell line, ROS are generated from NADPH oxidase enzyme system. The activation of this enzyme and the high level of ROS are implicated in angiotensin II-dependent hypertension associated with endothelial dysfunction (Rajagopalan et al., 1996). EA.hy926 cells were used as a model to measure the expression of eNOS enzyme after exposure to different phenolic compounds (Appeldoorn et al., 2009).

Chronic disease prevention and aging can be partly controlled through the dietary intakes of plant polyphenols. Therefore, it is crucial to improve our knowledge of polyphenol availability from diet. Although pomegranate components may offer ways of treatment and health benefits, extensive studies are still required before they can be recommended for regular human consumption.
As described previously, under the harmful effect of oxidative stress, high levels of ROS are produced in living cells, thus causing chronic diseases like cancer. Therefore, ROS scavenging is necessary in order to prevent cancer and protect the human body from ROS damage. Dietary antioxidants such as polyphenols are reported to have anti-carcinogenic activity (Malik et al., 2005, Zhang et al., 2008) and cardiovascular diseases prevention properties (Ignarro et al., 2006; Edirisinghe et al., 2011; Hidalgo et al., 2012) due to their antioxidant effects. In this research, the anti-carcinogenic effect of pomegranate punicalagin has been investigated through different mechanistic pathways.

1.6 Reactive Oxygen Species and Cancer Disease

Oxidative stress is a phenomenon resulting from the damage caused by free radicals on biological macromolecules like lipid, protein and nucleic acids (Florence, 1995; Becker, 2004). Free radicals are normally generated in the cell during metabolic processes. The imbalance in ROS production and degradation leads to initiation of several diseases such as cancer (Florence, 1995).

1.6.1 Cancer Definition

Cancer disease can be defined as uncontrolled cell growth and division. These cells can migrate and invade other tissues through the lymphatic system or bloodstream and affect any part of the body (Hanahan and Weinberg, 2000). Cancer occurs in several steps (Figure 1.14). The first is the initiation step when chemicals, metabolic, environmental factors and genetic factors, damage the DNA. Promotion is the second long step, which starts with the proliferation of the defected cells in the initiation
process. The final step is progression described as the metastasis of tumor cells developed in the proliferation step (Doll and Peto, 1981).

![Diagram of carcinogenesis](image)

**Figure 1.14: Illustration of the process of carcinogenesis.** Source: Dai and Mumper, 2010

### 1.6.2 Cancer Incidence and Morbidity

Globally, cancer is a leading cause of death, and was responsible for approximately 13% of deaths in 2008. The most common types of cancer globally are lung (1.37 million deaths), stomach (736 000 deaths), liver (695 000 deaths), colon (608 000 deaths), and breast (458 000 deaths) (Ferlay et al., 2011). All over the world, the incidence of colorectal cancer is the third most common type and it is the fourth most common type of death (Ferlay et al., 2011). Worldwide, there are several causes of this
disease such as ultraviolet radiation (Armstrong and Kricker, 2001), smoking (Secretan et al., 2009), drinking alcohol (Secretan et al., 2009), unhealthy diet (high calories intake and low fiber supply) (La Vecchia, 1992), and chronic infection from hepatitis B (HBV) (Buendia, 1992).

1.6.3 Treatment and Prevention Strategies

Several types of cancer can be treated if detected in the early stages (Duffy et al., 2010). The awareness of some cancer signs and symptoms helps in early diagnosis and can be treated before the disease metastases. Regular screening programmes for some organs (colon, breast, stomach and cervix) can be effective for preventing this disease (Smith et al., 2007). The treatment of this disease depends on the stages; the treatment can be either surgical, chemotherapy or radiotherapy, or in combination in advanced stages (Samee and Selvasekar, 2011). There are several side effects of these treatments such as appetite loss, hair loss or thinning, fatigue, nausea, vomiting, skin and nail problems, diarrhoea or constipation and effects on some organs like kidney, heart and sexual organs (Shapiro and Recht, 2001).

Risk of cancer can be reduced by avoiding risk factors such as smoking, increasing intake of fruits and vegetables, performing physical activities, reducing exposure to sunlight and decreasing the amount of alcoholic drinks (Anton-Culver, 1995).

1.6.4 Dietary Polyphenols and Cancer Prevention

Life style and diet are closely linked to the incidence of colon cancer. One of the prevention strategies of this disease is increasing fruit and vegetable intakes as they are rich in bioactive compounds (Potter et al., 1993). These bioactive compounds, which
are found in plant food (vegetables and fruit) or drink (tea, coffee and wine), are phenolic compounds that have beneficial health effects in addition to their basic nutrition effect (Hollman et al., 1997; Hartman et al., 2006; Kasimsetty et al., 2010a). They have the ability to change some biochemical processes involved in cancer disease (Pozo-Guisado et al., 2004; Afaq et al., 2005; Gonzalez-Sarrias et al., 2009).

These polyphenols may inhibit cell proliferation in different cancer cell types through induced apoptosis by arresting cell cycle in a specific phase (Seeram et al., 2005; Hafeez et al., 2008). The antioxidant activity of these compounds can inhibit the oxidative damage done by ROS scavenging (Lima et al., 2006). Several studies propose that the mechanisms used by these anticancer compounds include antioxidant and anti-proliferative activities by stimulating apoptosis and arresting the cell cycle through the activation of subcellular signaling pathways, (Yang et al., 2001).

1.6.5 Cell Cycle Process

Cancer cell growth and metastases can be prevented by specific cell cycle regulation. There are groups of proteins such as cyclin-dependent kinase groups that act in specific pathways to determine whether the cell cycle will go forward or remain between stages (Nigg, 1995). The cell cycle is a sequential process, which takes place in a cell, leading to its division and duplication. There are three stages for cell division; the first called the interphase, the second is the mitotic phase (M), and the final stage is cytokinesis (Alberts et al., 2002). The precise completion of one stage leads to activation of the following stage.

Interphase is the longest phase; it consists of four steps: Gap 0 phase (G0), Growth phase 1 (G1), S phase (synthesis) phase, and Growth phase 2 (G2). These steps take
around 12 to 24 hours in mammalian tissues by which the cell produces protein synthesizing RNA, and grows in size (Alberts et al., 2002).

**G0 phase** is the arresting time when the cell leaves the cycle and stops dividing.

**G1-phase** is when the cell size increases and starts producing RNA and synthesises protein. G1 checkpoint is an important control mechanism activated in this step to ensure that everything is prepared for DNA synthesis (Alberts et al., 2002).

**S-phase** is where the replication of DNA starts. The aim of this duplication is to produce exactly two identical chromosomes in the new daughter cells. Accuracy is important in this step to prevent any genetic abnormalities that often lead to cell death or disease (Alberts et al., 2002).

**G2-phase** is one in which cells remain growing and produce new proteins. By the end of this step another control checkpoint (G2 checkpoint) is used to check whether the cells are ready to enter the new stage, the mitosis phase (M), and divide (Alberts et al., 2002).

**Mitosis-phase** is the where the chromosomes split between the two daughter cells this phase is shorter than the interphase. Similar to G1 and G2, there is a checkpoint in the middle of this phase to ensure that the cell is ready to complete division (Alberts et al., 2002).

**Cytokinesis** is the final stage in the cell cycle initiated at the late stage of mitosis (Alberts et al., 2002). The whole cell cycle process is illustrated in Figure 1.15.
1.6.6 Cell Death Mechanisms

There are several cell death mechanisms, as illustrated in Figure 1.16. Apoptosis is a type of cell death programmed process, characterised by changing cell morphology leading to cell death (Rowan and Fisher, 1997). The apoptosis pathway is induced by anticancer therapy through the induction of the intracellular cysteine family (caspase family) (Persson et al., 2009). Cell apoptosis is controlled via intrinsic or extrinsic pathways. Caspase activation protein, which is released from mitochondria, is responsible for the intrinsic pathway. The extrinsic pathway is controlled by transmembrane death receptors like tumor necrosis factor (TNF-R) (Seeram et al., 2005). The other cell death type, called necrosis, is characterised by cell swallowing and injury. The cells spill all their contents in the intracellular cell space and induce inflammation around the dying cell (Edinger and Thompson, 2004a). Another non-
apoptotic cell death is autophagy meaning eat oneself. In autophagy, during the period of nutrient stress, the cell components are degraded to produce energy referred to as the catabolic metabolic programme (Edinger and Thompson, 2004a).

Figure 1.16: Representation of different cell death mechanism. Source: Zappavigna et al., 2013

1.6.7 Pharmacological Effect of Pomegranate on Mammalian Cell Line

1.6.7.1 Proliferation

The chemical components of pomegranate seeds, juice and peels are reported to have pharmacological effect in mammalian cells that may prevent or be used in the treatment
of cancer as discussed below. Epidemiological studies suggest a protective role of pomegranate as anticancer and anti-proliferative activities (inhibit cell growth). In 2006, Larrosa and colleagues found that ellagic acid (EA) produced from hydrolysable tannin and dietary ellagitannins (pomegranate punicalagin “PUNI”) both inhibit the proliferation of Caco-2 cell line in a dose-and time-dependent manner at level of 30 μmol/L and 100 μmol/L, respectively. Cell viability decreased at 24 hours, especially in the presence of PUNI (100 μM) (Larrosa et al., 2006b) and a further decrease was shown when a mixture of EA and PUNI was added (Larrosa et al., 2006b). Another in vitro study done by (Seeram et al., 2005) evaluated anti-proliferative activities of punicalagin, ellagic acid (EA), total pomegranate tannin (TPT) and pomegranate juice (PJ). The effects of these components were studied on human oral (KB, CAL27), colon (HT-29, HCT116, SW480, SW620) and prostate (RWPE-1,22Rv1) tumor cells. Pomegranate juice showed the greatest anti-proliferative effect against all cell lines.

1.6.7.2 Apoptosis

Several cancer research studies have focused on the types of cancer cell death mechanisms. A study of the apoptotic effect of punicalagin, ellagic acid (EA), total pomegranate tannin (TPT) and pomegranate juice (PJ) on (HT-29 and HCT116) colon cancer cell lines showed that apoptosis was induced by PJ, EA, punicalagin and TPT in HT-29 colon cells at 100 μg/ml. However, EA, punicalagin and TPT (but not PJ) induced the apoptosis in HCT116 colon cells (Seeram et al., 2005). PUNI and EA significantly increased apoptosis in Caco-2 cells; the highest early apoptotic cell population was at 24 and 48 h in the presence of EA and PUNI, respectively (Larrosa et al., 2006b). In addition, the anti-carcinogenic effect is mainly due to the pomegranate ellagitannins hydrolysis product, EA that induces apoptosis in human colon cancer cell
line, via activating caspase 9 and effector caspase 3 due to the release of cytochrome C into the cytosol. The cell cycle of Caco-2 cells was arrested in the S phase (Larrosa et al., 2006b).

From previous studies it is clear that pomegranate may have a cancer-chemo-preventive effect as well as a 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. Pomegranate extraction showed a significant anti-proliferative and pro-apoptotic activity against highly aggressive human prostate cancer PC3 cells at (10-100 μg/ml, 48 hours) and resulted in a dose-dependent inhibition of cell growth/cell viability and apoptosis induction (Li et al., 2002). Also, it depicted alteration in the regulatory molecules arresting in the G1 phase of the cell cycle (Malik et al., 2005). 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).

Punicalagin is an ellagitannin (type of phenolic compound). It is considered to be the main compound in pomegranate husk (Chen and Li, 2006; Kulkarni et al., 2007; Lee et al., 2008). Punicalagin is reported to have anti-cancer, anti-inflammatory and anti-atherosclerotic properties (Chen et al., 2000; Seeram et al., 2005; Kulkarni et al., 2007). Therefore, some bioactive properties of punicalagin were investigated in this research.
Figure 1.17: The chemical structure of punicalagin. Source: Larrosa et al., 2006a
1.7 Aim and Objectives

To this end, it is clear that there is a growing interest in plant products such as polyphenols because of their bioactive properties that may be used for disease protection. In the present study, more attention was focused on the mechanistic that promote antioxidant, anti-carcinogenic and antihypertensive activity. For this reason, the aim of this research was to investigate some of the bioactive properties of ellagitannin “punicalagin” class of polyphenols through different *in-vitro* mechanisms.

To achieve this goal the following was carried out:

1. The radical scavenging and antioxidant activity of pomegranate and punicalagin was determined.
2. The protective effects of punicalagin under oxidative stress conditions caused by tert-butyl hydroperoxide treatment in a Caco-2 intestine cell line were studied.
3. The inhibitory effect of punicalagin on angiotensin converting enzyme (ACE) and induction of nitric oxide (NO) production in the EA.hy926 endothelial cell line was examined.
4. The effect of the active component of pomegranate punicalagin on human colon cancer Caco-2 cells was investigated.
Chapter 2
2 Antioxidant Activity of Pomegranate Juice and Punicalagin

2.1 Introduction

Antioxidants are compounds that scavenge free radicals and convert them to non-reactive molecules. These compounds are converted to stable molecule after quenching or scavenging free radical (Young and Woodside, 2001).

Antioxidants are essential components of the human diet and recently there has been a great interest in using rich sources of natural antioxidants such as plants and food additives as they consist of vitamins (vitamin E, C and β-carotene) and plant polyphenols. Natural antioxidants found in nutraceuticals and functional food plants are more advantageous than synthetic antioxidants like butylated hydroxytoluene (BHT) because of their ability to protect food against free radicals and reactive oxygen species (ROS) damage and reduce risk of chronic disease (Finley et al., 2011).

It is well known that human health condition is partly controlled through the dietary intake of plant polyphenols. Antioxidants prevent food degradation and thus are used as food additives (Gulcin, 2012). It is, therefore, vital to improve our knowledge of polyphenol availability from diet.

Pomegranate fruit contains many phenolic compounds including flavonoids-anthocyanins, and other complex flavanoids and hydrolyzable tannins (punicalagin, gallic and ellagic acid), which are compounds with high antioxidant activity that may offer beneficial health properties. Around 92 % of pomegranate antioxidant activity was
comes from hydrolysable tannins (Passamonti et al., 2003). Punicalagin, ellagic acid and gallic acid are the polyphenols found in pomegranate (Gil et al., 2000). The main component of pomegranate husk is punicalagin (Chen and Li, 2006; Kulkarni et al., 2007; Lee et al., 2008). Punicalagin is reported to have anti-inflammatory, anti-cancer and anti-atherosclerotic properties (Chen et al., 2000; Seeram et al., 2005; Kulkarni et al., 2007).

Investigations on pomegranates show high antioxidant and anti-proliferative, anti-cancer and pro-apoptotic activity in various cancer cell lines and animal models (Chen et al., 2000; Seeram et al., 2005; Kulkarni et al., 2007). Pomegranate polyphenols are thus considered as agents capable of restraining the effect of ROS on the body (Chen et al., 2000, Seeram et al., 2005).

Radicals are molecules with unpaired electrons that are highly reactive, e.g. hydroxyl radical OH• and superoxide radical O2•-. Radicals form in all living organisms in normal metabolic pathways during oxidation reactions. The free radical concentration level increases under certain circumstances e.g. environmental stress, wounding and pathogen attack, and can damage the living organisms when left unchecked. Where cell membranes consist of unsaturated lipids (Halliwell, 1991), free radicals are reactive molecules that have the ability to react and damage all types of bio-molecules-lipid, proteins, carbohydrates and DNA. This damaging effect could lead to several diseases e.g. coronary heart disease, inflammation and cancer (Wolfe et al., 2008). Pomegranate juice was, nevertheless, found to exert potent antioxidant activity against lipid peroxidation (Malik et al., 2005).
The objectives of this chapter are:

- To determine the total polyphenol and flavonoid content in pomegranate juice.
- To determine the presence of punicalgin in pomegranate seed and husk.
- To determine the antioxidant activities of punicalagin and pomegranate juice by using four methods:
  1) Radical scavenging method determined by DPPH and H$_2$O$_2$ scavenging for punicalagin and pomegranate juice at various concentrations.
  2) Ferrous chelating activity of punicalagin and pomegranate juice at different concentrations.
  3) Determining the reducing power for each compound at several concentration levels.

2.2 Materials and Methods

2.2.1 Materials

Trolox, 2,2-Diphenyl-1-picrylhydrazyl (DPPH), FeCl$_2$.4H$_2$, Ethylenediaminetetraacetic acid (EDTA), Butylated hydroxytoluene (BHT), ferrozone ascorbate, H$_2$O$_2$, potassium ferricyanide, phosphate buffer, ferric chloride, gallic acid, catechin, Folin-Ciocalteu reagent, sodium nitrite, aluminum chloride, glacial acetic acid, acetonitrile, and formic acid were obtained from Sigma-Aldrich Chemical Co, (Pool, UK). Ethanol, methanol, trichloroacetic acid (TCA), sodium carbonate and sodium hydroxide were purchased from Fisher Scientific (Loughborough, UK). Phenomenex Synergi 4 μm Hydro-RP
Chapter 2: Antioxidant Activity of Pomegranate Juice and Punicalagin

80A column (250 mm x 4.6 mm x 5 µm) and Phenomenex C18 5 µm column (250 mm x 3.0 mm) were obtained from Torrance, CA, USA.

UVIKontron 860 spectrophotometer (Zurich, Switzerland) was used in all the experiments, described in this chapter, to measure the specific wave length for each experiment.

2.2.2 Methods

2.2.2.1 Sample Preparation

Fresh pomegranates were harvested in Spain in September, 2011 were purchased from the local store and peeled, and the edible portions (seeds and arils) were juiced then stored at -80 °C overnight. The resulting pomegranate juice (PJ) was then freeze-dried for 7 days. The freeze-dried pomegranate (powder) was stored at -80 °C until analysed.

2.2.2.2 Determination of Total Phenolic and Total Flavonoid Content

The total phenolic content of pomegranate juice was determined using the Folin-Ciocalteu method described by Kim et al., (2003). A sample of 1 ml pomegranate juice (40 mg/ml) was mixed with 10 ml of deionised water and 1 ml Folin-Ciocalteu reagent. After 5 minutes, 2 ml of 2% sodium carbonate (w/v) was added to the solution. The mixture was incubated in a dark place at room temperature for 1 hour, whereby the absorbance of the solution was measured at 750 nm. The standard curve was determined with gallic acid (0, 50, 100, 150, 200, 250 and 500 µg/ml) (w/v) dissolved in 50% methanol (v/v). The results were expressed as gallic acid equivalents. Zhishen et al., (1999) used an aluminum chloride colorimetric assay to determine the total
Chapter 2: Antioxidant Activity of Pomegranate Juice and Punicalagin

flavonoid. A sample (250 µl) from (60 mg/ml pomegranate juice) or from the standard (0, 50, 100, 150, 200, 250, 300 and 500 µg/ml of catechin) was added to 1.25 ml deionised water and 75 µl of 5% NaNO₂ (w/v). After 5 minutes, 150 µl of 10% AlCl₃ was added to the mixture and 0.5 ml of 1 M NaOH (w/v) and 275 µl deionised water were added to make up the total volume of the solution to 2.5 ml and measure the absorbance at 510 nm. The total flavonoid content of pomegranate juice was expressed in terms of catechin equivalents. Each set of the three separate experiments was repeated three times in order to verify the results obtained from the determination of both total phenolic and flavonoid contents of pomegranate.

2.2.2.3 Extraction of Sample for HPLC and LC-MS

HPLC and LC-MS were carried out in North Carolina State University, United State. Freeze dried pomegranate samples (1g) were extracted with 25 ml 50% methanol in water, vigorously vortexed at room temperature. Extracted samples were centrifuged at 1500 x g for 3 minutes using a Beckman GRP centrifuge. The supernatants were filtered through a Whatman No.1 filter paper in to a 50 ml volumetric flask. The precipitate was re-extracted with another 25 ml of 50% methanol then centrifuged, and the supernatant was added to the previously collected solution. In order to make up the volume of the volumetric flask, 50% methanol was used. Subsequently, 1ml of the extracted solution was filtered through 0.2 mm PTFE filters in HPLC number vial. Punicalagin standard was prepared by dissolving 0.5 mg in 1 ml of 100% methanol. Samples were then filtered through 0.2 mm PTFE filters into HPLC amber vial for HPLC and LC-MS analysis.
2.2.2.4 HPLC Analysis

HPLC analyses were conducted using Agilent Technologies 1200 series HPLC (Santa Clara, CA, USA) with a photodiode array (PDA) detector and an auto sampler. Chemstation software was used to control the experiment and for quantification of phenolic compounds. Hydrolysable tannin separation was undertaken using a Phenomenex Synergi 4 μm Hydro-RP 80A column (250 mm x 4.6 mm x 5 μm, Torrance, CA, USA). The mobile phase was 2% acetic acid in distilled H₂O (solvent A) and 0.5% acetic acid in 50% acetonitrile in water (solvent B). The flow rate was 1 mL/min with a step gradient of 10%, 55%, 100%, 10% and 10% of solvent B at 0, 10, 13, 15 and 20 min, respectively. Samples, filtered through 0.2 mm PTFE filters, were injecting (10 μL) on the HPLC column (25 °C). Peak areas recorded at 280 nm were quantified using a calibration curve obtained with punicalagin reference standard. (Gonzalez-Barrio et al., 2010).

2.2.2.5 LC-MS Analysis

Following HPLC analysis, the samples were injected on to the LC-MS Electrospray ionization ion-trap time-of-flight mass spectrometry (Shimadzu Scientific Instruments, Columbia, MD, USA) system for structural elucidation. PJE and standards were analysed on Phenomenex C18 column (250 mm x 3.0 mm x 5 μm, Torrance, CA, USA). The mobile phase consisted of 0.1% formic acid in distilled H₂O (solvent A) and 0.1 % formic acid in methanol (solvent B). The flow rate was set at 0.4 mL/min with a step gradient of 5%, 8%, 14%, 14%, 25%, 85% and 5% of solvent B at 0, 5, 15, 25, 30, 32, and 40 min, respectively. Samples were filtered through 0.2 mm PTFE filters before injecting 5 μL on the LC-MS column (25 °C). Quantification of the compound was
performed from the peak areas recorded at 250 nm to the calibration curve obtained with reference standards punicalagin (Lu et al., 2010).

2.2.2.6 DPPH Radical Scavenging Activity

The total radical scavenging capacity of pomegranate juice and punicalagin was determined by the Bersuder et al., (1998) method; this method was used to measure the reducing ability of antioxidants. Trolox and BHT were prepared with a concentration of 0.1 mg/ml dissolved in deionised water whereas alcoholic DPPH concentration was set to 0.02% (w/v) in 99.5% ethanol. Different concentrations of 0.05, 0.1 and 0.15 mg/ml were prepared from pomegranate juice and punicalagin. Both control and blank samples were prepared in triplicate. A sample/control of 500 μl was added to 500 μl 99.5% ethanol, and 125 μl of DPPH was then added to the solution and vortexed thoroughly. All samples were incubated in the dark for one hour; the absorption of the solution was read in the spectrometer calibrated with a phosphate buffer at 517 nm. A blank was also prepared where 500 μl of deionised water was used instead. The DPPH radical scavenging activity was then calculated as follows:

\[
\text{Radical Scavenging Activity(\%)} = \frac{AC_{\text{DPPH}} - AS_{\text{DPPH}}}{AC_{\text{DPPH}}} \times 100
\]

Where: \(AC_{\text{DPPH}}\) represents the absorbance of the control which contains DPPH, and \(AS_{\text{DPPH}}\) refers to the absorbance pomegranate / punicalagin in the presence of DPPH.

2.2.2.7 Scavenging of Hydrogen Peroxide (H\(_2\)O\(_2\))

The ability of pomegranate juice and punicalagin to scavenge hydrogen peroxide was determined by the Gulcin et al., (2005) method. Different concentrations from
experimental samples have been studied (0.05, 0.1 and 0.15 mg/ml water). H_2O_2 (40 mM) was prepared in phosphate buffer saline at pH 7.4 (v/v), and 0.6 ml was added to 1 ml of each pomegranate and punicalagin concentration (0.05, 0.1 and 0.15 mg/ml). The solutions were then incubated for 10 minutes and read at 230 nm. The absorbance of the positive controls of 0.0.1 mg/ml BHT and trolox were measured. The percentage inhibition activity was calculated as follows:

\[
\text{Inhibition Activity(\%)} = \frac{AC_{H_2O_2} - AS_{H_2O_2}}{AC_{H_2O_2}} \times 100
\]

Where \( AC_{H_2O_2} \) is the absorbance of the control with H_2O_2 and \( AS_{H_2O_2} \) is the absorbance of testing sample in the presence of H_2O_2.

2.2.2.8 Ferrous Chelating Activity

Ferrous ion was measured by inhibiting the formation of ferrous-ferrozine complex after adding the components under test (pomegranate juice and punicalagin) following a modified method (Dinis et al., 1994). The chelating activity of substances was measured at 562 nm. Concentrations of 0.05, 0.1 and 0.15 mg of pomegranate juice and punicalagin were investigated. Slight modifications were made to the published method, wherein 1.5 ml of deionised water and 50 \( \mu \)l of 2 mM FeCl_2 (w/v) were added to 500 \( \mu \)l of sample then vortexed. After 30 seconds, 100 \( \mu \)l of 5 mM of ferrozine (w/v) was added to the solution. The final solution was incubated for 10 minutes at room temperature, and its absorption was read at 562 nm. Each sample of the above concentrations was prepared in triplicates with a blank for each concentration. A 0.01% EDTA solution was used as a positive control in this experiment. The chelating activity of the pomegranate juice and punicalagin for Fe^{2+} were calculated as:
\[ \text{Chelating rate(\%)} = \frac{A_0 - A_1}{A_0} \times 100 \]

Where, \(A_0\) is the absorbance of the control that contains \(\text{FeCl}_2\) and ferrozine complex and \(A_1\) is the treated sample in the presence of \(\text{FeCl}_2\) and ferrozine complex.

2.2.2.9 Reducing Power Assay

The reducing power of pomegranate juice and punicalagin were quantified by the Yildirim et al., (2000) method. This method is based on determining the ability of the tested material to reduce \(\text{Fe}^{3+}(\text{CN})_6\) to \(\text{Fe}^{2+}(\text{CN})_6\), in which the formed Perl's Prussian Blue complex was measured at 700 nm. A solution of 1 ml from each sample (with concentrations of 0.05, 0.1 and 0.15 mg/ml of water) was added to 2.5 ml of 0.2 M pH 6.6-phosphate buffer (w/v) and 2.5 ml of 1% potassium ferricyanide (w/v). This mixture was incubated at 50 °C for 30 minutes in a water bath. The reaction mixture was subsequently acidified by adding 2.5 ml of 10% TCA (w/v) and centrifuged at 1600 rpm for 10 minutes at 10 °C. Finally, 2.5 ml of supernatant was mixed with 2.5 ml deionised water and 0.5 ml of 1% ferric chloride (w/v). The resultant mixture was then incubated for 10 minutes at room temperature, after which its absorbance was read at 700 nm; higher absorbance of the reaction mixtures indicates a higher reducing power. This experiment was repeated three times to verify the results.

2.2.2.10 Statistical Analysis

All experiments were presented as mean ± SD. All measurements were replicated three times. The data were statistically analysed using Graph Pad Prism. Differences between pomegranate arils and punicalagin were assessed by unpaired t-test. A one-
way analysis of variance followed by Bonferroni's test testing between treatments and controls was performed. Values of $p \leq 0.05$ were considered significant.

2.3 Results and Discussion

2.3.1 Total Phenol and Flavonoid Contents

Standard curves of gallic acid and catechin were produced, as depicted in Figure 2.1 and Figure 2.2, respectively, to estimate the total polyphenols and flavonoid contents in pomegranate juice and punicalagin.

![Graph](image_url)

**Figure 2.1: Gallic acid standard curve.** Standard curve represented as mean ± SD of gallic acid (0-500 µg/ml) for a total polyphenol content determination. $R^2 = 0.996$. 
The quantitative determination of the total phenolic content was expressed in mg of gallic acid corresponding to 40 mg dry weight of pomegranate arils. The content of polyphenols was 118.56 µg of gallic acid/40 mg and the total flavonoid content of the pomegranate juice was 31.5 µg of catechin/60 mg of dry weight of pomegranate arils.

2.3.2 HPLC and LC-MS Analysis

Methanol extracts of pomegranate husk acquired from a local store in the United States and freeze-dried pomegranate arils from United States and United Kingdom were applied to HPLC. The LC-MS analysis was undertaken for pomegranate husk, pomegranate juice methanol extracts and compared with punicalagin standard. The HPLC and LC-MS chromatograms are illustrated in Figures 2.3, 2.4 and 2.5, respectively. There was a high degree of similarity found between the punicalagin standard and methanol extract from pomegranate husk.
Figure 2.3: HPLC analysis of punicalagin and pomegranate husk. A = punicalagin standard, B = US pomegranate husk in 50% methanol. Separation conditions were: column Phenomenex Synergi 4 µm Hydro-RP 80A (250 mm x 4.6 mm x 5 µm, Torrance, CA, USA). Column temperature: 25 °C. Mobile phase: Solvent A = 2% acetic acid in distilled H₂O, Solvent B = 0.5% acetic acid in 50% acetonitrile in water. Gradient condition: 10, 55, 100, 10 and 10 % at 0, 10, 13, 15 and 20 min, respectively. The flow rate: 1 mL/min, recorded at 280 nm. A = punicalagin standard and B = methanol extract for pomegranate husk.

Figure 2.4: HPLC analysis of punicalagin and pomegranate juice. A = punicalagin standard, B = US handy pomegranate juice and C = UK handy pomegranate juice extracted in 50% methanol. Separation conditions were: column Phenomenex Synergi 4 µm Hydro-RP 80A (250 mm x 4.6 mm x 5 µm, Torrance, CA, USA). Column temperature: 25 °C. Mobile phase: Solvent A = 2% acetic acid in distilled H₂O, Solvent B = 0.5% acetic acid in 50% acetonitrile in water. Gradient condition: 10, 55, 100, 10 and 10 % at 0, 10, 13, 15 and 20 min, respectively. The flow rate: 1 mL/min, recorded at 280 nm.
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Figure 2.5: Identification of punicalagin in methanol extract of pomegranate husk by LC-MS. Identification conditions were: column C18 (250 mm x 3.0 mm x 5 μm, Torrance, CA, USA). Column temperature: 25 °C. Mobile phase: Solvent A= 0.1% formic acid in distilled H₂O, Solvent B= 0.1 % formic acid in methanol. Gradient conditions: 5-85% solvent B followed by 10 min re-equilibration. Flow rate: 0.4 ml/min monitored at 250 nm. A = punicalagin standard, B = methanol extract of pomegranate husk.

The retention time was 17 minutes for the total run of 20 minutes in HPLC and 40 minutes for a total run of 40 minutes. In contrast, the chromatograms resulting from the methanol extract for pomegranate juice from both countries did not show any peak for punicalagin.

Several analytical reports on extracted pomegranate juice manually found that the concentrations of anthocyanin are higher than tannin compounds present in
pomegranate husk such as punicalagin, gallic acid and ellagic acid (Gil et al., 2000; Fischer et al., 2011; Qu et al., 2012). These high concentrations of gallic acid, punicalagin and ellagic acid were found in both pomegranate husk and commercial juice because the majority of phenolic compounds were extracted during the pressing process (Gil et al., 2000; Fischer et al., 2011).

2.3.3 DPPH Radical Scavenging Activity

The mechanism of antioxidant action differs from one component to another; consequently it cannot be assumed that only one mechanism reflects the antioxidant activity of the compounds. For this reason, investigation of different antioxidant mechanisms has been adopted in this study.

DPPH exists as a stabilised free radical, which has a deep violet colour with an absorbance wavelength of 520 nm. In the presence of antioxidants, the DPPH radical form is converted to a DPPH-H non-radical form. The ability to bleach the purple colour to yellow indicates the efficacy of the antioxidant component. When the odd electron in DPPH accepts a hydrogen atom or electron from the antioxidant, the absorbance decreased proportionally due to the increase in the non-radical form of DPPH. The changes in the DPPH radical scavenging effects of pomegranate juice and punicalagin at different concentrations (0.05, 0.1 and 0.15 mg/ml) are shown in Figure 2.6.

The percentages of inhibition caused by pomegranate juice were 14.4, 27.5 and 37.9% for the concentrations (0.05, 0.1 and 0.15 mg/ml), respectively, while the scavenging activity in the presence of punicalagin at the same concentrations was (12.5, 23.9 and
30.8 %), respectively. The DPPH radical scavenging effects increased in proportion to the dose. It was noted that at 0.1 and 0.15 mg/ml pomegranate juice, radical scavenging was significant as compared with punicalagin at the same concentrations (p ≤0.05).

This radical scavenging activity of pomegranate juice and punicalagin at 0.1 mg/ml was compared to 0.1 mg/ml of trolox and BHT individually. DPPH radical scavenging activity was significantly increased by trolox compared to 0.1 mg/ml of pomegranate juice and punicalagin (p ≤ 0.0001) the percent of inhibition was 66, 27.5 and 23.9 % respectively. However, there was no significant difference in DPPH radical scavenging activity between BHT, pomegranate juice and punicalagin (p > 0.05), the percent inhibition was 24, 27.5 and 23.9 % respectively (Figure 2.7 A and B). It is likely that pomegranate juice and punicalagin have the ability to scavenge DPPH radical due to the presence of multiple phenolic hydroxyl groups (Chen and Ho, 1997; Bouchet et al., 1998).
Figure 2.7: Comparison between DPPH radical scavenging activity of punicalagin, pomegranate juice, trolox and BHT. Concentration was 0.1 mg/ml for all components. Values are mean ± SD of three determinations. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (*** = p < 0.0001).

In a research study conducted by Gulcin et al., (2010), tannic acid concentrations between (15-45 μg/ml) produced significant inhibition of DPPH. The scavenging effect for BHT was more significant than tannic acid, while the tannic acid DPPH scavenging activity was more than α-tocopherol and trolox. In contrast to our finding, the scavenging effect of DPPH was not significant when compared BHT with punicalagin or pomegranate juice. In contrast, trolox showed a significant difference when compared with punicalagin (which is hydrolysable tannin) or pomegranate juice (containing tannin). Methanolic extracts of phenolic compounds from peanut hulls (1.5 mg/ml) produced a significant inhibition of DPPH of about 90% scavenging activity. This inhibition of DPPH radicals was similar to the scavenging activity from BHA and catechin at 8 μM and 240 μM respectively (Yen and Duh, 1994).
2.3.4 Scavenging of Hydrogen Peroxide (H\textsubscript{2}O\textsubscript{2})

The ability of pomegranate juice and punicalagin to scavenge H\textsubscript{2}O\textsubscript{2} was measured at 320 nm. The presence of phenolic groups in pomegranate juice and punicalagin give them the ability to donate an electron to H\textsubscript{2}O\textsubscript{2} and convert it to H\textsubscript{2}O (Ebrahimzadeh et al., 2010). There was significant inhibition percent of pomegranate juice compared with punicalagin at the highest concentration (p < 0.001) as depicted in Figure 2.8.

![Figure 2.8: H\textsubscript{2}O\textsubscript{2} scavenging activity of pomegranate juice and punicalagin. Concentrations were 0.05, 0.1 and 0.15 mg/ml. Each value is expressed as mean ± SD of triplicate measurements. Comparisons of means were made using unpaired t-test (** = p < 0.001).](image)

The percent of inhibition activity of H\textsubscript{2}O\textsubscript{2} was 14%, 17% and 30% for 0.05, 0.1 and 0.15 mg/ml of pomegranate juice respectively. On the other hand, the percent of H\textsubscript{2}O\textsubscript{2} scavenging by 0.05, 0.1 and 0.15 mg/ml of punicalagin was 11%, 17% and 18% respectively. Both compounds showed scavenging of H\textsubscript{2}O\textsubscript{2} with increasing concentrations.
Figure 2.9 (A and B) illustrates the activity of 0.1 mg/ml trolox and BHT compared with 0.1 mg/ml pomegranate juice and punicalagin. Scavenging activity values for trolox, BHT, pomegranate juice and punicalagin were 19, 13, 17 and 17.8% respectively. The pomegranate juice and punicalagin showed a significant increase in the scavenging of H$_2$O$_2$ compared with BHT ($p < 0.001$). However, no significant difference was observed between trolox, pomegranate juice and punicalagin. Although H$_2$O$_2$ itself is a weak oxidant, it is sometimes toxic to the cell because it may give rise to a hydroxyl radical in the cell (Ebrahimzadeh et al., 2009), which results in lipid peroxidation as described in the introduction chapter (1.3.2).

**Figure 2.9**: Comparison of H$_2$O$_2$ scavenging activity of pomegranate juice, punicalagin, trolox and BHT. Concentration was 0.1 mg/ml. Values are mean ± SD of three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (**) = $p < 0.001$).

Gil et al., (2000) reported, that both experimental (hand pressing in the laboratory) and commercial pomegranate juice demonstrated antioxidant activity that was always higher than that of red wine and green tea. The activity of experimental juice was, however,
lower than that of the commercial juice (Gil et al., 2000). Hydrogen peroxide was significantly scavenging by 15 μg/ml tannic acid (a type of tannin polyphenols). This tannic acid has higher scavenging activity compared to other standards, i.e. (Tannic acid > α-tocopherol > BHT > trolox) (Gülçin et al., 2010). The above results are similar to our findings for H₂O₂ scavenging activity, i.e. pomegranate juice (containing tannins) and punicalagin (which is a hydrolysable tannin) were significantly higher than BHT (p < 0.001) and were similar to trolox (p > 0.05). Moreover, the methanolic extract of peanut hulls showed the ability to scavenge H₂O₂ at 1.2 mg/ml, similar to standards BHA and catechin (Yen and Duh, 1994).

2.3.5 Ferrous Chelating Activity

With regards to ferrous ion chelating ability, the formation of Fe²⁺-ferrozine complex is inhibited in the presence of antioxidant. The antioxidant that has the ability to inhibit the formation of this complex is expressed as Fe²⁺ chelatinon. The chelation of ferrous ions by pomegranate juice and punicalagin is shown in Figure 2.10. Both components chelate ferrous ion in a dose dependent-manner; at 0.15 mg/ml punicalagin was significantly higher than pomegranate juice as ferrous chelator (p ≤ 0.05). A standard metal chelating agent used in this experiment was EDTA. Ferrous chelating activity of EDTA was 97% while, for pomegranate juice and punicalagin, chelating activity were lower at 14% and 18% respectively (Figure 2.11).
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Figure 2.10: Ferrous ion chelating activity by pomegranate juice and punicalagin.

Concentrations were 0.05, 0.1 and 0.15 mg/ml. Each value is the mean ± SD of three measurements. Comparisons of means were made using unpaired t-test (* = p < 0.05).

Figure 2.11: Metal chelating activity of pomegranate juice, punicalagin and EDTA.

Concentration was 0.1 mg/ml. Values are mean ± SD of three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (*** = p < 0.0001).

Punicalagin and pomegranate juice that contain several OH groups are efficient to chelating ferrous ion. Yen and Duh (1994) found that, the methanolic extract of peanut hulls did not show any chelating activity for metal ions compared with EDTA. Gülçin
et al., (2010) examined ferrous chelation activity of tannic acid, BHT and \( \alpha \)-tocopherol. Tannic acid which is tannin polyphenols showed the ability to chelate ferrous ion at 15 \( \mu \)g/ml, the chelation percentage was found to be similar to that of BHT and higher than \( \alpha \)-tocopherol (Gülçin et al., 2010) which is similar to our finding since both pomegranate juice contain tannin and punicalagin is a type of hydrolysable tannin.

2.3.6 Reducing Power Assay

Reducing power reflects the electron donating capacity of bioactive compounds; a mechanism also known as antioxidant activity. It measures the reduction of \( \text{Fe}^{3+}/\text{Fe}^{2+} \) thiocyanide in the presence of antioxidants; the resulting ferrocyanids form a complex with ferric chloride. The results showed an increase in the absorbance at 700 nm and, therefore, an increase in the reductive ability of pomegranate juice and punicalagin (Gulcin et al., 2005). Figure 2.12 illustrates the reducing activities corresponding to the concentration range (0.05, 0.1 and 0.15 mg/ml) of pomegranate juice and punicalagin. The reducing activity for both treatments increased in a dose dependent manner. The reducing activity shown by punicalagin increased significantly compared with pomegranate juice \((p \leq 0.05)\) at all concentrations.
These results demonstrate that pomegranate juice and punicalagin have the ability to reduce ferric ions and donate electrons to neutralise free radicals by forming stable products (Gulcin, 2012). Reducing power is a significant indicator that pomegranate juice and punicalagin demonstrate antioxidant activity. As reported from Gill et al., (2000), punicalagin (type of tannin) contain 16 phenolic hydroxyls per molecule while the manually extracted pomegranate juice contains higher concentrations of anthocyanin than tannin group. This could explain the higher reducing activity of punicalagin than that of pomegranate juice extract.

2.4 Conclusion

The study confirmed that punicalagin was present in high concentrations in pomegranate husk compared to pomegranate juice, as measured using a punicalagin standard. All experiments on pomegranate juice and punicalagin to determine the antioxidant mechanism concluded that pomegranate juice has a significantly higher
radical scavenging activity in comparison with punicalagin ($p \leq 0.05$). However, punicalagin showed significant ferrous chelating activity ability as compared with pomegranate juice. Both of these tested samples had the ability to reduce $\text{Fe}^{3+}$ ion to $\text{Fe}^{2+}$. However, punicalagin showed significant reducing power ability in a dose dependent manner compared with pomegranate juice. Nonetheless, both the pomegranate juice and punicalagin depict the ability to scavenge $\text{H}_2\text{O}_2$. In addition, both punicalagin and pomegranate juice showed non-significant inhibition of DPPH radicals compared with BHT and significant scavenging of $\text{H}_2\text{O}_2$ compared with BHT. Although, the DPPH radicals significantly inhibited by trolox compared with punicalagin and pomegranate juice, there was no significant difference found in $\text{H}_2\text{O}_2$ scavenging for both pomegranate juice and punicalagin compared with trolox.
Chapter 3
3 Protective Effects of Punicalagin on Caco-2 Intestine Cell Line under Oxidative Stress Caused by Tert-butylhydroperoxide

3.1 Introduction

Oxidative damage to cellular components occurs if there is an imbalance between the antioxidant capacity of cellular antioxidants in a biological system and the quantity/activity of reactive oxygen species (ROS). Excessive ROS production leads to tissue injury and is the basis of several human diseases such as coronary heart disease and cancer (Wijeratne et al., 2005). Free radicals can alter the poly-unsaturated fatty acids present in the cell membrane and change the permeability of the membrane itself. The lipid bilayer exists in all cellular organisms and forms the plasma membrane, and the surrounding membrane of organelles such as the endoplasmic reticulum and mitochondria. In addition to causing direct cellular damage to the membrane, the lipid peroxidation products, such as malondialdehyde (MDA), are also very toxic (Devasagayam et al., 2003). For this reason, biological systems are normally protected from oxidation by their in-built antioxidant defence systems and by dietary antioxidant. One of the important antioxidant defence mechanisms at cellular level is the concentration of reduced glutathione (GSH).

Glutathione is a tri-peptide of glutamic acid, cysteine and glycine. It has the ability to work as an antioxidant, protecting the cell from damage caused by ROS (Castell et al., 1997). Reduced and oxidized forms of GSH are both present in the cell at the same
time. In healthy cells and tissues, the reduced form of GSH accounts for more than 90%, while the oxidized form (GSSG) accounts for less than 10%. A severe decrease in GSH level in the cell leads to the susceptibility of the cell to oxidative damage by radicals (Castell et al., 1997). Previous studies have indicated a relationship between polyphenols and GSH levels in the cell. Polyphenols have the ability to prevent the decrease of GSH levels induced by tert-butylhydroperoxide (T-BOOH) (Kedderis, 1996, Castell et al., 1997, Lima et al., 2006). Thus, natural products containing polyphenols have been proposed as antioxidants.

Epidemiological studies have shown that the intake of polyphenol may reduce the risk of some diseases such as cancer and cardiovascular disease (Steinmetz and Potter, 1996; Arts et al., 2001). Pomegranate juice has a high content of phenolic compounds (such as punicalagin isomers, ellagic acid) and anthocyanins (such as delphinidin, cyanidin, and pelargonidin) (Gil et al., 2000). Gallic and ellagic acid link to glucose molecules to form punicalagin. The high antioxidant properties of pomegranate juice are due to the presence of punicalagin (Gil et al., 2000). Punicalagin is a yellow water-soluble compound that is mainly present in the pomegranate husk and is extracted in the juice during fruit processing (Gil et al., 2000).

Previous studies on polyphenols and flavonoids demonstrate their ability to prevent lipid peroxidation in living cells by trapping free radicals (Cotelle et al., 1996; van Acker et al., 1996; Cao et al., 1997). The tissue with the highest exposure to dietary flavonoids and polyphenols is the intestine. For this reason, human intestinal Caco-2 cells were used as a model to investigate the protective effects of punicalagin under oxidative conditions caused by T-BOOH by measure, GSH, ROS and MDA levels.
The aims of this chapter are:

- To determine the cytotoxicity of dimethyl sulphoxide (DMSO) and methanol (MeOH) on Caco2 cells to choose an appropriate solvent for punicalagin.
- To study the antioxidant activity of different concentrations (1-200 μM) of punicalagin to determine if it is cytotoxic to Caco-2 cells by the MTT method.
- To determine the cytotoxicity of T-BOOH at different concentrations (2, 3, 5 and 10 mM) and to assess whether punicalagin treatment decreases lipid oxidation in Caco-2 cells by measuring cell viability.
- To determine MDA levels by the TBARS method.
- To determine the antioxidant effect of punicalagin as a ROS scavenger.
- To determine the GSH level with treatments before and after induction by T-BOOH.
- To assess cellular morphology with treatments before and after induction by T-BOOH.

3.2 Materials and Methods

3.2.1 Materials

The Caco-2 cell line was obtained from the European Collection of Cell Cultures (ECACC) Salisbury, UK. Phosphate buffered saline (PBS) tablets were obtained from Oxoid, Hampshire, UK. Punicalagin, T-BOOH, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye), thiobarbituric acid (TBA), trichloroacetic acid (TCA), 1,1,3,3-tetraethoxypropane (TEP), DMSO, and cell lysis buffer were obtained from Sigma-Aldrich Chemical Co, Poole, UK. Foetal bovine serum (FBS), trypsin-
EDTA solution, L-glutamine (200 mM), non-essential amino acids (NEAAs),
Dulbecco’s Modified Essential Medium (DMEM) and CM-H₂DCFDA were purchased
from Invitrogen, Paisley, UK. A total glutathione peroxidase kit was obtained from the
Cell Biolabs, INC, Cambridge, UK.

3.2.2 Methods

3.2.2.1 Solutions and Buffers for Tissue Culture

3.2.2.1.1 Phosphate Buffered Saline (PBS)

One tablet was dissolved in 100 ml of Milli-Q water and autoclaved to sterilise.

3.2.2.1.2 MTT Stock

MTT (5 mg) was dissolved in 1 ml PBS solution, sterilised and filtered, and then stored
at 4 °C.

3.2.2.1.3 FBS DMEM Media (Complete Media)

DMEM media was supplemented with 20% FBS, 1% glutamate, 1% NEAA and 1%
penicillin.

3.2.2.1.4 Freezing Media

FBS was supplemented with 10% DMSO.
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3.2.2.2 Cell Culture

3.2.2.2.1 Routine Cell Culture

Caco-2 cells were grown in DMEM complete media. Cells were grown in a monolayer starting with a cell concentration of $1 \times 10^6$ cells/ml in 75cm$^2$ flasks. Cells were sub-cultured at 3 day intervals by trypsinisation. Caco-2 cells were maintained in a humidified atmosphere in 5% CO$_2$ at 37°C.

3.2.2.2.2 Trypsinisation

Media was removed from cells when 70%-90% confluent and cells were washed with PBS. The PBS was aspirated and 5 ml trypsin was added to the 75cm$^2$ flask. Cells were incubated for 8 minutes in the incubator. Cells were then detached from the flask surface by tapping. Complete media (5 ml) was then added to inactivate the trypsin. Cells were then ready for counting, culturing and freezing.

3.2.2.2.3 Cell Count

A cell suspension (10 μl) was placed on a haemocytometer slide with a depth of 0.2 mm. The total cell number was counted in the four chambers using an inverted microscope. The number of cells in the chamber and the volume of suspension were used to calculate the cell concentration; this was then used to determine the volume of cell suspension required to seed cells at a particular density:

$$\text{Cell count} = \frac{\text{Number of cells needed} \times 10}{\text{Average number of counted cells} \times 10^4 \times 10}$$
3.2.2.4 Cell Freezing

Cells of 80% confluency were trypsinised and collected in a 50 ml centrifuge tube and centrifuged at $500 \times g$ for 5 minutes. The media was removed and the cell pellet re-suspended in the freezing media. Cell suspension (1.8 ml) was added to cryovial tubes. Vials were stored at 4 °C for 30 minutes, and then moved to a freezer at -80 °C. On the following day, vials were transferred to a liquid nitrogen container for longer-term storage.

3.2.2.5 Cell Thawing

Cell vials to be thawed were immersed in a 37 °C water bath and gently agitated until thawed. The contents of the vial were transferred to a 20 ml centrifuge tube containing complete media (8 ml) and the suspension was centrifuged at $500 \times g$ for 5 minutes. The media was removed and the cell pellet re-suspended in 15 ml complete media. The cell suspension was then transferred to a 75 cm$^2$ flask.

3.2.2.3 MTT Assay

3.2.2.3.1 Principle

The MTT assay is dependent on the conversion of the soluble, yellow MTT dye to the insoluble purple formazan by the mitochondrial enzyme, succinate dehydrogenase. This occurs in metabolically active cells. Succinate dehydrogenase cleaves the tetrazolium ring in the MTT structure and converts it to formazan. Water insoluble formazan is largely impermeable to cell membranes and, therefore, accumulates within healthy cells (Jouvet et al., 2000; Waterfield et al., 1998). When formazan is solubilised in DMSO it produces a purple colour, which is measured by simple colorimetric assay at 492 nm.
using a plate reader (Boehringer CO, Marburg, Germany). The intensity of the colour is proportional to the amount of living cells present:

\[
\text{% Cell Viability} = \frac{\text{Mean absorbance of sample} \times 100}{\text{Mean absorbance of untreated cells}}
\]

3.2.2.3.2 Determination of Methanol (MeOH) and Dimethyl sulfoxide (DMSO) Cytotoxicity by MTT Assay

Since the punicalagin used in these investigations has the ability to dissolve in methanol and DMSO, the cytotoxicity of different concentrations of the solvents (0.1% - 1%) was tested on Caco-2 cells. Caco-2 cells were seeded into 96-well tissue culture plates with 200 µl DMEM complete media at a density of 1 × 10⁴ cells/ well. Cells were incubated for 24 hours at 37 °C to allow attachment. The plate was removed from the incubator after 24 hours and the media was aspirated. The plate was washed with 200 µl PBS, which was then aspirated from the cells. Cells were then incubated with different concentrations of DMSO and methanol individually for 24 hours at 37 °C. The plate was removed from the incubator and the media was aspirated. The plate was then washed with 200 µl with PBS. Following removal of the PBS, 20 µl MTT dye was added to each well along with 50 µl complete media to cover the MTT dye. The plate was incubated for 2 hours 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 formazan crystals produced were dissolved completely by shaking the plate for 30 seconds at room temperature. The colour intensity was measured at 492 nm using a plate reader (Boehringer CO, Marburg, Germany).
3.2.2.3.3 Determination the Cytotoxicity of Punicalagin

The cytotoxic effect of different concentrations of punicalagin ranging from 1-200 μM on Caco-2 cells was studied. A stock solution (10 mM) of punicalagin was prepared in DMSO. Caco-2 cells were seeded into 96-well tissue culture plates at 1 x 10⁴ cells/200 μl DMEM complete media. After 24 hours, punicalagin was added to each well to give a final concentration of 1, 3, 5, 10, 50, 100, 150 and 200 μM. The MTT assay was performed after 24 hours incubation at 37 °C and 5% CO₂. The plate was removed from the incubator and the media was aspirated. Following this, the plate was washed with 200 μl with PBS. The buffer was aspirated and 20 μl MTT dye was added to each well. A small volume (50 μl) of complete media was then added to cover the MTT dye. The plate was incubated for 2 hours at 37 °C. At the end of incubation time, the culture media was aspirated and 50 μl of DMSO was added to each well. The formazan crystals produced were dissolved completely by shaking the plate for 30 seconds at room temperature. The colour was measured at 492 nm using a plate reader (Boehring CO, Marburg, Germany)

3.2.2.3.4 Determination the IC₅₀ for Tert-butylhydroperoxide (T-BOOH)

The effect of different concentrations of T-BOOH on Caco-2 cells was investigated to determine the IC₅₀. Caco-2 cells were incubated with different concentrations of T-BOOH (1, 3, 5 and 10 mM). The T-BOOH concentrations were prepared in complete media. Caco-2 cells were seeded into 96-well tissue culture plates at a density of 1x10⁴ cells/200 μl DMEM complete media. After 24 hours, each of the four experimental concentrations were incubated with Caco-2 cells for 2 hours. The MTT
assay (described above) was performed to determine cell viability. Absorbance was read at 492 nm using a plate reader (Boehringer CO, Marburg, Germany).

3.2.2.3.5 Determination the Protective Concentrations of Punicalagin in Caco-2 Cells Treated with T-BOOH

The antioxidant activity of punicalagin in cells treated with T-BOOH was measured by MTT method. Caco-2 cells were seeded into 96-well tissue culture plates at a density of 1 \times 10^4 cells/200 µl DMEM complete media. After 24 hours, the cells were incubated at 37 °C for 24 hours with 1, 3, 5 and 10 µM punicalagin. Then the media was aspirated and the cells washed with 200 µl of PBS. T-BOOH (3 mM) was added to the pre-treated cells and incubated for 2 hours at 37 °C and 5% CO₂. Cell viability was then determined by the MTT assay (see section 3.2.4.2). Absorbance was read at 492 nm using a plate reader (Boehringer CO, Marburg, Germany).

3.2.2.4 Determination of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS are considered a marker of lipid peroxidation (Liu et al., 1997; Devasagayam et al., 2003). The spectrophotometric method of Ohkawa et al., (1979) was used with some modifications to detect TBARS in the biological system. The oxidation of poly-unsaturated fatty acid present in cell membranes produces MDA. Caco-2 cells at a density of 1\times 10^6 cells/ml were seeded in 25cm² tissue culture flasks. Once 50% confluency was achieved, cells were treated with 5 and 10 µM punicalagin and incubated for 24 hours. Trolox, at a concentration of 0.01%, was used as positive control. After 24 hours, the media was aspirated and the cells were washed with 5 ml
PBS. T-BOOH (3 mM) was added to the treated cells and incubated for 2 hours. At the end of incubation time, the cells were scraped with 3ml of 20% TCA. Cells were then collected with 2 ml of 0.7% TBA and incubated in a water bath at 100 °C for 1 hour. After cooling, the tubes were centrifuged at 1500 x g for 10 minutes. The intensity of the pink colour was determined by reading the absorbance at 535 nm using a spectrophotometer (UNICAM Spectronic, Leeds, UK). A stock solution of TEP (200 µg/ml) was prepared to obtain a standard curve with a range of 0-200 µg/ml. The MDA concentration was expressed in nmol of MDA/mg protein.

3.2.2.5 Measurement of Cellular Reactive Oxygen Species (ROS)

Dichlorofluorescein dye (non-fluorescent CM-H2DCFDA) has the ability to diffuse through cell membranes. In the cytoplasm, this dye is hydrolysed enzymatically by intracellular esterases and in rapidly converted to fluorescein dye (DCF) in the presence of ROS. The fluorescence intensity of DCF is proportional to the ROS content (Osseni et al., 1999). The ROS levels in Caco-2 cells incubated with 5 and 10 µM punicalagin were measured by flow cytometry using the CM-H2DCFDA dye. Caco-2 cells were seeded in 25cm² flasks at a density of 1x10⁶ cells/ml. The treatments were applied for 24 hours after the cells reached 60-70% confluence. Following incubation, the media was aspirated and the cells washed with 5 ml PBS. T-BOOH (3 mM) was added to the treated cells and incubated for 2 hours. At the end of the incubation time, the cells were trypsinised and the cell suspension centrifuged for 3 minutes at 150 x g using a Beckman GRP centrifuge. The supernatant was discarded and the cells were washed and centrifuged with 3 ml PBS. Again the supernatant was discarded and 1 ml PBS was added. The cells were incubated with 5 µM of CM-H2DCFDA (prepared in DMSO) for
30 minutes at 37 °C and 5% CO₂. At the end of the incubation time, the cells were kept on ice under low light conditions due to the high susceptibility of the dye to photo-oxidation. DCF 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 520 nm. Trolox, at a concentration of 0.01%, was used as positive control.

3.2.2.6 Preparation of Cell Lysates

Caco-2 cells at a density 1× 10⁶ cells/ml were seeded in 25cm² tissue culture flasks. Once 50% confluency was achieved, cells were treated with 5 and 10 µM punicalagin and incubated for 24 hours. Trolox (0.01%) was used as a positive control. After 24 hours, the media was aspirated and the cells washed with 5 ml PBS. T-BOOH (3 mM) was added to the treated cells and incubated for 2 hours. At the end of the incubation period, the culture media was decanted and cells were washed with 5 ml PBS. The cells were trypsinised and the cell suspensions centrifuged at 1500 × g for 3 minutes. Pellets were washed with 5 ml PBS. Supernatants were removed and the cell pellets were lysed by adding 300 µl of lysis buffer (Sigma-Aldrich Chemical Co, Poole, UK). The sample was then immersed in ice for 20 minutes and stored at -80 °C until protein concentration was measured and total GSH was determined.

3.2.2.6.1 Protein Quantification

The protein concentration of each sample was measured by the Bradford, (1976) method using a BioRad assay following the manufacturer’s instructions. Briefly, samples were diluted 1:10 and 50 µl of each was added to 96-well plate. To each
sample, 25 μl working solution (20 μl reagent S per ml reagent A) was added followed by 200 μl of reagent B into each well. The intensity of the blue colour formed was measured at 690 nm using a plate reader (Boehringer CO, Marburg, Germany).

Bovine serum albumin (BSA) was used as a protein standard to generate a standard curve. Six dilutions of BSA prepared in reverse osmosis (RO) water ranging from 0.1 to 1 mg/ml were used. The standard samples were treated in the same way as the samples. The absorbance of the solutions was measured at 690 nm.

3.2.2.7 Determination of Total Glutathione (GSH)

A total GSH assay kit from Cell Biolabs was used to measure the total concentration of GSH. Oxidized GSH (GSSG) can be reduced to GSH in the presence of NADPH and the GSH reductase enzyme. Consequently, the thiol group of GSH reacts with the chromogen to produce a yellow colour that absorbs light at 405 nm. The GSH concentration in the sample is proportional to the absorbance value of the chromophore. The unknown concentration of GSH was determined by generating a GSH standard curve. Briefly, 25 μl GSH reductase was added to a 96-well plate followed by the addition of 25 μl of NADPH. Samples and standards of volume 100 μl were applied to the mixture followed by adding 50 μl of chromogen. The absorbance was recorded immediately at 405 nm.
3.2.2.8 Morphology

Cell morphology was assessed through microscopic observation of the Caco-2 cell line (treated with control, trolox, 5 and 10 μM punicalagin) with and without incubation with 3 mM T-BOOH for 2 hours. Cells were seeded in T 25 flasks at a concentration 1 x 10^6 cells/ml. After 24 hours, treatments (trolox or punicalagin) were applied. Cells were then incubated with 3 mM T-BOOH for 2 hours. A photograph of the cells was taken using a Nikon Eclipse microscope at 10x magnification.

3.2.2.9 Statistical analyses

All experiments were performed at least three times. For the 96-well micro-titer tissue culture plates, 4 replicate wells were used per category. The data were analyzed using Graphpad Prism software version 6. Significant differences between control and experimental values were determined by one way analysis of variance followed by the Bonferroni test using a significance level of ≤ 0.05.

3.3 Results and Discussion

3.3.1 Cell Viability by MTT Results

3.3.1.1 Cytotoxicity of MeOH and DMSO

The cytotoxic effects of different concentrations of solvents ranging assay 0.1% - 1% were determined. The maximum solvent concentrations used in the cell should not exceed 1%. DMSO as a solvent was found to be non-toxic to the Caco-2 cells at all examined concentrations. In contrast, methanol showed a significant toxic effect that
started from 0.25% (p value ≤ 0.05). These results are illustrated in Figure 3.1. Based on this result DMSO has been used to dissolve punicalagin.

![Figure 3.1: The viability of Caco-2 cells treated with methanol and DMSO at different concentrations. A) methanol and B) dimethyl sulfoxide (DMSO) concentrations were (0.1-1%). These data represent the mean ±SD of at least 3 independent experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (* = p < 0.05, ** = p < 0.01 and *** = p < 0.0001).](image)

### 3.3.1.2 Cytotoxicity of Punicalagin on Caco-2 by MTT Assay

The cytotoxicity of punicalagin at different concentrations (1-200 μM) is displayed in Figure 3.2. The average cell survival at concentrations between 1-10 μM was more than 95%. There was no significant difference between the viability of control Caco-2 cells and those incubated with punicalagin at 1, 3, 5 and 10 μM. However, there was a significant decrease with higher punicalagin concentrations compared to the control (P < 0.0001). The non-toxic dose was assessed to examine the protective effect of those doses under oxidative stress generated by T-BOOH.
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Figure 3.2: Cell viability of Caco-2 cells treated with different concentrations of punicalagin (0-200 μM) for 24 hours. Viability of Caco-2 cells treated with punicalagin concentrations (0-200 μM) for 24 hours were determined by MTT assay. These data represent the mean ± SD of at least 3 independent experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (ns= non significant, ***= P <0.0001).

3.3.1.3 The IC50 of T-BOOH

Different doses of T-BOOH (1, 3, 5 and 10 mM) were incubated with Caco-2 cells for 2 hours to determine the IC50 of T-BOOH. The dose response curve in Figure 3.3 showed that the IC50 of T-BOOH, when applied to Caco-2 cells, was 3 mM. Consequently, this concentration was used to induce oxidative stress in Caco-2 cells and to measure the protective effect of punicalagin.
Figure 3.3: Dose-response curve for different concentrations of T-BOOH
(1, 3, 5 and 10 mM) incubated for 2 hours with Caco-2 cells. Cell viability was measured by the MTT assay. Values represent the mean ± SD, n=3, the IC 50 value was 3 mM T-BOOH.

3.3.1.4 Protective Effect of Punicalagin Against the Cytotoxicity of T-BOOH

The protective effect of punicalagin (1, 3, 5 and 10 µM) against the oxidative stress induced by 3 mM T-BOOH is illustrated in Figure 3.4. No significant difference was seen between the cells incubated with 3 mM T-BOOH (58% viability) and those pretreated with 1 µM (55% viability) and 3 µM (62% viability) of punicalagin. However, there was a significant difference between the cells incubated with 3 mM T-BOOH and pretreated with 5 and 10 µM punicalagin (p < 0.001 and p <0.0001, respectively). Concentrations of punicalagin that have the ability to protect Caco-2 cells against 3 mM T-BOOH were 5 and 10 µM; the cell viability with punicalagin was 71.5 and 64.4 % respectively, versus 58 % for 3 mM T-BOOH only.
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3.3.2 Determination of MDA concentration

The effect of different concentrations of punicalagin on the production of the secondary lipid oxidation product MDA was studied in Caco-2 cells. The MDA concentration was expressed as µg/ml using the standard curve shown in Figure 3.5. When trolox and different concentrations of punicalagin were incubated with Caco-2 cells without T-BOOH, non-significant differences in MDA concentration were observed in control cells compared with treated cells. Malondialdehyde concentrations were 37 µg/ml for control, 35.5 µg/ml for trolox, 35 µg/ml for 5 µM punicalagin and 34 µg/ml for 10 µM punicalagin respectively as presented in Figure 3.6 A.
There was a significant decrease in the production of MDA in cells pretreated with trolox, or 5 and 10 μM punicalagin following induction of oxidative stress using T-BOOH (Figure 3.6 B). The most protective cell concentration against oxidative stress was 10 μM punicalagin followed by trolox and then 5 μM punicalagin compared with cells treated with T-BOOH alone (p value ≤ 0.05). Concentrations of MDA were 27.7, 38.7, 41.4, and 64.6 10 μg/ml after treatment with either 10 μM, trolox, 5 μM, or 3 mM T-BOOH alone, respectively.

Figure 3.5: Standard curve of MDA concentration. Data represent the mean ± SD of MDA standard concentrations (0-200 μg/ml). Absorbance was measured at 532 nm. Linear regression performed in Graphpad Prism version 6.0 yielded a correlation coefficient of $R^2 = 0.998$. 
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Figure 3.6: Concentration of MDA in Caco-2 cells treated with punicalagin and trolox individually with/without 3 mM T-BOOH for 2 hours. A) Caco-2 cells were incubated with trolox and different punicalagin concentrations (5 and 10 μM); B) pretreated cells incubated with 3 mM T-BOOH for 2 hours. Values are the mean ± SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (**p < 0.0001). Trolox 0.1 mg/ml was used as antioxidant positive control.

MDA is one of the main products of lipid peroxidation (Suttnar et al., 1997). It is produced in high quantities in various diseases and it is related to free radical damage (Suttnar et al., 2001). Quercetin (a flavonol from a class of flavonoids) of 5 and 10 μM incubated for 2 and 20 hours in HepG2 cells significantly inhibited the production of MDA under oxidative stress conditions induced by 200 μM T-BOOH after a 3 hour incubation period (Alia et al., 2006b). Another study by Peng and Kuo in 2003, discovered that 0.1 μM quercetin, 10 μM luteolin and 1 μM epigallocatechin gallate had the ability to inhibit lipid peroxidation in Caco-2 cells under 30 μM H₂O₂ + 30 μM FeSO₄ inductive conditions (Peng and Kuo, 2003). Results from a study by Lima et al., (2007) on phenolic compounds extracted from Salvia officinalis (an aromatic plant)
showed a significant inhibition in MDA concentration in HepG2 cells under oxidative stress. The water and methanolic extracts at 250 and 16 μg/ml, respectively, incubated for 40 hours afforded protection to 80% of HepG2 cells and had the ability to prevent lipid peroxidation produced by 2 mM T-BOOH for 5 hours (Lima et al., 2007). The most abundant phenolic compounds present in the *Salvia officinalis* plant were rosmarinic acid and luteolin-7-glucoside (Lima et al., 2007). All of the previous findings discussed above are similar to our result that punicalagin has the ability to prevent production of MDA in the Caco-2 cell line when under stress from 3 mM of T-BOOH. The ability of phenolic compounds to scavenge peroxides and prevent chain-breaking reaction of cell membrane could explain the observed reduction in MDA as lipid peroxidation marker (Brown et al., 1998).

### 3.3.3 Measurement of Cellular ROS Levels

The ROS levels in Caco-2 cells without T-BOOH in control and treated cells with trolox or punicalagin concentrations for 24 hours were measured using the CM-H₂DCFDA dye. There was no significant difference in ROS levels as illustrated in Figure 3.7 A between control and tested components.

Punicalagin (5 and 10 μM) reduced cellular ROS levels compared with control in 24 hours in pretreated Caco-2 cells exposed to 3 mM T-BOOH for 2 hours (Figure 3.7 B). ROS production was assessed by flow cytometric analysis using CM-H₂DCFDA dye. Non-fluorescent CM-H₂DCFDA dye was converted to fluorescent DCF in the presence of ROS, as described in the method section (3.2.6). The pre-treated cell line with trolox and punicalagin showed a significant decrease in fluorescence intensity compared with T-BOOH treated group (p <0.001). This result indicates that punicalagin
has the ability to scavenge ROS and thereby protect cellular macromolecules such as lipids and proteins from ROS-mediated damage.

Figure 3.7: Cellular ROS levels in Caco-2 cells treated with punicalagin and trolox individually with/without 3 mM T-BOOH for 2 hours. A) Caco-2 cells were incubated with trolox (0.01%) or different punicalagin concentrations (5 and 10 µM) for 24 hours; B) pre-treated Caco-2 cells for 24 hours were incubated with 3 mM T-BOOH for 2 hours. Measurement of cellular ROS was by flow cytometric method. Values are the mean ± SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (ns= non-significant, ***P < 0.0001).

Previous studies have shown that the antioxidant activity of phenolic compounds occurs via ROS scavenging (Yokomizo and Moriwaki, 2006). The ability of different flavonoids (quercetin, kaempferol and luteolin) to decrease the amount of ROS was measured by Yokomizo and Moriwaki (2006). In their study, each flavonoid (50 µM) was incubated for 1 hour with the Caco-2 cell line. The pre-treated cell line was stressed by 50 µM H₂O₂ for 1 hour. The three flavonoids caused a significant decrease in ROS levels. Quercetin (54%) was the most effective, followed by luteolin (34%) and...
then kaempferol (26%) (Yokomizo and Moriwaki, 2006). ROS production was also significantly reduced in the HepG2 liver cell line when incubated with quercetin and rutin (1-100 μM) for 24 hours when stressed with 200 μM H₂O₂ or 3 hours compared with cells without pre-treatment (Alia et al., 2006a).

The cellular ROS scavenging activity of apple juice extract and apple peel extract has also been examined in the Caco-2 cell line (Bellion et al., 2010). Different concentrations (1-100 μg/ml) of each apple-based sample were tested. The apple peel extract caused a significant inhibition in ROS production in a dose-dependent manner, while the apple juice extract showed a significant reduction of ROS levels at the highest concentration only (Bellion et al., 2010). In agreement with previous results, the ability of examined phenolic compound (punicalagin) to decrease cellular ROS levels was observed. This finding may explain the importance of the presence of the phenolic hydroxyl group that scavenges radicals (Yokomizo and Moriwaki, 2006). The antiradical activity of punicalagin against DPPH and H₂O₂ is shown in the second chapter of this thesis (section 2.3.3 and 2.3.4).

3.3.4 Determination of Total Glutathione (GSH)

In Figure 3.8 B, the total GSH level was significantly increased in the pre-treated cells with trolox, 5 and 10 μM punicalagin under 3 mM T-BOOH oxidative induction compared with cells exposed to 3 mM T-BOOH only. Levels of GSH were similar for all pre-treated cells (0.3 μM). The GSH level of incubated cells with T-BOOH was only 0.18 μM. In the case of the trolox, 5 and 10 μM punicalagin treated cells without T-BOOH (Figure 3.8 A), the level of GSH was significantly higher with trolox
treatment ($p < 0.01$) whereas no significant difference was observed with punicalagin treatment compared with the control.

Figure 3.8: Glutathione (GSH) level in Caco-2 cells treated with punicalagin and trolox individually with/without 3 mM T-BOOH for 2 hours. A) Caco-2 cells were incubated with trolox (0.1 mg/ml) or different punicalagin concentrations (5 and 10 μM) for 24 hours; B) pre-treated Caco-2 cells for 24 hours were incubated with 3 mM T-BOOH for 2 hours. Values are the mean ± SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (ns= non-significant, **$p < 0.001$ and ***$p < 0.0001$).

Levels of GSH in Caco-2 cells decreased under oxidative stress conditions induced by 3 mM T-BOOH for 2 hours. This depletion was significantly reduced by 5 and 10 μM of punicalagin ($p < 0.0001$). Research by Lima et al., (2006) indicates that polyphenols provide protection against oxidative damage induced by T-BOOH. Luteolin 200 μM, 45 μM quercetin, 370 μM caffeic acid and 180 μM rosmarinic acid all had the ability to inhibit the GSH depletion in the HepG2 liver cell line (Lima et al., 2006). Lima et al., (2007) reported the protective effect of polyphenols extracted from the Salvia plant,
using the same liver cell line. GSH depletion was significantly prevented by pre-treating cells with methanolic and water extract of salvia. In all of Lima’s research, the oxidative stress induced in the liver cell line was caused by treatment with 2 mM T-BOOH for 5 hours (Lima et al., 2007). GSH has an important role in the protection of cells against ROS and free radicals. A severe reduction in GSH levels increases the susceptibility of cell damage by free radicals. Depletion of GSH levels has been suggested as one of the important mechanisms through with T-BOOH induces cell toxicity (Martin et al., 2001). Therefore, the potential effect of punicalagin against T-BOOH as radical scavenging activity could maintain the GSH levels and contribute to their cytoprotective effects.

3.3.5 Morphological Changes to Caco-2 Cells Treated with Punicalagin

The protective effect of different concentrations of punicalagin (5 μM and 10 μM) on Caco-2 cells subjected to 3 mM T-BOOH oxidative stress was investigated by microscopy for 24 hours. Figure 3.9 shows the morphological changes observed in Caco-2 cell lines stressed with T-BOOH compared with control cells. In contrast, cells pre-treated with different punicalagin concentrations and then stressed with T-BOOH appeared healthy, with morphology appearing the same as the control (Caco-2 cells) as well as cells exposed to punicalagin only. These observations confirm the protective effect of punicalagin. Trolox was used as the positive control. Trolox and different punicalagin treatments showed the same cytotoxic protection of Caco-2 cells stressed by T-BOOH. The cells looked healthy which demonstrates the protective effect of that antioxidants against oxidative stress.
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Figure 3.9: Effect of trolox and punicalagin on a Caco-2 cell line with/without 3 mM T-BOOH for 24 hours. Cells were treated with trolox and punicalagin for 24 hours. The pre-treated cells incubated with 3 mM T-BOOH for 2 hours. Images were captured using light microscopy (magnification 10x).

3.4 Conclusion

In conclusion, punicalagin at low concentrations has a protective effect against oxidative damage induced by 3 mM T-BOOH in a Caco-2 cell line, and may therefore be a useful natural antioxidant. From our experiments, we observed that punicalagin strongly protects Caco-2 cells against cell death, by cellular ROS scavenging and preventing of lipid peroxidation. Moreover GSH depletion caused by 3 mM T-BOOH was prevented by treating Caco-2 cells with punicalagin. These findings suggest that punicalagin may afford protection against the development of some diseases that are caused by excessive ROS production. This conclusion supports the importance of fruit and vegetable intake in our diet.
Chapter 4
Chapter 4: Punicalagin Inhibits Angiotensin Converting Enzyme and Induces Nitric Oxide (NO) Production in the EA.hy926 Endothelial Cell Line

4 Punicalagin Inhibits Angiotensin Converting Enzyme and Induces Nitric Oxide (NO) Production in the EA.hy926 Endothelial Cell Line

4.1 Introduction

High blood pressure is a major risk factor for some chronic diseases such as stroke, renal disease, and cardiovascular disease (Mittal and Singh, 2010). In patients with hypertension, angiotensin II is produced from angiotensin I by the catalytic effect of angiotensin converting enzyme in higher amounts than normal (Aviram and Dornfeld, 2001; Clarke and Turner, 2012). Angiotensin II acts as vasoconstrictor, increasing blood pressure. For this reason, inhibition of ACE activity is a pharmacological target for the treatment of hypertension (Persson et al., 2009). The ACE enzyme has a Zn$^{2+}$ ion in its two active sites (Riordan, 2003). Substrate binding and catalysis of ACE indicate the mechanism of Zn$^{2+}$ ion (Reeves and O'Dell, 1986). One of the mechanisms of ACE inhibitors is the ability to bind the Zn$^{2+}$ ion (Sturrock et al., 2004).

High and unregulated production of reactive oxygen species (ROS) is another risk factor for heart disease (Lobo et al., 2010; Sugamura and Keaney, 2011). In endothelial cells, enzyme systems involving NADPH oxidase, xanthine oxidase, and the mitochondrial respiratory chain are responsible for ROS production (Zhang et al., 2001). ROS production should be regulated or adverse effects like oxidation will be cause to cell macronutrients such as proteins, lipids, and nucleic acids. Balance in ROS concentration can be achieve by the antioxidant system that exists in the human body.
and by antioxidant supplement (Himaya et al., 2012). Moreover, ROS have the ability to oxidize nitric oxide (NO) produced from endothelial cells, leading to endothelial dysfunction and the initiation and development of cardiac disease (Li et al., 2002).

Nitric oxide (NO) is known as relaxing factor because it acts as a vasodilator, increases blood flow, and inhibits platelet aggregation and adhesion (Kelly et al., 1996). Calcium-dependent endothelial nitric oxide synthase (eNOS) is one of the important factors responsible for the production of NO in endothelial cells. Increased levels of NO in the endothelial cells are often due to the increased protein expression of the eNOS enzyme or by scavenging the ROS produced within the cells (Ignarro et al., 2001; Napoli and Ignarro, 2001).

Several research groups have reported a significant interaction between biological systems and dietary polyphenols from vegetables and fruits. These include benefits such as anticancer (Meyers et al., 2003; Zhao et al., 2004), anti-inflammatory (Havsteen, 2002), antioxidant (ROS scavenging or metal chelating) (Kähkönen and Heinonen, 2003), and antibacterial properties (Viljanen et al., 2004). Pomegranate is a fruit that contains a high quantity of different polyphenols e.g. tannins, ellagic tannins, anthocyanins, catechins, gallic, and ellagic acid (Ben Nasr et al., 1996; Gil et al., 2000). Punicalagin (of the ellagitannin class) is one of the phenolic compounds present in pomegranate. The effect of punicalagin as antioxidant and as an anti-hypertensive was investigated in the EA.hy926 cell line.
Chapter 4: Punicalagin Inhibits Angiotensin Converting Enzyme and Induces Nitric Oxide (NO) Production in the EA.hy926 Endothelial Cell Line

The objectives of this chapter were:

1. To determine the cell viability of EA.hy926 cells after treatment with different concentrations of punicalagin.
2. To determine NO production of EA.hy926 cells after treatment with different concentrations of punicalagin.
3. To determine the inhibitory effect of punicalagin on the ACE.
4. To determine the antioxidant effect of punicalagin as a ROS scavenger.
5. To determine the ability of punicalagin to induce eNOS activity.
6. To determine the effect of punicalagin on cellular Ca$^{2+}$ concentration.
7. To determine the effect of punicalagin on the eNOS enzyme expression by Western blotting.

4.2 Materials and Methods

4.2.1 Materials

The human endothelial-like immortalized cell line Ea.hy926 was kindly donated by Dr. Bodman-Smith University of Surrey, UK. Ethylene glycol tetraacetic acid (EGTA), hippuryl-histidyl-leucine (H-H-L) and angiotensin II were obtained from Sigma-Aldrich Chemical Co, Poole, UK. Griess Reagent Kit for nitrite quantitation, Hanks buffer saline solution (HBSS) buffer with calcium and magnesium, o-phthaldialdehyde, high purity, fura-2, AM, dihydroethidium dye, foetal bovine serum (FBS), trypsin-EDTA solution, Dulbecco’s Modified Essential Medium (DMEM), penicillin were obtained from Invitrogen, Paisley, UK. Antibody eNOS and β-actin mouse mAb were purchased from cell signaling technology, Hertfordshire, UK. EnzyChrom™ Nitric Oxide
Synthase Assay Kit was obtained from Universal Biological, Cambridge, UK. Chemiluminescent Kit–Anti-Rabbit, Chemiluminescent Kit–Anti-Mouse, NuPAGE® Novex 4-12% Bis-Tris Gel 1.0 mm (15 well), Novex® Sharp Pre-stained Protein Standard -2 × 250 μl, Invitrolon™ PVDF/Filter Paper Sandwiches, NuPAGE® Transfer Buffer (20X) (1 L), NuPAGE® MOPS SDS Buffer Kit (for Bis-Tris Gels), NuPAGE® Sample Reducing Agent (10X) (250 μl) and NuPAGE® LDS Sample Buffer (4X) (10 ml) were obtained from Invitrogen, Paisley, UK. Fixer and developer solutions champion amfix were obtained from First Call Photography, West Monkton, UK.

4.2.2 Methods

4.2.2.1 Cell Viability and Nitric Oxide (NO) Release

The EA.hy926 cell line was seeded in DMEM with high glucose content (4.5 g/L), and supplemented with 10% FBS and 5% penicillin. Different punicalagin concentrations (1-100 μM) were tested on Ea.hy926 cells to measure the cell viability and nitric oxide (NO) production. Briefly, a stock solution of punicalagin (10 mM) was prepared in DMSO. Ea.hy926 cells were seeded into 96 well tissue culture plates at a density of 1 × 10⁶ cells/200 μl DMEM serum media. Once the cells reached 60-70% confluence, they were treated with punicalagin for 12, 24, or 48 hours. Different incubation times were tested to determine the optimal time for nitrite production, as a marker of nitric oxide production and cell viability. Nitrite levels in the treated cell supernatant were measured by Griess kit and cell viability was measured by MTT assay, at each experimental time.
According to the Griess kit instructions, 100 µl of medium supernatant was mixed with 100 µl Griess reagent (0.1% N-naphthylethylethylenediamine dihydrochloride in 5% phosphoric acid and 1% sulfanilamide, mixed in a 1:1 ratio). The formation of a yellow colour was measured at 550 nm using a plate reader (Boehringer CO, Marburg, Germany); the colour intensity was proportional to nitrite concentration. A sodium nitrite standard curve was obtained using a sodium nitrite standard solution supplied with the kit. This was used to calculate the NO concentration in the test samples.

Cells pretreated with different concentrations of punicalagin were incubated with 20 µl of MTT dye (5 mg/ml PBS) at 37 °C and 5% CO₂ to measure cell viability after different incubation times. At the end of each experimental time, the culture medium was aspirated and 100 µl of DMSO was added to each well. The formazan crystals produced were dissolved completely by shaking the plate for 30 seconds at room temperature. The purple colour produced was measured at 492 nm using a plate reader (Boehringer CO, Marburg, Germany).

4.2.2.2 Measuring ACE Activity in Ea.hy926

Angiotensin converting enzyme activity was measured in the Ea.hy926 cell line after 24 hours of exposure to different concentrations of punicalagin (1, 20, 40 and 60 µM) using a modified fluorometric method (Kapiloff et al., 1984). Briefly, Ea.hy926 cells were seeded to confluence in 25cm² flasks (1x10⁶ cells /ml) and then incubated with punicalagin (1, 20, 40 and 60 µM) for 24 hours. Treated cells were washed three times with 3 ml HBSS buffer. The cells were scraped from the flask with 1 ml HBSS and frozen at -20 °C until assayed. For the assay, frozen cells were thawed and sonicated.
then 20 µl samples were added to 80 µl of H-H-L (5 mM prepared in HBSS buffer) and incubated at 37 °C for 3 hours. The incubated cells were then mixed with 1.4 ml NaOH (0.5 N) to stop the reaction. The fluorescent dye, O-phthaldialdehyde (100 µl of 10 mg/ml in methanol) was used to detect the histidyl-leucine reaction product. The reagents were incubated for 5 minutes at room temperature followed by the addition of 250 µl of HCl (6 N). A fluorimeter (Varian, USA) was used to measure the fluorescence of the samples using an excitation wavelength of 365 nm and an emission wavelength of 495 nm. Captopril (1 µM) was used as positive control in place of punicalagin.

4.2.2.3 Measurement of ROS by Flow Cytometry

Dichlorofluorescein dye (non-fluorescent CM-H$_2$DCFDA) has the ability to diffuse through the cell membrane (Osseni et al., 1999). In the cytoplasm, this dye is hydrolysed enzymatically by intracellular esterases and rapidly converted to fluorescent dye (DCF) in the presence of ROS. The fluorescence intensity is proportional to the ROS content (Osseni et al., 1999). The ROS level in the Ea.hy926 cell line was determined by flow cytometry, using CM-H$_2$DCFDA dye after incubation with 1, 20, 40, and 60 µM punicalagin. Ea.hy926 cells were seeded in 25cm$^2$ flasks at 1x10$^6$ cells/ml. The treatments were added when cells reached the desired cell confluence (60-70%). After 24 hours incubation time, half of pre-treated cells were exposed to 10 nM angiotensin II for 1 hour, while the other left untreated. The cells were trypsinised and centrifuged for 3 minutes at 150 x g using a Beckman GRP centrifuge (Shropshire, UK). The supernatant was removed, the pellets washed with 2 ml of HBSS buffer and centrifuged. The supernatant was discarded and the cells re-suspended in
1 ml HBSS. Re-suspended cells were then incubated with 5 μM CM-H₂DCFDA (prepared in DMSO) for 30 minutes at 37 °C and 5% CO₂. At the end of the incubation time, cells were kept on ice under low light conditions due to the high susceptibility of the dye to photo-oxidation. DCFDA fluorescence was then 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.

4.2.2.4 Measurement of ROS by Fluorometric Method

The fluorescent dye, dihydroethidium can penetrate the cell membrane and is oxidized by superoxide radicals to form 2-hydroxyethidium; a red fluorescent product (Sudheesh and Vijayalakshmi, 2005). This intercalates with DNA to enhance intracellular fluorescence (Bindokas et al., 1996; Li et al., 2003). Ea.hy926 cells were seeded in 25cm² flasks at a density of 1×10⁶ cells/ml. Different concentrations of punicalagin (1, 20, 40 and 60 μM) were incubated with the cells once 60-70% confluent. After 24 hours, a group of treated cells was incubated with angiotensin II (10 nM) for 1 hour and another group were left untreated (control). All cells were then washed with HBSS buffer. Cells were covered with 3 ml HBSS buffer and incubated with 25 μM of DHE dye for 30 minutes at 37 °C and 5% CO₂. The treated cells were washed with 3 ml HBSS and incubated with 5 ml HBSS for 1 hour at 37 °C and 5% CO₂. HBSS buffer was discarded and the cells were scraped from the flask with 1ml cold methanol. The cell suspension was sonicated and filtered through a 0.22 μM membrane filter. The 2-hydroxyethidium was detected by fluorimetry (Varian, USA) with excitation and emission wavelengths of 480 nm and 580 nm, respectively.
4.2.2.5 Determination of Cellular Calcium Concentration by Fluorescence Method

Intracellular calcium concentrations were measured by fluorometry after loading the cells with fura-2/AM dye. Free intracellular calcium will bind with the membrane diffusible fluorescent dye fura-2/AM (Gryniewicz et al., 1985). Briefly, Ea.hy926 cells were cultured in 25cm² flasks at 1x10⁶ cells/ml concentration. Different concentrations of punicalagin (1, 20, 40 and 60 µM) were incubated with the cells after they reached 60-70% confluence. Following 24 hours incubation, cells were loaded with 5 µM fura-2/AM dye and incubated for 45 minutes at 37 °C and 5% CO₂. Consequently, the dye was removed and the cells were washed and scraped from the flask with 2 ml HBSS buffer. The calcium concentration was detected by fluorimetry (Varian, USA) with excitation and emission wavelengths of 340 nm and 510 nm, respectively. Ethyleneglycoltetraacetic acid (EGTA) at 1 mM was used as negative control instead of punicalagin.

4.2.2.6 Determination of Endothelial Nitric Oxide Synthase Enzyme (eNOS) Activity in the Ea.hy926 Cell Line

Cells treated with different concentrations of punicalagin (1, 20, 40 and 60 µM) for 24 hours were lysed as described previously in chapter 3 section 3.2.7, and frozen at -20 °C until eNOS activity was measured. EGTA (1 mM) was used as a negative control in place of punicalagin.

eNOS synthase activity was measured using a nitric oxide synthase assay colorimetric kit (Bioassay System, ENOS-100) using a direct and non-radioactive method.
According to the manufacturer’s instructions, this kit is designed to measure eNOS activity in two steps. The first step is an oxidation reaction by an eNOS enzyme that oxidises the amino acid arginine to NO. The second step is detection of NO via a reduction reaction. NO is rapidly oxidised to nitrite and nitrate after production, thus the reduction of nitrate to nitrite by Griess method was used to measure NO production. The yellow colour produced was measured at 540 nm using a plate reader (Boehringer CO, Marburg, Germany). A NO synthase standard curve was obtained using a eNOS standard solution supplied with the kit.

4.2.2.7 Assessment of eNOS Expression by Western Blot

4.2.2.7.1 Preparation of Whole Cell Lysate

Ea.hy926 cells at a density of $1 \times 10^6$ cells/ml were seeded in 25cm$^2$ tissue culture flasks. Once 50% confluence was achieved, cells were treated with 1, 20, 40 and 60 μM punicalagin and incubated for 24 hours. 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 $1500 \times g$ for 3 minutes with 5 ml PBS to wash. Supernatants were removed and the cell pellets were lysed by adding 300 μl of lysis buffer (Sigma Aldrich Chemical Company). The last step for cell lysis was to keep the cells on ice for 20 minutes and then store them at -80 °C until protein determination and western blot experiment were carried out.
4.2.2.7.2 Protein Quantification

The protein concentration for each sample was measured as described previously in chapter 3 section (2.3.7.1) with the Bradford method using BioRad assay (Bradford, 1976).

4.2.2.7.3 Gel Electrophoresis

For gel electrophoresis, 4 μl sample buffer was mixed with each sample containing 20 μg of protein this was followed by the addition of 2 μl of reducing agent and completed to a final volume of 20 μl with distilled water. The prepared samples were heated at 70 °C for 10 minutes and then directly put on ice for a few minutes. An Invitrogen NuPAGE 4-12% Bis-Tris gel was used for protein electrophoresis. An Invitrogen Xcell SureLock Mini-cell chamber was filled with NuPAGE® SDS Running Buffer (50 ml 20x MOPS and 950 ml RO water). In each gel well, 20 μl of each sample was loaded and the last well was loaded with pre-stained protein ladder. The electrophoresis running time was 2.5 hours at 100 V at room temperature.

4.2.2.7.4 Blotting and Developing

At the end of the gel electrophoresis running time, the gel cassette was removed, washed with water and opened with a gel knife. The gel, together with PDVF membrane pre-soaked in methanol, was placed in an Invitrogen transfer chamber. The chamber was filled with transfer buffer (50 ml of 20x transfer buffer + 100 ml methanol to a final volume of 1 L with RO water). The running time for transfer was 2 hours at 30 V.
Following transfer, 20 ml sterile water was used to wash the membrane twice for 5 minutes. After that, transferred membrane was blocked with 10 ml of blocking solution (0.5 g BSA, 2 ml blocker/diluent (Part A), 3 ml blocker/diluent (Part B) and 5 ml RO water) for 1 hour on the rotary shaker. The next step after blocking was adding 10 ml primary antibody diluents (0.5 g bovine serum albumin, 5 ml RO water 2 ml blocker/ diluent (Part A) and 3 ml blocker/ diluent (Part B), and 10 µl primary antibody) and incubated with the membrane on a rotary shaker overnight at 4 °C. On the following day, 20 ml antibody washing solution (10 ml Ab Wash Solution (16x) + 150 ml RO water) was used 3 times to wash the incubated membrane. The PDVF membrane was then incubated with a secondary antibody solution (anti-mouse or anti-rabbit) for 1 hour to allow binding of the secondary antibody to the primary antibody. Using a Chemiluminescent Western Blot Kit Immunodetection the presented band of protein on the PDVF membrane was developed. In a dark room, 2.5 ml chemiluminescent substrate was applied to the membrane surface and incubated for 5 minutes. The excess of chemiluminescent reagent was removed with a filter paper and cling film was used to cover the membrane. The membrane was then inserted into a cassette with Amersham film paper placed on top of the membrane and the cassette closed.

The exposure time for membrane to film was different depending on the antibody used and ranged from 30 seconds to 15 minutes. At the end of this exposure, the film was developed and fixed using developing and fixing solutions (Champion Amfix. First call Photography, West Monkton, UK), respectively.
4.2.2.8 Statistical Analyses

All experiments were performed at least in triplicate. For the 96-well microtiter tissue culture plates, 4 replicate wells were used per category. The data were analyzed by Graphpad Prism version 6. For significant differences between control and experimental values, the P-value between groups was determined by one-way analysis of variance followed by Bonferroni test. The significance level was set at $P \leq 0.05$.

4.3 Results and Discussion

4.3.1 Cell Viability and NO Production

Cell viability and nitric oxide production was measured over a time course to determine the optimal experimental exposure time. The data in Figure 4.1 A show that cell viability increased significantly ($p \leq 0.05$) after treatment with 1, 20, 40, and 60 μM punicalagin for 12 hours, but that NO production is similar compared with the untreated control (Figure 4.1 B). In contrast, there was a significant decrease in cell viability at 80 and 100 μM punicalagin concentrations ($p < 0.0001$) with a corresponding significant decrease in NO production at 12 hours ($p < 0.0001$). After 24 hours incubation time with the same range of punicalagin concentrations, cell viability and NO production were significantly increased ($p \leq 0.05$) compared with untreated cells (control) as shown in Figures 4.2 A and B. As for the 12-hours incubation, concentrations of 80 and 100 μM punicalagin caused a significant decrease in cell viability and NO production compared with untreated cells ($p \leq 0.05$).
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After 48 hours incubation significant decreases in cell viability ($p \leq 0.05$) and NO production ($p < 0.001$) were observed at all punicalagin concentrations (1, 20, 40, 60, 80 and 100 μM) Figure 4.3 A and B. Based on the previous cell viability and NO production results, punicalagin concentrations in the range 1 – 60 μM were selected for exposure to cells for 24 hours in future experiments because higher punicalagin concentrations 80-100 μM produced a toxic effect on Ea.hy926 cells. The significantly enhanced release of NO due to the activation of eNOS enzyme is described below in section 4.3.5.

![Figure 4.1: Cell viability and NO production after exposure of Ea.hy926 cells to different concentrations of punicalagin (1-100 μM) for 12 hours. A) Cell viability; B) NO production. Data represent mean ± SD of three individual experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test ($*=p<0.05$; $**=p<0.001$; $***=p<0.0001$ versus control).](image)
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Figure 4.2: Cell viability and NO production after exposure of Ea.hy926 cells to different concentrations of punicalagin (1-100 μM) for 24 hours. A) Cell viability; B) NO production. Data represent mean ± SD of more than three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (* = p < 0.05; ** = p < 0.001; *** = p < 0.0001 versus control).

Figure 4.3: Cell viability and NO production after exposure of Ea.hy926 cells to different concentrations of punicalagin (1-100 μM) for 48 hours. A) Cell viability; B) NO production. Data represent mean ± SD of more than three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (* = p < 0.05; ** = p < 0.001; *** = p < 0.0001 versus control).
Several research studies the effect of polyphenol to produce NO in agreement with our findings. Leikert et al.,(2002) examined the effect of red wine polyphenol extract on EA.hy926 cell line. EA.hy296 cell line incubated with different concentrations of extract (100-600 µg/ml) for 18 hours produced a significant increase in NO production (Leikert et al., 2002). Dihydrocaffeic acid (caffeic acid metabolite) at different concentrations (0-200 µM) significantly increased the production of NO after being added to EA.hy926 cells for 18 hours in a dose-dependent manner (Huang et al., 2004).

Another study was performed on NO production after incubating the bovine pulmonary artery endothelial cell line with 50 and 100 µM pomegranate juice for 24 and 48 hours. The observed result showed an increased NO production (Ignarro et al., 2006). Punicalagin and o-galloyl punicalagin extracted from Terminalia calamansanai plant leaves (50 µM each) produced a significant increase in NO production in bovine aortic cell line (Chen et al., 2008). NO production was also significantly increased in a dose-dependent manner after incubating the EA.hy926 cell line for 24 hours with different polyphenols (resveratrol, epicatechin gallate and epigallocatechin gallate) (Appeldoorn et al., 2009). Different concentrations (0.1-10 µM) of polyphenols extracted with 70% acetone from black currant were exposed to EA.hy926 cell line for 10 minutes and produced a significant increase in NO production a dose-dependent manner (Edirisinghe et al., 2011).

4.3.2 Measuring ACE Activity in Ea.hy926 Cells

In this study, incubation of Ea.hy926 with different concentrations of punicalagin (1, 20, 40 and 60 µM) for 24 hours caused significant and dose-dependent inhibition of ACE activity compared with the control (Figure 4.4) (P < 0.0001). The inhibition
percent was 62, 30, 41, 44 and 51% achieved from 1 \mu M captopril, and 1, 20, 40 and 60 \mu M punicalagin, respectively.

![Bar chart A](image1)

![Bar chart B](image2)

**Figure 4.4:** ACE activity in Ea.hy926 cell exposed to punicalagin (1-60 \mu M) for 24 hours.  
A) Represent fluorescence intensity for negative control (untreated cells), positive control (1 \mu M captopril) and treated cells with different punicalagin concentrations, the fluorescent intensity proportional to enzyme activity.  
B) Represent the ACE inhibition activity for positive control and treated cells with different punicalagin concentrations in relative to negative control

\[
\text{ACE inhibition} \% = \frac{(\text{control fluorescence} - \text{sample fluorescence}) \times 100}{\text{control fluorescence}}
\]

Inhibition of enzyme activity was observed in a dose dependent manner. Data represent mean ±SD of more than three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (*=p<0.05, **p<0.001 and *** = p < 0.0001). Captopril was used as a positive control.

Numerous studies have been conducted on endothelial cell lines to examine the effect of polyphenols as ACE inhibitors. Black tea, green tea and rooibos tea, epicatechin, epigallocatechin, epicatechin gallate and epigallocatechin gallate were incubated with HUVEC cell line at different concentrations for 10 min. All experimental components
inhibited the activity of ACE at all concentrations except rooibos tea. Rooibos tea did not contain any catechin compounds (Persson et al., 2006). In 2009, another study was performed on HUVEC cell line to investigate the inhibition of ACE enzyme after exposure to aqueous phenolic extract from Vaccinium myrtillus (bilberry). The bilberry extract contained several polyphenols such as quercetin, stilbene, resveratrol, ferulic acid and coumaric acid; these components (0.000625-0.1 mg/ml) significantly inhibited ACE activity in a dose dependent manner in HUVEC cell line treated for 10 minutes (Persson et al., 2009).

Aviram and Dornfeld (2001) found that serum ACE enzyme activity was significantly inhibited by 36% in seven hypertensive patients who consumed pomegranate juice for 2 weeks. Each treated patient's serum was incubated with pomegranate juice (50-350 µM) for 15 minutes at 37 °C. The enzyme activity was significantly inhibited in a dose dependent manner (Aviram and Dornfeld, 2001). Thus, all the previous research results show similar effects to punicalagin in the present study as an inhibitor for ACE activity on EA.hy926 cell line. The ACE inhibitory activity may be due to metal chelation by punicalagin as zinc ion is present in the active site of ACE. This metal chelating activity of punicalagin is discussed in Chapter 2, Section 2.3.5.

4.3.3 Measurement of ROS by Flow Cytometry and Fluorometric Methods

Angiotensin (Ang) II has the ability to stimulate ROS production in endothelial cells through activation of a redox-sensitive signaling system. In an endothelial cell line, NADPH oxidase was considered as a source of ROS, responding to Ang II with the
donation of an electron to reduce a molecule of oxygen to produce the superoxide anion, 
$O_2^-$ (Rey et al., 2001). ROS production in the Ea.hy926 cell line, treated with different concentrations of punicalagin for 24 hours, in the absence of Ang II stimulation, was not significantly different to ROS production in cells not treated with punicalagin. This was true using either flow cytometry (Figure 4.5A) or fluorimetry (Figure 4.6 A) as the ROS determination method.

The ability of different concentrations of punicalagin added to Ea.hy296 cells for 24 hours to reduce ROS production was assessed by flow cytometer analysis after treating cells with 10 nM Ang II for 1 hour (Figure 4.5 B). As described in the methods section, the non-fluorescent CM-H$_2$DCFDA dye is converted to fluorescent DCF in the presence of ROS. The cells treated with Ang II showed a significant decrease in fluorescence intensity as the concentration of punicalagin increased compared with cells treated with Ang II alone (positive control) (Figure 4.5 B: $p \leq 0.001$). The percent of inhibition was 75, 60, 51, 49 and 48% for the positive control, and 1, 20, 40 and 60 μM punicalagin, respectively.

The results were confirmed by a second method, measuring the conversion of DHE 2-hydroxyethidium in the presence of ROS, by fluorimetry (Figure 4.6 B). The fluorescence intensity in Ea.hy926 cells incubated for 24 hours with punicalagin (1, 20, 40 and 60 μM) and then treated with 10 nM Ang II also decreased in a dose-dependent manner compared with cells treated with Ang II only (positive control). The fluorescence intensity was 0.83, 0.59, 0.54, 0.48 and 0.46 for positive control, and 1, 20, 40 and 60 μM punicalagin, respectively. These findings are in contrast with cells not
stimulated with Ang II, in which no significant difference was found in ROS levels between punicalagin-treated cells and control by flow cytometry or fluorescence methods (Figure 4.5 A and 4.6 A, respectively). These results illustrate that punicalagin has the ability to scavenge ROS and thereby protect cellular macromolecules from ROS-mediated damage. The antioxidant properties of punicalagin showing radical scavenging activity were examined previously in Chapter 2, Section 2.3.3 and Section 2.3.4 and Chapter 3, Section 3.3.3.

![Graph A](image1.png)

![Graph B](image2.png)

**Figure 4.5:** ROS production measurement by flow cytometry in the Ea.hy926 cell line incubated with different punicalagin concentrations with/without 10 nM angiotensin II for 1 hour. **A)** ROS production in the Ea.hy926 cell line treated with different punicalagin concentrations (1-60 μM); **B)** pre-treated cells with punicalagin incubated with 10 nM angiotensin II for 1 hour. Values are mean ± SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (ns = non significant, ** = p < 0.001 and *** = P < 0.0001 when compared with control).
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Figure 4.6: ROS production measurement by DHE fluorescence method in the Ea.hy926 cell line incubated with different punicalagin concentrations with/without 10 nM angiotensin II for 1 hour. A) ROS production in the Ea.hy926 cell line treated with different punicalagin concentrations (1-60 μM); B) pre-treated cells with punicalagin incubated with 10 nM angiotensin II for 1 hour. Values are mean ± SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni’s test (ns= non significant, **= p < 0.001 and ***p < 0.0001 when compared with control).

As reported previously from several in vitro studies, the presence of the OH group in phenolic compounds plays an important role in scavenging ROS (Chen and Ho, 1997; Bouchet et al., 1998; Khan et al., 2007). The Chinese medicine, Seabuckthorn, contains different flavonoids e.g. quercetin, isorhamnetin and kaempherol. Seabuckthorn at different concentrations (9.38-37.5 μg/ml) and 15 μg/ml quercetin was incubated with EA.hy926 cell line for 20 minutes separately before adding 100 μg/ml oxidized LDL as a ROS inducer for a 24 hours incubation time. A protective effect against superoxide anion in EA.hy926 cells was found (Bao and Lou, 2006). ROS production was
significantly decreased in liver cell line (HepG2) exposed to different concentrations of quercetin and rutin (1-100 μM) for 24 hours followed by incubation with 200 μM H2O2 for 3 hours (Alia et al., 2006). Pomegranate juice, concord juice and blueberry juice antioxidant activities were examined and showed scavenging of superoxide anion and protection of NO destruction from ROS action. Pomegranate juice, diluted 6-fold and using a small volume of 3 μl, showed very high antioxidant activity while the same effect was only possible from 300 μl undiluted blueberry or 1000 μl undiluted concord grape (Ignarro et al., 2006). In this study, punicalagin concentrations (1-60 μM) showed a significant reduction of ROS levels in Ea.hy926 cell line after stimulation by Ang II (p < 0.001).

4.3.4 Determination of Cellular Calcium Concentration by Fluorescence Method

Different concentrations of punicalagin (1, 20, 40 and 60 μM) exposed to Ea.hy926 cell line for 24 hours caused a significant dose-dependent increase in intracellular Ca2+ concentration p < 0.001 (Figure 4.7). Since eNOS activity is dependent on Ca2+, the cytoplasmic Ca2+ concentration was investigated in response to different punicalagin concentrations. EGTA (a Ca2+ inhibitor) was used as the negative control.

This study demonstrates that the punicalagin from pomegranate (1-60 μM) produced a significant increase in calcium concentration in EA.hy926 cell line after incubation for 24 hours. Polyphenols can affect intracellular Ca2+ store and release it or increase the entrance of Ca2+ through the cell membrane (Martin et al., 2002). This suggestion could explain the significant induction of Ca2+ concentration by different punicalagin
concentrations in the endothelial cell line (p < 0.001). Previous research results on polyphenols as calcium inducer were similar to our findings.

![Graph showing calcium concentration in EA.hy926 cells exposed to punicalagin (1-60 μM) for 24 hours. Calcium concentration was increased in a dose-dependent manner. Fura 2/AM dye was used to measure calcium concentration. Data represent mean ± SD of more than three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (*=p < 0.05; **=p <0.001 and *** = p < 0.001. EGTA 1 mM was used as a negative control.]

As eNOS enzyme is calcium-dependent, several researchers measured the calcium concentration in EA.hy926 cell line after incubation with different polyphenols. EA.hy926 cell line was incubated for 20 hours with 100-600 μg/ml red wine polyphenols water extract. The experimental dose showed a significant increase in calcium concentration in EA.hy926 cell line, which increased the eNOS protein level, and showed a significant release of NO (Leikert et al., 2002). Another study was performed on calcium concentrations in EA.hy926 after incubation with punicalagin
and o-galloylpunicalagin extracted from *Terminalia Calamansanai* plant leaves; EA.hy926 cell line, exposed to 50 μM of each component for 12 hours produced a significant increase in calcium concentration (Chen et al., 2008).

### 4.3.5 Determination of eNOS Enzyme Activity in Ea.hy926 Cell Line

The effects of punicalagin at different concentrations (1, 20, 40, and 60 μM) on activation of the eNOS enzyme were determined in Ea.hy926 cells after a 24 hours exposure time. As shown in Figure 4.8, punicalagin caused a significant increase in eNOS enzyme activity that was dose-dependent $p \leq 0.05$. EGTA (1 mM) was used as the negative control.

![Figure 4.8: Effect of different concentrations of punicalagin (1-60 μM) incubated for 24 hours on eNOS activity in the Ea.hy926 cell line. The enzyme activity was measured by using eNOS activity detection kit. Enzyme activity was increased in a dose-dependent manner. Data represent mean ± SD of more than three experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test ($* = p < 0.05$; $**=p < 0.001$; and $*** = p < 0.0001$). EGTA 1 mM incubated for 3 hours was used as a negative control.](image-url)
Western blot analysis using a specific antibody against eNOS revealed no change in eNOS protein expression in Ea.hy926 cells treated with punicalagin compared with untreated control cells (Figure 4.9). The calcium concentration for 1 mM EGTA (negative control) was significantly decreased (p ~ 0.05); therefore, the protein expression level of eNOS enzyme was down regulated and eNOS enzyme activity decreased. Punicalagin may increase eNOS activity via eNOS phosphorylation, which is enhanced by the activation of redox-sensitive phosphatidylinositol-3 (PI3)/ protein kinase B (AKT) pathways (Edirisinghe et al., 2011; Hidalgo et al., 2012).

![Western blot of eNOS expression in Ea.hy926 cells treated with different punicalagin concentrations.](image)

Figure 4.9: Western blot of eNOS expression in Ea.hy926 cells treated with different punicalagin concentrations. Protein was extracted from Ea.hy926 cells after treatment with 1-60 μM punicalagin for 24 hours. EGTA 1 mM incubated for 3 hours was used as a negative control. Cells were harvested and lysates were prepared. 20 μg protein per lane 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. MW= 140 kDa.

Similar to this research outcome, pomegranate juice (50 and 100 μM) was examined on bovine pulmonary artery endothelial cell line for 24 and 48 hours. The treatment did not show any significant effect on protein expression on eNOS enzyme at any concentration (Ignarro et al., 2006). Chen et al., (2008) examined the effect of
punicalagin and o-galloylpunicalagin extracted from *Terminalia Calamansanai* leaves on eNOS expression. Protein expression of eNOS enzyme in bovine aorteic cell line was not affected after incubation for 12 hours with 50 μM of punicalagin and o-galloylpunicalagin extracted from *Terminalia Calamansanai* leaves (Chen *et al.*, 2008).

In contrast, Leikert *et al.*, (2002) found that red wine polyphenols water extract (100-600 μg/ml) had increased eNOS protein levels after incubation in EA.hy926 cell line for 20 hours. Huang *et al.*, (2004) found a significant increase in protein expression and activity of eNOS enzyme in EA.hy926 cell line after incubation with dihydrocaffeic aicd (caffeic acid metabolite) at different concentrations (0-200 μM) for 18 hours. The effect of resveratrol, epigallocatechingallate and epicatechingallate on eNOS enzyme was investigated on EA.hy926 cell line at different concentrations (0-100 μM) for 24 hours. The protein level of eNOS enzyme was significantly increased in a dose dependent manner (Appeldoorn *et al.*, 2009). Edirisinghe *et al.*, (2011) studied the black currant polyphenols 70% acetone extract on protein expression of eNOS. Different extract concentrations (0.1-10 μM) were applied on EA.hy926 cell line for 10 minutes. The treated cells produced a significant increase in eNOS enzyme expression associated with the concentration increase (Edirisinghe *et al.*, 2011).

### 4.4 Conclusion

In conclusion, all of the results presented in this chapter support the proposition that punicalagin is a type of polyphenol that could play a role in reducing the risk of cardiovascular disease. As observed previously, punicalagin incubated with EA.hy926
cells at different concentrations for 24 hours produced a significant inhibition of ACE enzyme activity and increased NO production. Increased NO production was via increased eNOS activity due to an increase in calcium concentration. There was no induction of eNOS enzyme expression observed. This NO level is protected from destruction by ROS through punicalagin scavenging activity. The above findings indicate that punicalagin may be helpful for lowering blood pressure; this could be achieved through dietary intervention or by the production of new anti-hypertensive treatments from a natural product.
Chapter 5
5 The Effect of Pomegranate Active Component (Punicalagin) on Caco-2 Human Colon Cancer Cells

5.1 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 (Syed et al., 2007). The carcinogenesis process (initiation, promotion and progression) is enhanced due to high ROS level in the organs (Khan et al., 2008). Xenobiotics, toxins and ingested food are some sources for ROS production in the small intestine (Herring et al., 2007).

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 (Surh, 2003). The third most common type of cancer incident in the world is colorectal cancer. However, in terms of morbidity, colorectal cancer is the fourth most common type of cancer death globally (Parkin et al., 2001). Around 610,000 people have been estimated to die due to colorectal cancer in 2008 (Ferlay et al., 2011). According to the International Agency for Research on Cancer (2010), the United Kingdom colorectal cancer mortalities were 47th highest of male and 48th highest for females out of 184 countries worldwide (Ferlay et al., 2011). In Saudi Arabia, according to the tumor registry annual report (2011) from King Faisal Special Hospital and Research Center,
colorectal cancer was the most common cancer among male population and the third most common among female population; males were more affected by this disease than females (Mehmood et al., 2012). Some risk factors of this type of cancer are human lifestyle (smoking and heavy alcohol drink) and dietary factors. This risk is associated with an increased fat consumption and decreased consumption of cereal grains and dietary fibre (Potter et al., 1993). Therefore, the incidence of colorectal cancer all over the world is varied depending on the dietary style in each country (Parkin et al., 2001; Bingham et al., 2005; Moghaddam et al., 2007).

Due to concerns for general health, chronic disease prevention and aging have increased the consumers interest in phytonutrients because of their potential curative, preventive and nutritive values (Guhr and Lachance, 1997). Phytonutrients have functionally been demonstrated as antioxidants, as well as modulators of enzyme activity, cell proliferation and apoptosis (Balentine et al., 1999). 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 polyphenols content. The suggested mechanisms of polyphenol action as anticancer agents include antioxidant, anti-inflammatory and anti-proliferative activities as well as their effect on subcellular signaling pathways, stimulation of cell cycle arrests and apoptosis (Middleton et al., 2000; Yang et al., 2001). Pomegranate is widely consumed as fruit, juice and as wine (Gil et al., 2000). Pomegranate contains pectin, sugars, fiber 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
and punicalagin. Around 92% of pomegranate antioxidant activity is due to the presence of hydrolysable tannins (Khan et al., 2010).

One of the anticancer 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 has been characterised by cell shrinkage (nuclear chromatin condenses and cytoskeleton collapses) and engulfed 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 and Thompson, 2004). Some dietary polyphenols such as EGCG and genistein have the ability to induce apoptosis in various cancer cell lines but not to normal cell lines (Ahmad et al., 1997; Chang et al., 2009).

According to a number of studies on the anticancer properties of polyphenols, increasing the consumption of fruits, vegetables and dietary fibre may reduce the risk of colon cancer (Steinmetz and Potter, 1996; Briviba et al., 2002; Larrosa et al., 2006a). It is, therefore, the purpose of this study to investigate the mechanism in which pomegranate active component (punicalagin) acts as an anticancer agent. To achieve this goal, the colon carcinoma cell culture model (Caco-2) was used and the results were compared with a normal colon cell line (HCEC).
Chapter 5: The Effect of Pomegranate Active Component (Punicalagin) on Caco-2 Human Colon Cancer Cells

The objectives of this chapter are therefore:

- To determine the cytotoxicity of punicalagin at different concentrations (1-500 \( \mu \text{M} \)) on Caco-2 cells and the HCEC cell line by MTT method.

- To study the morphological changes in Caco-2 cell line and HCEC cells induced by punicalagin.

- To determine the level of ROS by flow cytometry using CM- \( \text{H}_2\text{DCFDA} \).

- To determine the cell death type on Caco-2 cell line by Annexin V-FITC.

- To examine the effect of punicalagin treatment on the Caco-2 cell cycle at a range of concentrations.

5.2 Materials and Methods

5.2.1 Materials

The human colorectal carcinoma cell line Caco-2 was obtained from the European Collection of Cell Cultures (ECACC), Salisbury, UK. The normal colon cell line (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 lytic buffer were obtained from Sigma-Aldrich Chemical Co, 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$_2$DCFDA, Mitosox WesternBreeze Antibody were obtained from Invitrogen, Paisley, UK. PARP, caspase 9,8 and 3 were purchased from Cell Signaling Technology, UK.

5.2.2 Methods

5.2.2.1 Cytotoxicity of Punicalagin on Colon Cells

Colon cancer cell line (Caco-2) was grown in DMEM media with high glucose content without sodium pyruvate. Media was supplemented with 20% FBS, 5% penicillin and 5% glutamine. The cytotoxic effect of different concentrations ranging from 1-500 μM of punicalagin on Caco-2 cells was studied. In brief, a stock solution of 10 mM punicalagin was prepared in DMSO. Caco-2 cells were seeded into 96 well tissue culture plates at 1×10$^4$ cells/200 μl DMEM serum media. After 24 hours, punicalagin of different concentration was added to each well. The MTT determination was performed after 24 hours of incubation at 37 °C and 5% CO$_2$. 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 hours 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 seconds at room temperature. The colour intensity was measured at 492 nm using a plate reader (Boehringer CO, Marburg, Germany).

The HCEC cell line was seeded in DMEM with high glucose content without sodium pyruvate, supplemented with 10% of FBS. Tissue culture plates (96 wells) were coated
for 15 minutes with a coating solution before seeding HCEC cells. The coating solution contained 50 ml serum-free media (without FBS), 65 µl 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 minutes, the coating solution was collected and reused for 5 times only. The HCEC seeding density was 1×10^4 cells per well. The same concentrations of punicalagin were examined using HCEC as with 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.

5.2.2.2 Morphological Change in Caco-2 and HCEC Treated with Punicalagin

Caco-2 cells and HCEC cell line were seeded at concentrations of 1×10^6 cells/ml in 25cm² flasks. After 24 hours, two different concentrations of punicalagin (50 µM and 75 µM) were applied to each cell line. Cellular morphology was determined by microscopy after 24 and 48 hours. 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.

5.2.2.3 Measurement of Cellular Reactive Oxygen Species (ROS)

Dichlorofluorescein dye (non-fluorescent CM-H2DCFDA) has the ability to diffuse across cell membranes (Osseni et al., 1999). In the cytoplasm, this dye is hydrolysed enzymatically by intracellular esterases and is rapidly converted to fluorescein dye (DCF) in the presence of ROS (Osseni et al., 1999). The fluorescence intensity was 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 concentration were measured by flow cytometry using CM-H2DCFDA dye. Caco-2 cells were seeded in 25cm² flasks at 1×10⁶ cells/ml. The treatments were applied once the cells reached 60-70% confluence. Following that, the cells were trypsinised and centrifuged for 3 minutes at 150 x g using a Beckman GRP centrifuge. The supernatant was discarded and the cells were washed and centrifuged with 3 ml PBS. Again, the supernatant was discarded and 1 ml PBS was added. The cells were incubated with 5 μM of DCFDA (prepared in DMSO) for 30 minutes at 37 °C and 5% CO₂. At the end of the incubation time, the cells were kept on ice under low light conditions due to the high susceptibility of the dye to photo-oxidation 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. Experiments were carried out in triplicate and repeated three times to ensure reproducibility.

5.2.2.4 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 occurs in living cells. Only once this dye is oxidised by superoxide ion, does it produce red fluorescence that can be determined by flow cytometry (Liu et al., 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. Caco-2 cells were seeded in 25cm² flasks at a density of 1×10⁶ cells/ml. The treatments were applied after 24 hours of seeding. Following incubation for 24 hours with treatments, the cells were trypsinised and centrifuged for 3 minutes at 150 x g using a Beckman GRP centrifuge. The supernatant was removed and cells were washed by
centrifugation with PBS for 3 minutes at 150 x g. The supernatant was discarded and
1 ml Hank’s Buffered Salt Solution (HBSS) containing calcium and magnesium was
added. The cells were then incubated with 5 μM of Mitosox (prepared in DMSO) for
10 minutes at 37 °C and 5% CO₂. At the end of the incubation time, the cells were kept
on ice under low light conditions, due to the high susceptibility of the dye to photo-
oxidation, until measurements were taken. Red florescence was measured using BD
FACSCanto flow cytometer (California, USA). At least 10,000 events were acquired in
the gated regions using an emission wavelength of 620 nm. Experiments were carried
out in triplicate and repeated three times to ensure reproducibility.

5.2.2.5 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 apoptosis programme and was
measured by using annexin V–FITC apoptosis detection kit from Merck Millipore. To
detect early and late apoptosis, Caco-2 cells were cultured in 25cm² flasks at a
concentration of 1×10⁶ cells/ml and incubated in 5% CO₂ at 37 °C. Treatments were
added 24 hours later and cells were incubated for 24 hours while exposed to different
concentrations of punicalagin (50 μM and 75 μM). Before trypsinisation, the media was
collected in falcon tubes from each flask; this media contained floating cells produced
during the cell death process; the trypsin was inhibited by re-suspending the detached
cells in the collected media. All cells were then centrifuged for 3 minutes at 150 x g
using a Beckman GRP centrifuge. Chilled binding buffer (500 μl) was added to each
tube followed by 1.25 μl conjugated Annexin V-FITC. The cells were then incubated
for 15 minutes in the dark at room temperature. At the end of this incubation, the cells
were centrifuged for 3 minutes at 150 x g using a Beckman GRP centrifuge. Supernatant was removed and 500 µl cold binding buffer was added with 10 µl from propidium iodide (PI) and mixed gently. The cells were kept on ice until analysed by BD FACSCanto flow cytometry (California, USA). A minimum of 10,000 events was acquired in the gated regions. The emission was 520 nm for cells labelled with Annexin V-FITC and 620 nm for cells labelled with 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. Experiments were carried out in triplicate and repeated three times to ensure reproducibility.

5.2.2.6 Cell Cycle and DNA Fragmentation

To study the effect of punicalagin, 50 µM and 75 µM on the cell proliferation of Caco-2 cells. Cells were seeded in 25 cm² flasks at a concentration of 1×10⁶ cells/ml. Each punicalagin concentrations were added once the cells reached 50-60% confluence using a 24 hours exposure time. The cells were trypsinised and centrifuged for 3 minutes at 150 x g using a Beckman GRP centrifuge. 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 minutes to achieve (1) full fixation and (2) complete dissociation of clumped cells. The cells were incubated at 4 °C for 24 hours. On the following day, the cells were centrifuged to remove the fixation buffer. Next, the cells were re-suspended in PBS with 10 µM of RNAse and incubated for 30 minutes at 37 °C. At the end of this incubation, the cells were then stored on ice and 10 µl of 1mg/ml PI was added to each sample and analysed by BD FACSCanto flow cytometry.
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.

5.2.2.7 Assessment of Caspases Family by Westren Blot

5.2.2.7.1 Preparation of Whole Cell Lysate

Caco-2 cells at a density of $1 \times 10^6$ cells/ml were seeded in 25 cm$^2$ tissue culture flasks. Once 50% confluence was achieved, cells were treated with 50 μM and 75 μM punicalagin and incubated for 24 hours. 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 $1500 \times g$ for 3 minutes with 5 ml PBS to wash. Supernatants were removed and the cell pellets were lysed by adding 300 μl of lysis buffer from (Sigma Aldrich Chemical Co.). The last step for cell lysis was to keep the cells on ice for 20 minutes and then store them at -80 °C until which time for protein determination and western blot experiment were determine.

5.2.2.7.2 Protein Quantification and Western Blot

The protein concentration for each sample was measured, as described previously in Chapter 3 Section 3.2.2.6.1, with Bradford method using BioRad assay (Bradford, 1976). The Western blot technique was performed as described previously in Chapter 4, Section 4.2.2.7.3.
5.2.2.8 Statistical Analyses

All experiments were performed in triplicate. For the 96-well microtiter tissue culture plates, 4 replicate wells were used per category. The data were analyzed by Graphpad Prism software 6. Significant differences between the control and the experimental values were evaluated using P-values determined by one way analysis of variance followed by Bonferroni test. Significance was set at a cut-off level of ≤0.05.

5.3 Results and Discussion

5.3.1 Dose Response Curve for Punicalagin

To measure the effect of punicalagin on the Caco-2 cell lines, cell viability was assessed by the MTT assay as shown in Figure 5.1.

![Dose response curve for Punicalagin](image)

**Figure 5.1:** Dose response curve for different concentrations of punicalagin incubated for 24 hours in Caco-2 cell Line. Punicalagin concentrations were (1, 5, 10, 25, 50, 100, 150, 200 and 500 μM) incubated for 24 hours in Caco-2 cell Line. Cell viability was measured by the MTT assay. Log concentrations were plotted in Graphpad Prism to obtain a sigmoidal dose response curve and the curve was fitted using a standard IC$_{50}$ equation. The data represent the mean of three independent experiments (n=3 ± SD).
Punicalagin doses of 1-500 µM cause a significant dose-dependent inhibition of cell survival. The IC\textsubscript{50}, determined after a 24-hour incubation time, was 75 µM.

### 5.3.2 Comparison of the Effect of Punicalagin on Caco-2 and HCEC Cells

Figure 5.1 revealed that a concentration of 75 µM was the IC\textsubscript{50} of punicalagin concentration on 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.

![Cell viability graph](image)

**Figure 5.2**: The effect of different punicalagin concentrations on Caco-2 and HCEC cells for 24 hours. Cells were treated with punicalagin 50, 100, 150, 200 and 500 µM. The cell viability was monitored for 24 hours by the MTT assay. 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 test (** = p < 0.001; *** = p < 0.0001).
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The cell viability for Caco-2 cells was 152%, 141%, 131%, 95%, 67%, 53%, 46%, 37%, and 19% at punicalagin concentrations of 1, 5, 10, 25, 50, 100, 150, 200, and 500 µM respectively. Different punicalagin concentrations (50-500 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 presented in Figure 5.2. In contrast to Caco-2 cells, the cell viability for HCEC cells was 95%, 93%, 70%, 62%, and 43% at 50, 100, 150, 200 and 500 µM punicalagin concentrations, respectively.

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). Seeram et al., (2005) observed a greater anti-proliferative effect for pomegranate juice at 100 µg/ml, compared with ellagic acid, punicalagin and total pomegranate tannin when using different human tumour cell lines (oral, colon and prostate). In their research, the cell proliferation in all tumour cell lines tested was inhibited by 30% to 100% at 12.5 µg/ml to 100 µg/ml in a dose-dependent manner (Seeram et al., 2005). Similarly, Larrosa et al., (2006) found that the cell viability of Caco-2 cells exposed to ellagic acid (1, 10 and 30 µM) or punicalagin (1, 10 and 100 µM) for 24 hours was significantly reduced in a dose dependent-manner, by both compounds. Additionally, another research study performed on a 70% acetone extract of pomegranate, similarly showed anti-proliferative effects on a lung cancer cell line (A549) at different concentrations (50, 100 and 150 µg/ml) in a dose-dependent manner (Khan et al., 2007). 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).
5.3.3 Morphological Changes of Caco-2 and HCEC Cells Induced by Punicalagin

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 48 hours. Figure 5.3 shows a morphological change in the Caco-2 cell line in response to punicalagin treatments compared with the control. Conversely, the same concentrations of punicalagin had little effect on HCEC comparable to the control, i.e. the cells appeared as healthy as the control (Figure 5.4). This confirms the anti-proliferative effect of punicalagin on the colon cancer cell line but not on normal colon cell line.

![Figure 5.3: Effect of different punicalagin concentrations on the Caco-2 Cell line for 24 and 48 hours. Caco-2 cells were treated with 50 µM and 75 µM punicalagin for 24 and 48 hours. Images were captured using light microscopy at 10x magnification.](image-url)
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5.3.4 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%) (Figure 5.5 A) (p < 0.001). Mitochondrial dysfunction may have been caused by different punicalagin concentrations; this may be the reason for the increased production of superoxide anion. In section 5.4 we show the activation of caspase 9 which support the hypothesis that punicalagin caused mitochondrial dysfunction. When mitochondria lose their function they release cytochrome C which activates caspase 9 (Larrosa et al., 2006a). In contrast to mitochondrial ROS production discussed above, reduction in cytoplasmic ROS production was observed with 50 μM and 75 μM punicalagin compared with the control (Figure 5.5 B). The percent ROS inhibition was 69% at 50 μM and 54% for 75 μM, while the control ROS production was 81%.

Figure 5.4: Effect of different punicalagin concentrations on the HCEC cell line for 24 and 48 hours. HCEC cells were treated with 50 μM and 75 μM punicalagin for 24 and 48 hours. Images were captured using light microscopy at 10x magnification.
**Figure 5.5:** Measurements of mitochondria and cellular ROS in Caco-2 cells treated with different punicalagin concentrations for 24 hours. 

A) Punicalagin-Induced mtROS Production in Caco-2 Cells. Caco-2 cells were treated with 50 µM and 75 µM punicalagin for 24 hours. 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 24 hours. Cells were loaded with CM-H2DCFDA and DCF and 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 test (** = p < 0.001*** = p < 0.0001).

In this research, the DCF assay method was applied to assess ROS production or scavenging activity by phenolic compounds to induce cell death. According to several studies in which ROS levels in tumour cell lines after exposing them to polyphenols or flavonoids were measured, the induction or reduction of ROS levels leads to cell death (Yamamoto *et al.*, 2003; Qanungo *et al.*, 2005; Renis *et al.*, 2008; Singh *et al.*, 2011). Mertens-Talcott *et al.*, (2006) reported that tannins namely ellagic acid (0.4 µmol/g) and ellagitannine (0.8 µmol/g) from red muscadine grapes, caused a significant increase in ROS production when applied to the Caco-2 cell line at different dilutions (1:10, 1:100 and 1:1000). However, the total red muscadine grapes phenolic extract (7.45 µmol/g)
caused a significant reduction in ROS level at the lowest dilution (1:10), while the other dilutions from red muscadine grapes (1:100 and 1:1000) did not change the generation of ROS (Mertens-Talcott et al., 2006).

In contrast, cellular ROS levels in Caco-2 cells was decreased significantly by anthocyanins, cyanidin at 25, 50, 100 and 200 μM, while, cyanidin-3-o-β-glucopyranoside had the ability to decrease ROS level only at the lowest concentration (25 μM) (Renis et al., 2008). In a different study, also on anthocyanins, ROS levels decreased significantly in Caco-2 cells and in a metastatic colorectal cell line (lovo cell line) after exposure to different concentrations (25, 50 and 100 μM) of cyanidin and delphinidin (Cvorovic et al., 2010). The above results are in agreement with our findings, and the high antioxidation potential of punicalagin due to the presence of phenolic groups may explain this decrease in cytoplasmic ROS (Mertens-Talcott et al., 2006).

5.3.5 Apoptosis in Caco-2 Cells

In the presence of 50 μM and 75 μM punicalagin concentrations for 24 hours, apoptosis was significantly induced in the Caco-2 cell line. Compared with the negative control, the percentage of apoptotic cells, in both the early apoptotic (single Annexin V) and the late apoptotic (Annexin V plus PI) stages were significantly increased (p <0.0001). The proportion of cells in the early apoptotic stage was 5%, 18%, 20%, and 28% for control, 50 μM, 75 μM punicalagin, and staurosporine (positive control) while the proportion in the late apoptotic stage was 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 value >0.05. The proportion of cells undergoing
necrosis was 6.5%, 7.5%, and 8% for negative control, 50 μM and 75 μM punicalagin, and staurosporine respectively (Figure 5.6 A & B).

Figure 5.6: Detection of Caco-2 cell death treated with different punicalagin concentrations for 24 hours by flow cytometric analysis. A) flow cytometric analysis of Annexin V in Caco-2 Cells treated with 50 μM and 75 μM punicalagin for 24 hours. Cells were harvested and stained with propidium iodide (PI) and Annexin 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 24 hours. 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).

Several studies have been conducted using the Annexin V assay to determine the type of cell death programme induced by polyphenols and anthocyanins as chemo-preventive
agents in different cell lines (Seeram et al., 2005; Larrosa et al., 2006a; Hafeez et al., 2008). In a study conducted by Seeram et al., (2005), significant induction of apoptosis in a colon cancer cell line (HT-29) was observed mostly by pomegranate juice followed by ellagic acid, punicalagin, and lastly by total pomegranate tannins at 100 μg/ml. However, pomegranate juice did not have any apoptotic effect on a different colon cancer cell line (HCT 116), although ellagic acid, punicalagin and total pomegranate tannins induced apoptosis at the same concentration 100 μg/ml (Seeram et al., 2005). Larrosa et al., (2006) found that both ellagic acid at 1, 10 and 30 μM and punicalagin at 1,10 and 100 μM significantly induced apoptosis in Caco-2 colon cancer cells in a dose-dependent manner. In contrast, no apoptotic effect was observed in a normal colon cell line (CCD 112) at the tested concentrations (Larrosa et al., 2006a). Hafeez et al., (2008) found the significant effect of delphinidin at 60, 120 and 180 μM as a cell inhibitor in a prostate cancer cell line (PC3) (Hafeez et al., 2008).

In this research, Annexin V and morphological changes to the Caco-2 cell line in the presence of 50 μM and 75 μM punicalagin concentrations confirmed the apoptosis cell death pathway.

5.3.6 Effect of Punicalagin on Cell Cycle

Figure 5.7 illustrates Caco-2 cell cycle phases after treatment with 50 μM and 75 μM punicalagin. 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 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 45%, 54%, and 60% for control, 50 μM and 75 μM, (p ≤ 0.01), respectively. The percentage of
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cells in the S-phase was 26%, 19% and 14%, while in the G2-phase, the percentage was 7%, 7.4% and 7.5% for the control and 50 μM and 75 μM (p ≤ 0.001), respectively.

Figure 5.7: Cell cycle arrest in Caco-2 cells treated with different punicalagin concentrations for 24 hours by flow cytometry. A) Caco-2 cells were treated with 50 μM and 75 μM Punicalagin and fixed in 70% ethanol for 24 hours. 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 24 hours 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 ± SD). Comparisons of means were made using one-way ANOVA followed by Bonferroni’s test (*=p < 0.01, **= p < 0.001 and ***= p < 0.0001).

In the present research, punicalagin at different concentrations may down-regulate cyclin E. Cyclin E is involved as a checkpoint for the cells to transfer from G1- to S-
phase; thereby any alteration in cyclin E may arresting the cell cycle (Lazze et al., 2004). Caco-2 cells significantly reduced in the S-phase and consequently increased significantly in the G1-phase.

Cell cycle arresting has been considered as a target for cancer therapy (Hartwell and Kastan, 1994; Dickson and Schwartz, 2009). Phenolic compounds showed different arresting phases in different cancer cell lines (Lazze et al., 2004). In this research, the significant reduction of Caco-2 cells in to S-phase and the accumulation the G1-phase caused by 50 μM and 75 μM punicalagin is 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., 2006a). In a separate study, ellagic acid at 10 μM and urolithins (A and B) at 40 μM have individually been shown to cause accumulation of Caco-2 cells in the S-phase (Gonzalez-Sarrias et al., 2009). An extract of pomegranate 70% acetone extract has also been shown to have a chemotherapeutic potential against a lung cancer cell line (A549) (Khan et al., 2007). 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).

Anthocyanins delphinidin, as a class of flavonoid, have also shown efficacy in arresting the cell cycle of prostate cancer cell line (PC3) (Hafeez et al., 2008). The PC3 cell line was exposed to 60, 120 and 180 μM delphinidin concentrations. Delphinidin induced apoptosis and arrested cells in the G2-M-phase in a dose-dependent manner (Hafeez et al., 2008).
5.3.7 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 (Figure 5.8) 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 both lead to the activation of caspase 3. Subsequently, the poly (ADP-ribose) polymerase protein (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 PARP protein as represented by Western blotting (Figure 5.8). Therefore, the effect of punicalagin as an inhibitor of colon cancer cell growth is mediated by the activation of the caspase family.

The caspase activation results confirm the findings of previous research conducted on 100 μM punicalagin and 30 μM ellagic acid (Larrosa et al., 2006a), except for the activation of caspase 8 which was only demonstrated in the present research wherein neither punicalagin nor ellagic acid activated caspase 8. Other polyphenols like pomegranate delphinidin (60, 120, and 180 μM) also induced apoptosis through the activation of caspase9 and 3 enzymes in prostate cancer cell line (PC3). (Hafeez et al., 2008).
Figure 5.8: 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 24 hours. 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 (MW=45 kDa). Panel A protein expression for caspase 9 (MW=47 kDa). Panel B protein expression for caspase 8 (MW=57 kDa), panel C protein expression for caspase 3 (MW=35 kDa) and panel D protein expression for PARP-1 (MW=116, 89 kDa).

5.4 Conclusion

According to the results obtained from all the previous experiments, punicalagin at 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 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, 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. Punicalgin may be a potential anticancer agent.
Chapter 6
6 General Discussion and Conclusions

6.1 General Discussion

In recent years there has been increased attention on phenolic compounds and flavonoids due to evidence from several research groups that diets rich in vegetables and fruits can reduce the risk of developing several diseases (Borges et al., 2010). In particular, pomegranate fruit has received attention because it contains many different phytochemical compounds. Pomegranates are a rich source of phenolic compounds that include hydrolysable tannins, condensed tannins, and flavonoids (flavonols, flavanols and anthocyanins) (Malik et al., 2005). Punicalagin, ellagic acid, and gallic acid are polyphenolic tannins found in pomegranate (Gil et al., 2000). Punicalagin (ellagitannin) is the main polyphenol in pomegranate husk (Chen and Li, 2006; Kulkarni et al., 2007; Lee et al., 2008); this compound is reported to have anti-cancer, anti-inflammatory, and anti-atherosclerotic properties (Chen et al., 2000; Seeram et al., 2005; Kulkarni et al., 2007). Therefore, this research aimed to study the detection of commercial punicalagin in pomegranate husk and juice and to determine some of the bioactive properties of punicalagin.

6.1.1 Punicalagin Detection in Pomegranate (husk and juice) and its Antioxidant Activity

In this study, the presence of punicalagin in pomegranate husk (US) and pomegranate juice (US & UK) was compared with a punicalagin standard using high performance liquid chromatography (HPLC) and liquid chromatography- mass spectroscopy (LC-MS) which are highly sensitive and selective analytical methods for the separation
and identification of phenolic compounds and anthocyanins (Gil et al., 2000; Fischer et al., 2011; Qu et al., 2012). The present study shows a high degree of similarity of HPLC and LC-MS results between the punicalagin commercial standard (Sigma Aldrich) and US pomegranate husk extracted with methanol. In contrast, in the methanol juice extract obtained from US and UK pomegranates, punicalagin was not detected using the same detection methods. Most research on pomegranate has established that phenolic compounds such as punicalagin, gallic acid, and ellagic acid are in high quantities in pomegranate husk, whereas the concentration of anthocyanins like delphinidin, cyanidin and pelargonidin is high in pomegranate juice (Gil et al., 2000; Fischer et al., 2011; Qu et al., 2012).

6.1.2 Punicalagin Antioxidant Activity

In the present work, the antioxidant mechanism of punicalagin and pomegranate water extract was investigated. The DPPH radical scavenging activity assay has previously been used to measure the free radical scavenging effectiveness of different polyphenols (Bersuder et al., 1998; Murcia et al., 2001; Gülçin et al., 2010). In the present study, inhibition of DPPH· by pomegranate juice or punicalagin was increased with increasing concentration (0.05-0.15 mg/ml). Pomegranate juice showed significant inhibition of DPPH· compared with punicalagin at concentrations between 0.1 and 0.15 mg/ml (p < 0.05). Thus, pomegranate juice exhibits greater ability to donate hydrogen atoms to reduce the stable radical DPPH· to its non-radical form (DPPH-H) than punicalagin. The inhibition of DPPH radical activity caused by natural (trolox) and synthetic (BHT) antioxidants (positive controls) at 0.1 mg/ml was
compared with pomegranate and punicalagin at the same concentration. Compared with BHT, pomegranate juice and punicalagin inhibited the DPPH• radical activity to a similar extent. In contrast, an equivalent concentration of trolox 0.1 mg/ml significantly inhibited DPPH• to a greater degree than punicalagin or pomegranate juice (p < 0.0001 for both examples components). Several polyphenols such as tannins and anthocyanins have demonstrated antioxidant properties through scavenging the DPPH• radical (Ozcelik et al., 2003).

The second radical scavenging mechanism for punicalagin and pomegranate juice as H2O2 scavenger was examined. H2O2 has an effect on lipid peroxidation; however, it can sometimes cause cytotoxicity if it generates hydroxyl radicals (Namiki, 1990). The hydroxyl radical is a very reactive free radical, which can initiate lipid peroxidation (Halliwell and Gutteridge, 1984). In this study, both punicalagin and pomegranate juice showed scavenging activity towards H2O2, which was dose dependent. Pomegranate juice demonstrated significant scavenging of H2O2 compared with punicalagin at 0.15 mg/ml (p < 0.001); the percent inhibition was 30% and 18% for pomegranate juice and punicalagin, respectively. In addition, pomegranate juice and punicalagin showed significant inhibition of H2O2 at 0.1 mg/ml compared with BHT at the same concentration (p <0.001 for both experimental components). However, no significant difference was observed when punicalagin and juice were compared with trolox. The percent of inhibition was 19%, 17%, and 17% for trolox, pomegranate juice, and punicalagin, respectively.
The ability of punicalagin and pomegranate juice to act as ferrous chelating agents was examined in order to study their antioxidant effects further. Ferrous metal (Fe$^{2+}$) ions are reactive and can induce free radical formation via the Fenton reaction: 

$$\text{Fe}^{2+} + \text{H}_2\text{O}_2 \rightarrow \text{Fe}^{3+} + \text{OH} + \cdot\text{OH}$$

(Halliwell and Gutteridge, 1984). Consequently, this radical can initiate lipid peroxidation (Yu et al., 1988). The amount of Fe$^{2+}$-ferrozin complex was significantly reduced in a dose-dependent manner in the presence of punicalagin and pomegranate juice at concentrations of 0.05-0.15 mg/ml. At 0.15 mg/ml concentration ferrous chelating activity of punicalagin was significantly higher than for pomegranate juice (p < 0.05). The positive control (EDTA) showed 97% complex inhibition, while punicalagin and pomegranate juice showed 18% and 14% inhibition at 0.1 mg/ml, respectively. It has been established that, due to the presence of several hydroxyl groups, many phenolic compounds can bind with metal ions such as Fe$^{2+}$ or Cu$^{2+}$ and prevent free radical formation (Yoshino and Murakami, 1998). Moreover, moderate free-radical scavenging components often have strong metal chelation capacity (Wu et al., 2006).

The potential of pomegranate juice and punicalagin to act as reducing agents was also tested. Punicalagin and pomegranate juice appeared to have reducing activity associated with increased concentrations (0.05-0.15 mg/ml). Reduction of ferric (Fe$^{3+}$) to ferrous (Fe$^{2+}$) iron by pomegranate juice and punicalagin has been demonstrated in this study. Both compounds have the ability to reduce Fe$^{3+}$ to Fe$^{2+}$ in a dose-dependent manner. Punicalagin was a more powerful reducing agent than pomegranate juice at all concentrations (0.05-0.15 mg/ml; p ≤ 0.05). As reported by Gill et al., (2000) punicalagin contains 16 phenolic hydroxyls per molecule, while the manual extract of pomegranate juice contains higher concentrations of anthocyanins.
than the tannin compounds. This may help to explain the high reducing activity of punicalagin compared with the pomegranate juice extract.

Increased levels of ROS in the cells, results in the phenomena of oxidative stress that can lead to cell death. Oxidative stress can be involved in the initiation of different diseases such as cancer (Zhao et al., 2004; Hafeez et al., 2008) and cardiovascular diseases (Paravicini and Touyz, 2006; Tabassum and Ahmad, 2011). Therefore, in vitro cyto-protective effects of punicalagin against T-BOOH damage were investigated through different mechanisms such as MDA, glutathione and ROS levels.

6.1.3 Protective Effect of Punicalagin on Caco-2 Intestine Cell Line under Oxidative Stress caused by Tert-butylhydroperoxide

Antioxidants are compounds that have the ability to scavenge ROS and reduce oxidative stress. The damaging effects of ROS on cell macronutrients such as lipids, proteins and nucleic acids can be prevented by antioxidants (Lima et al., 2006). Our data from experiments using the Caco-2 cell line incubated with different concentrations of punicalagin (1-200 μM) for 24 hours showed non-significant decreases in cell viability at 1, 3, 5 and 10 μM punicalagin concentration. However, even at these concentrations, cell viability was >95%. A concentration of 3 mM T-BOOH exposed to cells for 2 hours was used as an oxidative inducer. The protective effect of punicalagin (after a 24-hour incubation period) was observed on Caco-2 cell viability after exposure to T-BOOH. Cell viability was significantly greater at 5 and 10 μM concentrations of punicalagin, compared with 1 and 3 μM concentrations (P <0.001). The percent cell viability was 55, 62, 65 and 72% for 1, 3, 5 and 10 μM respectively, versus 58 % for 3 mM T-BOOH only. This protective effect of
punicalagin was confirmed by microscopy, where adverse morphological cell changes were less apparent with antioxidant treatment.

Malondialdehyde levels (MDA) were measured as an indicator of lipid peroxidation (Suttnar et al., 2001). In several diseases that are associated with free radical damage, the MDA level is increased (Suttnar et al., 1997). In this study, the effectiveness of punicalagin to reduce the level of MDA under oxidative stress induced by T-BOOH was tested. The level of MDA after induction by T-BOOH was significantly reduced when Caco-2 cells were pretreated for 24 hours with 5 and 10 μM punicalagin concentrations (p < 0.0001). Therefore, the protective effect of punicalagin against T-BOOH is assumed to be due to its ability to scavenge ROS that would otherwise oxidize the polyunsaturated fatty acids in cell membranes (Mora et al., 1990). This effect is similar to the effects of other polyphenols that protect cell membrane lipids from the effects of ROS (Alia et al., 2006b).

Several studies reported that polyphenols could decrease the level of ROS in a liver cell line (HepG2) (Alia et al., 2006a). The effect of punicalagin as a ROS scavenger was studied in the current research. Caco-2 cells were incubated with punicalagin at 5 and 10 μM for 24 hours and then treated with 3 mM of T-BOOH for 2 hours. In the presence of 3 mM T-BOOH, the cells pretreated with trolox (positive antioxidant control) or punicalagin showed a significant decrease in ROS levels compared with cells treated only with T-BOOH (p < 0.0001). Punicalagin clearly showed antioxidant activity by inhibiting ROS generation induced by T-BOOH. The presence of several hydroxyl groups in the chemical structure of punicalagin may account for this property (Dziedzic and Hudson, 1983).
Glutathione (a non-enzymatic antioxidant) has an important protective role against ROS in the cell (Dringen, 2000). Increased intracellular oxidation leads to depletion of glutathione (Buc-Calderon et al., 1991; Martin et al., 2001). Conversely, an increased level of glutathione protects the cell from the harmful effect of ROS. Therefore, the level of glutathione in Caco-2 cells pretreated with punicalagin under T-BOOH oxidative stress conditions was measured. Our data showed that glutathione significantly increased to 0.3 μM in pretreated cells compared with untreated cells under oxidative stress conditions. Untreated cells had a glutathione level of 0.18 μM, which indicates significant depletion due to increased ROS levels. From this point of view, the protective effect of polyphenols as radical scavengers could help to maintain glutathione levels under oxidative stress conditions as demonstrated in other published research concerning the effect of polyphenols on human health (Alia et al., 2006a; Lima et al., 2007).

Several studies have shown that high intakes of polyphenols may protect the human body from cardiovascular diseases (Aviram and Dornfeld, 2003; Adams et al., 2006). Possible protection against cardiovascular diseases could be mediated by polyphenol effects, namely anti-platelet aggregation (Appeldoorn et al., 2009), vasodilator properties (Leikert et al., 2002; Acharya et al., 2003), and prevention of LDL oxidation. In this study, the ability of punicalagin to act as an antihypertensive agent has been studied because hypertension is a potential risk for cardiovascular disease. Different pathways have been investigated as follows.
6.1.4 Punicalagin Inhibits Angiotensin Converting Enzyme and Induces Nitric Oxide Production in Endothelial Cell Line EA.hy926

Major research indicates that polyphenols have the ability to reduce the risk of cardiac disease such as atherosclerosis as well as hypertension (Leikert et al., 2002; Huang et al., 2004; Persson et al., 2009). A wide concentration range of punicalagin (1-100 μM) was examined using an exposure time of 24 hours on EA.hy926 cells. At concentrations of 1-60 μM, no cytotoxicity was observed, while NO production increased dose-dependently. In contrast, at 80 and 100 μM punicalagin, a reduction in cell viability and consequently reduction in NO was observed, indicating toxicity at high levels of punicalagin on EA.hy926 cells.

To investigate whether punicalagin has the potential to reduce hypertension via ACE inhibition, the activity of ACE was examined. This research showed significant inhibition of ACE activity associated with increasing punicalagin dose (p < 0.0001 for all concentrations 1-60 μM). As a consequence of enzyme inhibition, levels of angiotensin II were decreased. Angiotensin II acts as a vasoconstrictor leading to increased blood pressure (Aviram and Dornfeld, 2001), thus reduction of angiotensin II levels should decrease blood pressure. The mechanism of inhibition has been suggested by Persson et al., (2009) to involve polyphenol binding to Zn²⁺ ions in the active site of ACE. As reported by Kulkarni et al., (2004) punicalagin has the ability to chelate metal ions like Fe²⁺ and Zn²⁺.
Protection of endothelial NO from ROS destruction is important since the NO acts as a vasodilator. Therefore, the antioxidant activity of punicalagin (1-60 μM) was studied in the EA.hy926 cell line after a 24-hour incubation period. The superoxide production that was induced after incubation of cells with angiotensin II was significantly inhibited at all concentrations of punicalagin when investigated by flow cytometry and fluorescence methods. This result, i.e. inhibition of superoxide levels was expected due to the presence of several hydroxyl groups within the chemical structure of punicalagin (Dziedzic and Hudson, 1983). The structure-activity relationship of polyphenols as radical scavengers has been well demonstrated (Bao and Lou, 2006; Alia et al., 2006b).

The NO-release response in endothelial cells after exposure to punicalagin (1-60 μM) was also studied. The NO level significantly increased in a dose-dependent manner in response to punicalagin treatment. As seen in other research on polyphenols, the increase in NO can be mediated by increased activity and protein expression of the eNOS enzyme (Leikert et al., 2002; Chen et al., 2008) or by scavenging of ROS that would normally degrade it (Ignarro et al., 2006; Edirisinghe et al., 2011; Hidalgo et al., 2012).

Punicalagin, at experimental doses, showed a significant increase in eNOS enzyme activity but there was no increase in the protein expression level of the eNOS enzyme. Punicalagin may have increased the eNOS activity via eNOS phosphorylation, which enhances activity via the activation of the redox-sensitive phosphatidylinositol-3 (PI3)/ protein kinase B (AKT) pathways (Edirisinghe et al., 2011; Hidalgo et al., 2012).
eNOS enzyme activity is calcium-dependent (Leikert et al., 2002), therefore, the calcium concentration in EA.hy926 cells treated with punicalagin was measured. At the same examined concentrations (1-60 μM), punicalagin caused a significant increase in Ca^{2+} concentrations measured by flow cytometry. Polyphenols can affect intracellular Ca^{2+} levels by releasing ions from intracellular stores or by increasing the entrance of Ca^{2+} through the cell membrane (Martin et al., 2002). One or both of these mechanisms may help to explain the significant increase of Ca^{2+} concentration caused by punicalagin in the endothelial cell line (p < 0.001).

Under the harmful effects of oxidative stress, high levels of intracellular ROS are produced in living cells that cause chronic diseases like cancer. Therefore, ROS scavenging is necessary in order to prevent cancer and protect the human body from ROS damage. The dietary antioxidants such as polyphenols are reported to have anti-carcinogenic (Malik et al., 2005, Zhang et al., 2008) and cardiovascular diseases prevention properties (Ignarro et al., 2006; Edirisinghe et al., 2011; Hidalgo et al., 2012) due to their antioxidant effects. In this research, the anti-carcinogenic effect of pomegranate punicalagin was investigated to determine the potential mechanism and pathways involved as discussed below.

6.1.5 The Effect of Punicalagin on Human Colon Cancer Cell Line Caco-2

Several studies on polyphenols have indicated that they can inhibit the proliferation of cancer cell lines obtained from various tissues such as lung (Khan et al., 2007), cervix (McDougall et al., 2008), colon (Seeram et al., 2005) and prostate (Malik et al., 2005). 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. A similar dose of 50 and 100 μM was also tested on a normal colon cell line (HCEC) and 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, the high antioxidant potential of punicalagin due to the presence of phenol groups may explain the decrease in cytoplasmic 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. This result was confirmed by the activation of caspase 9, which supports our 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 hours showed apoptotic responses as measured by
flow cytometry using an Annexin V kit. Annexin V dye binds with phosphatidyl serine that has been externalized 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 poly (ADP-ribose) polymerase protein (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 are 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 hours. Flow cytometry assays showed that cells were arrested in the S-phase, and accumulated in the G1-phase. The percentage of cells in the G1-phase was 45%, 54%, and 60% for control, 50 and 75 μM punicalagin, respectively. The percentage of cells in the S-phase was 26%, 19% and 14% for the treatments (stated in the same order). It has been reported that arresting cells in 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 G1-, S-, S-G2 and G2-phases (Fresco et al., 2006; Renis et al., 2008; Hafeez et al., 2008).

6.2 General Conclusions

**Characterisation**

- Commercial punicalagin standard used in this study and analysed by HPLC and LC-MS, revealed an identical peak in samples extracted from a methanol extract of pomegranate husk (US), whilst no punicalagin peak was observed in a methanol extract of pomegranate juice (US and UK).

**Antioxidant Activity and Mechanisms**

- In vitro experimental solutions of punicalagin and pomegranate juice (0.05, 0.1 and 0.15 mg/ml) showed antioxidant activity by radical scavenging, hydrogen peroxide scavenging, metal chelating, and reducing power mechanisms. These antioxidant properties were similar to the standard synthetic antioxidant BHT.

**Antioxidant Activity in Cells**

- Punicalagin at 5 and 10 μM concentrations significantly scavenged ROS in Caco-2 cells produced by added tert butyl hydroperoxide (T-BOOH).

- The formation of malondialdehyde (MDA) as a secondary metabolite product of lipid peroxidation was significantly inhibited by 5 and 10 μM punicalagin in Caco-2 cells stressed with T-BOOH.
• Punicalagin at 5 and 10 μM minimized the depletion of glutathione levels in Caco-2 cells treated with T-BOOH.

• Morphological changes indicating decrease in viability of Caco-2 cells stressed with T-BOOH were protected in the presence of 5 and 10 μM punicalagin.

• Therefore 5 and 10 μM punicalagin have good antioxidant properties.

**Antihypertensive Effect**

• Punicalagin at 1, 20, 40 and 60 μM concentrations showed a significant inhibition of ACE activity in the EA.hy926 cell line after a 24 hours incubation period; ACE inhibition is important for reducing hypertension, a risk factor for cardiovascular disease.

• Punicalagin caused a significant decrease in ROS levels in EA.hy926 cells under oxidative stress induced by angiotensin II. ROS scavenging can prevent destruction of NO which is a vasodilator.

• The cytoplasmic calcium concentration was significantly increased in EA.hy926 cells treated with different concentrations of punicalagin, thereby increasing activity of calcium dependent eNOS.

• The significant increased activity of eNOS was confirmed in EA.hy926 cells treated with punicalagin. Therefore the level of NO production was significantly increased.

• The above findings indicate that punicalagin is a potential antihypertensive agent.
Anticancer Effect

- Cytotoxicity in the Caco-2 cell line was induced by punicalagin with an IC$_{50}$ value of 75 μM. In contrast, there was no cytotoxicity observed in the normal colon cell line (HCEC) at the same concentration. Morphological changes observed in cells incubated with punicalagin confirmed these results. Therefore, punicalagin (50-100 μM) killed Caco-2 cancer cells but not normal colon cells.

Mechanism of Cell Death in Cancer Cells

- As markers of apoptosis, caspase (9, 8 and 3) protein expression was significantly increased in the Caco-2 cell line following incubation with 50 and 75 μM punicalagin for 24 hours; consequently PARP cleavage was detected. Therefore, the apoptosis mechanism of cell death in the Caco-2 cancer cell line was confirmed.

- Increased mitochondrial superoxide anions were detected in the Caco-2 cell line following incubation with different concentrations of punicalagin.

- A decreased proportion of the Caco-2 cell populations were in the S-phase because more remained in the G1-phase after incubation with different punicalagin concentrations.

- The above findings indicate that punicalagin arrested the cell cycle and caused cell death by apoptosis in Caco-2 cancer cells
6.3 Future Work

- To study the effect of punicalagin as antihypertensive agent \textit{in vivo} in animal models.

- To study the effect of punicalagin as a colon cancer treatment agent \textit{in vivo} in animal studies.

- To study the effect of punicalagin as an anticancer agent for different types of cancer cell lines, such as breast, liver, pancreas and prostate cancer cell lines.
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