

Protective Effects of Punicalagin on Caco-2 Intestine Cell Line under Oxidative Stress Caused by Tert-butyl hydroperoxide

Ulfat Omar^{a,b,*}, Akram Aloqbi^{a,c}, Marwa Youسر^a and Nazlin Howell^a

^aUniversity of Surrey, Faculty of Health and Medical Sciences, Division of Nutrition and Metabolism, Guildford, Surrey, GU2 7XH, UK

^bKing Abdulaziz University, Faculty of Science, Biochemistry Department, Jeddah, PO Box 40288, zip code 21499, Saudi Arabia

^cPresent Address University of Jeddah, Faculty of Sciences and Arts - Alkamel, Biology Department, Jeddah, PO Box 80327, Jeddah 21589, Saudi Arabia

Abstract: Hydrolysable tannin polyphenols from pomegranate (punicalagin) have been reported to show a wide range of health properties correlated to their high antioxidant and free radical scavenging activities. The objective of the current study was to investigate the protective effect of punicalagin on cell viability and redox status of cultured Caco-2 cells exposed to oxidative stress induced by tert-butyl hydroperoxide. The production of malondialdehyde, and total glutathione levels, as well as the generation of reactive oxygen species were used as markers of cellular oxidative status. Pretreatment of Caco-2 cells with 5 and 10 μ M punicalagin for 24 hours significantly protected cell viability after exposure to tert-butyl hydroperoxide $IC_{50} = 3$ mM for 2 hours. The examined doses prevented the decrease of total glutathione and the increase of malondialdehyde induced by tert-butyl hydroperoxide in Caco-2 cells. Reactive oxygen species generation provoked by tert-butyl hydroperoxide was significantly reduced at the same concentrations. Finally, cell morphology with treatments before and after induction by tert-butyl hydroperoxide showed irreversible effect of the oxidizing agent. The results of the biomarkers analyzed showed that treatment of Caco-2 cells with the natural dietary antioxidant punicalagin protected the cells against oxidative stress.

Keywords: Polyphenols, Malondialdehyde, Glutathione, Reactive oxygen species, morphology.

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 it is the basis of several human diseases such as coronary heart disease and cancer [1]. 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 [2]. For this reason, biological systems are normally protected from oxidation by their antioxidant defence systems and by 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 [3]. 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 [3]. Increased level of intracellular ROS leads to depletion of glutathione [3]. 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-butyl hydroperoxide (t-BOOH) [3, 4, 5]. Thus, natural products containing polyphenols have been proposed as antioxidants.

Epidemiological studies have shown that the intake of polyphenols may reduce the risk of some diseases such as cancer and cardiovascular disease [6,7]. Pomegranate juice has a high content of phenolic compounds (such as punicalagin isomers, ellagic acid) and anthocyanins (such as delphinidin, cyanidin, and pelargonidin) [8]. 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 [8]. Punicalagin is a yellow

*Address correspondence to this author at the King Abdulaziz University, Faculty of Science, Biochemistry Department, Jeddah, PO Box 40288, zip code 21499, Saudi Arabia; Tel: +966503675685; E-mail: uomer@kau.edu.sa

water-soluble compound that is mainly present in the pomegranate husk and is extracted in the juice during fruit processing [8].

Previous studies on polyphenols and flavonoids demonstrated their ability to prevent lipid peroxidation in living cells by trapping free radicals [9, 10, 11]. 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 measuring GSH, ROS and MDA levels.

The objective of the present study was to investigate the potential protective effect of different punicalagin concentrations against oxidative stress induced by t-BOOH in Caco-2 cells. Cell viability and several markers of oxidative damage were used to examine the effect of punicalagin on cell viability and their effect on the antioxidant system of Caco-2 cells stressed by 3mM of t-BOOH. Levels of malondialdehyde (MDA) as a marker of lipid peroxidation; scavenging of reactive oxygen species (ROS); concentration of total glutathione (GSH) and cell morphology were parameters that were determined in the present study.

2. MATERIALS AND METHODS

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 to stress Caco-2 cells, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT dye) to measure cell viability, 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 (dye for ROS measurement) were purchased from Invitrogen, Paisley, UK. A total glutathione peroxidase kit was obtained from the Cell Biolabs, INC, Cambridge, UK.

2.2. Methods

2.2.1. Cell Culture

Caco-2 cells were grown in DMEM supplemented with 20% FBS, 1% glutamate, 1% NEAA and 1% penicillin. Cells were sub-cultured when reach 70%-

90% confluent in a humidified atmosphere in 5% CO₂ at 37 °C.

2.2.2. Determination the Cytotoxicity of Punicalagin by MTT Assay

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. Succinate dehydrogenase cleaves the tetrazolium ring in the MTT structure and converts it to formazan [12, 13]. Different concentrations of t-BOOH 1,3,5 and 10 mM were assessed to determine the IC₅₀. The cytotoxic effect of different concentrations of punicalagin ranging from 1-100 µM on Caco-2 cells was studied. Caco-2 cells were seeded into 96-well tissue culture plates at 1×10^4 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, and 100 µM. The non-toxic dose 1, 3, 5, and 10 µM followed by 2 hours incubation with 3 mM t-BOOH after determination the IC₅₀ [14]. The MTT assay was performed by adding 20 µl MTT dye (5mg/ml) to each treated well. 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).

2.2.3. Determination of Thiobarbituric Acid Reactive Substances (TBARS)

TBARS are considered as a marker of lipid peroxidation [2,15]. 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. Briefly, the treated cells were scraped with 3ml of 20% TCA. Followed by adding 2 ml of 0.7% TBA and heated at 100 °C for 1 hour. After cooling, the tubes were centrifuged at 1500 × 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). The MDA concentration was expressed in nmol of MDA/mg protein. Trolox at a concentration of 0.01%, was used as positive control.

2.2.4. 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 rapidly converted to fluorescein dye (DCF) in the presence of ROS. The fluorescence intensity of DCF is proportional to the ROS content [17]. Caco-2 cells were seeded in 25cm² flasks at a density of 1×10⁶ cells/ml. The treatments were applied for 24 hours after the cells reached 60-70% confluence. Following incubation, the cell incubated with t-BOOH 3 mM for 2 hours. At the end of the incubation time, the cells were trypsinised and the cell suspension centrifuged for 3 minutes at 150 × *g* using a Beckman GRP centrifuge. The supernatant was discarded and the cells were re-suspend in 1 ml PBS and 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 and the DCF fluorescence was measured using a BD FACSCanto flow cytometer (California, USA).

2.2.5. Preparation of Cell Lysates

The pre-treated Caco-2 cell with different punicalagin concentration and t-BOOH were trypsinised and the cell suspensions centrifuged at 1500 × *g* for 3 minutes. 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 used. Trolox (0.01%) was used as a positive control

2.2.6. Determination of Total Glutathione (GSH)

A total GSH assay kit from Cell Biolabs was used to measure the total concentration of GSH by following the manufacturer's instructions.

2.2.7. 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. A photograph of the cells was taken using a Nikon Eclipse microscope at 10× magnification.

2.3. 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. RESULTS

3.1. Cytotoxicity of Punicalagin on Caco-2 by MTT Assay

The cytotoxicity of punicalagin at different concentrations (1-100 μM) is displayed in Figure 1. 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.

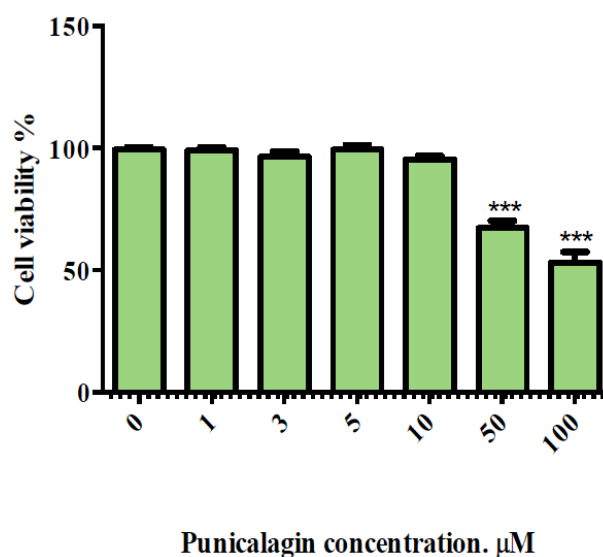


Figure 1: Cell viability of Caco-2 cells treated with different concentrations of punicalagin (0-100 μM) for 24 hours. These data represent the mean ± SD of at least 3 independent experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (***= *P* < 0.0001).

3.2. 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 2 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.

3.3. 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

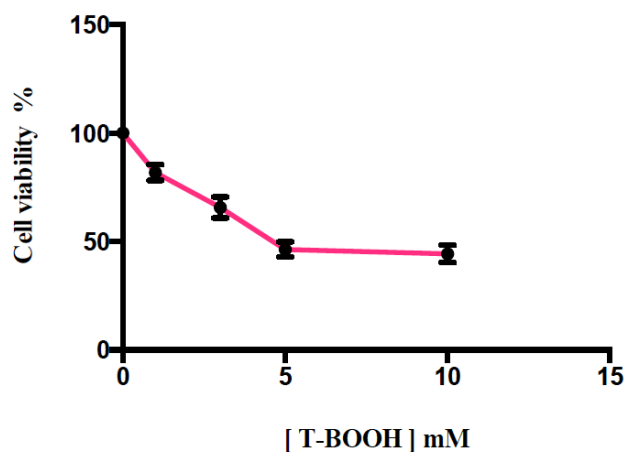


Figure 2: Dose-response curve for different concentrations of t-BOOH (1, 3, 5 and 10 mM) incubated for 2 hours with Caco-2 cells. Values represent the mean \pm SD, $n=3$, the IC_{50} value was 3 mM t-BOOH.

t-BOOH is illustrated in Figure 3. No significant difference was seen between the cells incubated with 3 mM t-BOOH (58% viability) and those pretreated with 1 μ M (55% viability) and 3 μ M (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 μ M 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 μ M; the cell viability with punicalagin was 64.4 and 71.5 % respectively, versus 58 % for 3 mM t-BOOH only.

3.4. Determination of MDA Concentration

The effect of different concentrations of punicalagin on the production of secondary lipid oxidation product MDA was studied in Caco-2 cells. 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 nmol/mg for control, 35.5 nmol/mg for trolox, 35 nmol/mg for 5 μ M punicalagin and 34 nmol/mg for 10 μ M punicalagin respectively as presented in Figure 4.

There was a significant decrease in the production of MDA in cells pretreated with trolox, or 5 and 10 μ M punicalagin following induction of oxidative stress using t-BOOH Figure 4. The most protective cell concentration against oxidative stress was 10 μ M punicalagin followed by trolox and then 5 μ M punicalagin compared with cells treated with t-BOOH alone (p value ≤ 0.0001). Concentrations of MDA were 27.7, 38.7, 41.4, and 64.6 10 nmol/mg after treatment with 10 μ M, trolox, 5 μ M, or 3 mM t-BOOH alone, respectively.

3.5. Measurement of Cellular ROS Levels

The ROS levels in Caco-2 cells exposed to t-BOOH in control and treated cells with trolox or punicalagin concentrations for 24 hours were measured using the CM-H₂DCFDA dye.

Punicalagin (5 and 10 μ M) 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 5. 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 previously. The pre-treated cell line with trolox and punicalagin showed a significant decrease in fluorescence intensity compared with t-BOOH treated group ($p < 0.0001$). This result indicates

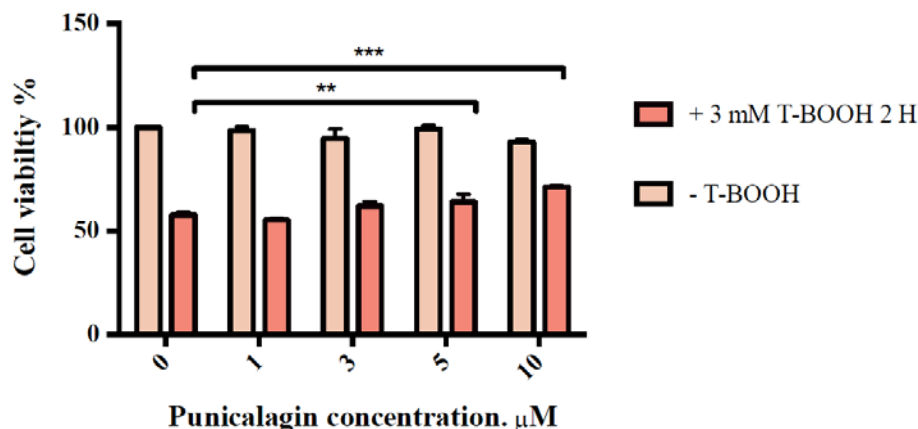


Figure 3: The viability of Caco-2 cells treated with different concentrations of punicalagin (1, 3, 5 and 10 μ M) with and without 3mM t-BOOH after 2 hours. These data represent the mean \pm SD of at least 3 independent experiments. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (** = $p < 0.001$ and *** = $p < 0.0001$).

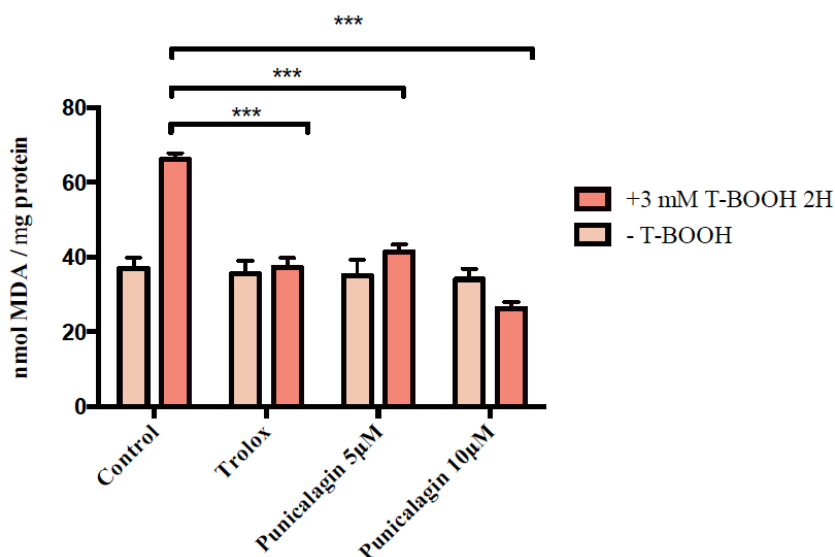


Figure 4: Concentration of MDA in Caco-2 cells treated with punicalagin and trolox individually with/without 3 mM t-BOOH for 2 hours. Values are the mean \pm SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (***) ($P < 0.0001$). Trolox 0.1 mg/ml was used as antioxidant positive control.

that punicalagin has the ability to scavenge ROS and thereby protect cellular macromolecules such as lipids and proteins from ROS-mediated damage.

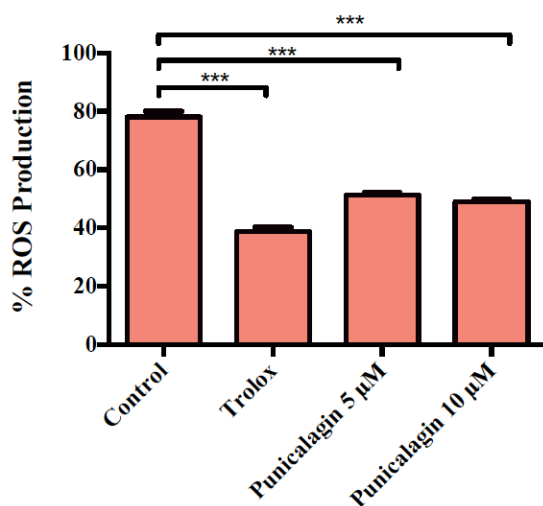


Figure 5: Cellular ROS levels in Caco-2 cells treated with punicalagin and trolox individually with 3 mM t-BOOH for 2 hours. Values are the mean \pm SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (***) ($P < 0.0001$).

3.6. Determination of Total Glutathione (GSH)

In Figure 6, the total GSH level was significantly increased in the pre-treated cells with trolox, 5 and 10 μ M 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 nmol/mg). The GSH level of incubated cells with t-BOOH was only 0.18 nmol/mg. In

the case of the trolox, 5 and 10 μ M punicalagin treated cells without t-BOOH Figure 6; the level of GSH was significantly higher with trolox treatment ($p < 0.0001$) whereas no significant difference was observed with punicalagin treatment compared with the control.

3.7. Morphological Changes to Caco-2 Cells Treated with Punicalagin

The protective effect of different concentrations of punicalagin (5 μ M and 10 μ M) on Caco-2 cells subjected to 3 mM t-BOOH oxidative stress was investigated by microscopy for 24 hours. Figure 7 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.

4. DISCUSSION

Pomegranate punicalagin, a hydrolysable tannin polyphenol compound protected Caco-2 cells against the IC_{50} (3 mM) of t-BOOH at 5 and 10 μ M. Different defence mechanisms in pretreated Caco-2 cells stressed with 3 mM of t-BOOH for 2 hours has been investigated. This protective effect of punicalagin was confirmed by microscopy, where adverse morphological cell changes were less apparent with antioxidant treatment. The cells looked healthy which demonstrates the protective effect of punicalagin against oxidative stress.

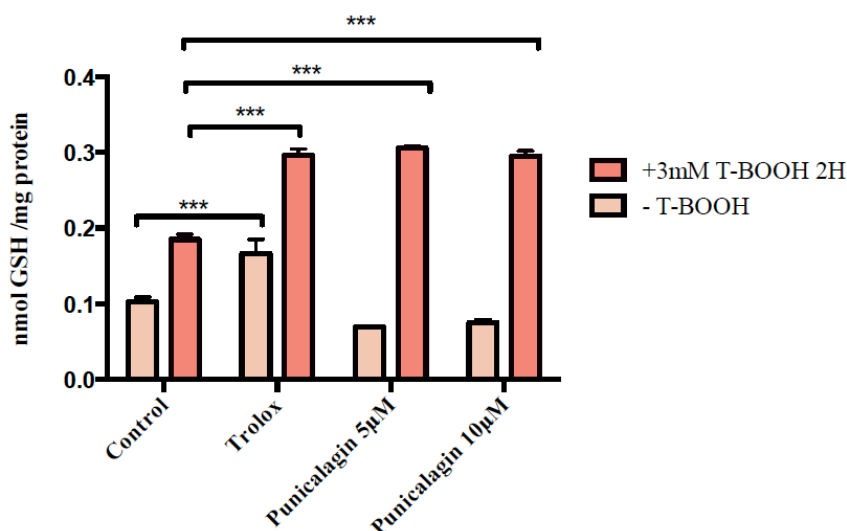


Figure 6: Glutathione (GSH) level in Caco-2 cells treated with punicalagin and trolox individually with/without 3 mM t-BOOH for 2 hours. Values are the mean \pm SD, n=3. Comparisons of means were made using a one-way ANOVA followed by Bonferroni's test (**P < 0.0001).

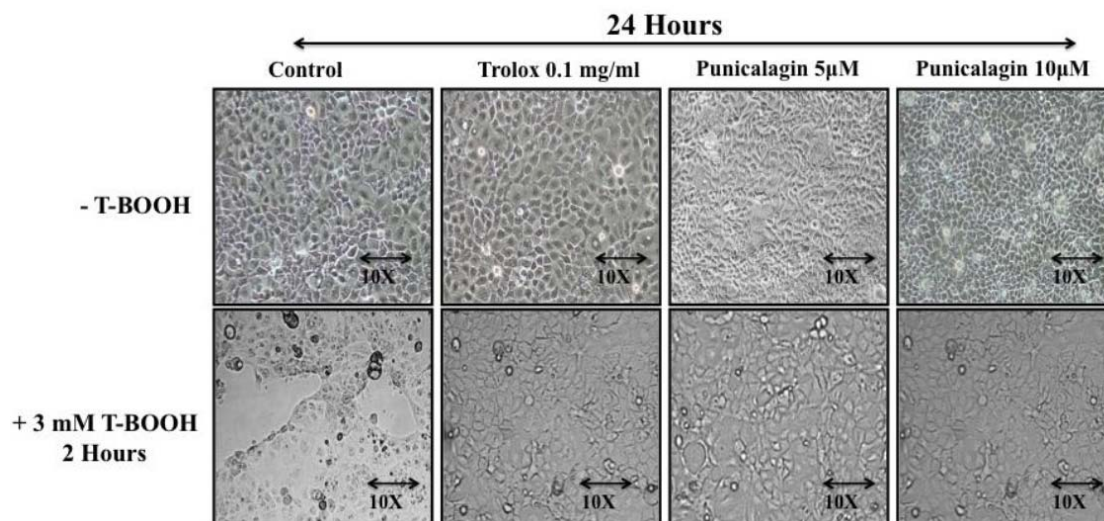


Figure 7: Effect of trolox and punicalagin on a Caco-2 cell line with/without 3 mM t-BOOH for 24 hours. Cells were treated with trolox and punicalagin for 24 hours. The pre-treated cells incubated with 3 mM t-BOOH for 2 hours. Images were captured using light microscopy (magnification 10x).

Malondialdehyde (MDA) is one of the main products of lipid peroxidation [18]. It is produced in high quantities in various diseases and it is related to free radical damage [19]. Quercetin (a flavonol from a class of flavonoids) of 5 and 10 μ M incubated for 2 and 20 hours in HepG2 cells significantly inhibited the production of MDA under oxidative stress conditions induced by 200 μ M t-BOOH after 3 hours incubation period [20]. Another study by Peng and Kuo in 2003, discovered that 0.1 μ M quercetin, 10 μ M luteolin and 1 μ M epigallocatechin gallate had the ability to inhibit lipid peroxidation in Caco-2 cells under 30 μ M H_2O_2 + 30 μ M $FeSO_4$ inductive conditions [21]. 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 μ g/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 [14]. The most abundant phenolic compounds present in the *Salvia officinalis* plant were rosmarinic acid and luteolin-7-glucoside [14]. 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 lipid peroxidation reaction of cell membrane could explain the observed reduction in MDA as lipid peroxidation marker [22].

Previous studies have shown that the powerful of antioxidant compounds occurs via ROS scavenging [23]. Therefore, The effect of punicalagin as a ROS scavenger was studied in the current research on Caco-2 cells. 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 μ M) was incubated for 1 hour with the Caco-2 cell line. The pre-treated cell line was stressed by 50 μ M 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%) [23]. ROS production was also significantly reduced in the HepG2 liver cell line when incubated with quercetin and rutin (1-100 μ M) for 24 hours when stressed with 200 μ M H₂O₂ or 3 hours compared with cells without pre-treatment [24].

In agreement with previous results, the ability of examined concentration 5 and 10 μ M phenolic compound (punicalagin) to decrease cellular ROS levels in stressed Caco-2 with 3 mM t-BOOH was observed. This finding may explain the importance of the presence of the phenolic hydroxyl group that scavenges radicals [23].

Glutathione (a non-enzymatic antioxidant) has an important protective role against ROS in the cell [18]. Increased intracellular oxidation leads to depletion of glutathione [17, 25]. 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 nmol/mg in pretreated cells compared with untreated cells 0.18 nmol/mg under oxidative stress conditions, 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 [14, 24]. Research by Lima *et al.*, (2006) indicates that polyphenols provide protection against oxidative damage induced by t-BOOH. Luteolin 200 μ M, 45 μ M quercetin, 370 μ M caffeic acid and 180 μ M rosmarinic acid all had the ability to inhibit the GSH depletion in the HepG2 liver cell line [5]. 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 [14]. 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 [25]. Therefore, the potential effect of punicalagin against t-BOOH as radical scavenging activity could maintain the GSH levels and contribute to their cytoprotective effects.

Trolox is partial hydrophobic in nature therefore its transfer through the cell is possibly hampered. Consequently, trolox might be kept partially within the cell membranes. Therefore, it will be near to the tight junction complexes where it can more easily defuse the direct oxidative effect of t-BOOH on these protein structures and prevent their effect [26].

In conclusion, punicalagin at low concentrations had 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 protected 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 excessive ROS production.

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Received on 11-11-2015

Accepted on 27-12-2015

Published on 08-01-2016

DOI: <http://dx.doi.org/10.6000/1927-5951.2015.05.04.5>

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