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Full Length Research Paper

Attenuation of *t*-Butylhydroperoxide induced oxidative stress in HEK 293 WT cells by tea catechins and anthocyanins

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The health promoting properties of catechins and anthocyanins have been of great interest to researchers in the recent past due to their significant *in vitro* and *in vivo* antioxidant activities. Most research on anthocyanins has been based on berry anthocyanins. These potentially health enhancing pigments are also found in some Kenyan tea cultivars. An *in vitro* study was carried out to determine the effects of pure catechins (EGCG and EC) and tea anthocyanin extract from cultivar TRFK 306/1 on *t*-Butylhydroperoxide (*t*-BHP) induced oxidatively stressed HEK 293 cells. The effects of the catechins and tea anthocyanin extract on untreated cells (without *t*-BHP) cells and cells treated (with *t*-BHP, 500 µM) were determined by measuring cytotoxicity (lactate dehydrogenase (LDH) leakage from the cells) and depletion of cellular glutathione (GSH). Cells were preincubated with the antioxidants for 30 min before addition of *t*-BHP (500 µM) and additional incubation for 6 h. The results showed that epigallocatechin gallate (EGCG) and epicatechin (EC) as well as tea anthocyanin extract significantly ($p<0.001$) attenuated *t*-BHP induced LDH leakage in a concentration dependent manner in treated cells. One way ANOVA analysis showed significant ($p<0.001$) differences in the various effective concentrations of the catechins and tea anthocyanin extract used. Intracellular GSH content was also increased in a dose dependent manner. From these results, it is concluded that anthocyanin rich tea from selected Kenyan cultivars may have cytoprotective effects against oxidative stressors.

Key words: Anthocyanins, catechins, oxidative stress, lactate dehydrogenase, glutathione, *t*-BHP, attenuate, antioxidant, reactive oxygen species (ROS).

INTRODUCTION

Kenya is one of the most important producers of black tea in Africa. In fact, Kenya supplies 22% of the world's black tea and this crop is a major foreign exchange earner for the country (TBK, 2010), contributing about 26% of all foreign exchange earnings and 4% of the gross domestic product (GDP). In 2010, tea earnings in Kenya was Kshs 98 billion compared to Kshs 69 billion earned in 2009 (TBK, 2010). Tea production in 2010 was 399 million Kg compared to 314 million Kg in 2009, accounting for much of the increase in revenue. Despite high production, local consumption of tea in Kenya is very low. For example, the estimated national per capita consumption of tea was only 0.46 Kg made tea/person/year in 2009 (TBK, 2010). To spur local use of Kenyan tea, it may be necessary to add nutraceutical value to the existing black tea products or to diversify into other products that can use tea and tea products as a raw material. Some specialty tea products are particularly sought after because they are considered to be healthful,

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often because they contain or are believed to contain a higher content of biologically active antioxidants. Alternatively, teas can be more appealing to consumers because they contain colour additives or flavours.

The pharmacological properties of the tea plant (*Camellia sinensis*) have historically been ascribed to its polyphenols particularly the catechins; (catechin (+)-C, epicatechin (EC), epigallocatechin (EGC), epigallocatechin gallate (EGCG), gallocatechin (GC) and epicatechin gallate (ECG) in green tea; and theaflavins (TFs) and thearubigins (TRs) in black tea. Tea catechins have been reported to exhibit the following pharmacological properties: radical scavenging of reactive oxygen species (ROS) and reactive nitrogen species (RNS) *in vitro* (Muzolf et al., 2008; Lee et al., 2008); metal chelation (Khokhar and Owusu, 2003; Tang et al., 2002); anticarcinogenic (Roy et al., 2005; Seely et al., 2005); antiinflammatory (Karori et al., 2008); antimicrobial (Almajano et al., 2008); antioxidant (Gramza et al., 2006; Luczaj and Skrzydlewska, 2005; Maurya and Rizvi, 2008; Karori et al., 2007); antidiabetic (Cabrera et al., 2006); pro-apoptotic in monocytes (Kawai et al., 2004) and protection of cells against oxidative stress-induced apoptosis (Yao et al., 2007). The most abundant and bioactive green tea catechin is EGCG (Cabrera et al., 2006). The antioxidant effectiveness of a tea therefore depends on the tea variety in concert with its content of EGCG (Katalinic et al., 2006). Though pharmacological properties of tea have traditionally been attributed to catechins in green tea and theaflavins in black tea (Leung et al., 2001), anthocyanins which occur in grapes, have also occur in some tea plants (Terahara et al., 2001; Kerio et al., 2011).

In the plant kingdom, anthocyanins occur ubiquitously conferring red, blue and purple colours to fruits, vegetables and some grains (Kong et al., 2003; Prior, 2003). Anthocyanins are most abundant in berries (*Vaccinium* sp.), grapes, apples, purple cabbage, eggplant, black carrots, purple fleshed sweet potato and grains like black rice and purple corn. In plants anthocyanins are localized in the cell vacuole (epidermis and peripheral mesophyll cells), in the leaves, stems, roots, flowers and fruits. In nature, over 600 different anthocyanins have been identified to date, though only six are predominant; cyanidin, peonidin, petunidin, pelargonidin, delphinidin and malvidin. A recent study we carried out on anthocyanin rich tea germplasm revealed that malvidin was the most predominant in Kenyan processed black and green tea (Kerio et al., 2011). The different anthocyanidins found in nature differ in their substitution patterns in the B-ring (hydroxylation and methylation), which also affects their biological activities.

Anthocyanins have been found to exhibit superior antioxidant activities to some other polyphenols although just like the catechins in the tea plant, they have been reported to have a wide range of biological effects which include antioxidant (Bae and Suh, 2007; Orak, 2007; Choi et al., 2007), anti-inflammatory (Dai et al., 2007; Arli and Cau, 2007) antimicrobial (Viskelis et al., 2009; Heinonen et al., 2007), antiartherosclerotic (Mazza, 2007) and anticarcinogenic (Wang and Stoner, 2008) activities. Anthocyanins have also been implicated in induction of apoptosis in cancer cells (Hafeez et al., 2008; Lee et al., 2009) as well as with the chemoprotection of cells against oxidative stress-induced apoptosis (Elisia and Kitts, 2008), improvement of vision (Lee et al., 2005) and neuroprotective effects (Tarrozia et al., 2007). Anthocyanins have also been linked with the modulation of oxidative stress in cells which has long been recognized as a mediator of cell death either by necrosis or apoptosis (Yao et al., 2008; Elisia and Kitts, 2008). Anthocyanins do this by preventing the accumulation of intracellular ROS that cause loss of mitochondrial membrane potential ($\Delta\Psi m$) (Haidara et al., 2002; Gogvadze et al., 2006). Apoptosis is an important physiological process of cell death that plays a critical role in tissue homeostasis (Krysko et al., 2008) but that also has been implicated in a number of pathological conditions for example Parkinson's Syndrome and Alzheimer's disease (Jellinger and Bancher, 1998; Erdem et al., 1998).

Though numerous potential pharmacological properties have been described for anthocyanins, little work has been carried out to establish the bioactivity of tea-derived anthocyanins. A cell culture assay based on human embryonic kidney (HEK 293) cells was used in this study to determine the ability of a tea anthocyanin extract and catechins to attenuate oxidative stress. Oxidative stress in cultured cells results in damage to three major categories of essential macromolecules, causing lipid peroxidation (Duthie et al., 2005) protein oxidation (Halliwell and Whiteman, 2004) and DNA damage due to formation of oxidized nucleotide derivatives (Lazze et al., 2003). To detoxify ROS, cells largely depend on innate antioxidants like glutathione (GSH), dietary antioxidants such as Vitamin C and E and enzymic antioxidants including superoxide dismutase, catalase, and the peroxiredoxins. The innate antioxidant glutathione (GSH), the oxidized form (glutathione disulphide) of which is a marker of oxidative stress, is a tripeptide (γ-glutamyl-cysteinyl-glycine) responsible, in significant part, for maintaining the redox balance of most cells, where it is the most abundant non-protein thiol (Lapenna et al., 1998). GSH can scavenge ROS directly or act as a co-factor for enzymatic defenses (Franco and Cidlowski, 2009). Glutathione is found almost exclusively in a reduced state in cells and the enzyme glutathione reductase, which is constitutively active and inducible upon oxidative stress, converts glutathione disulphide (GSSG) to GSH (Shih et al., 2005). The ratio of GSH: GSSG within cells is often used as a measure of cellular oxidative stress (Atakisi et al., 2010). If oxidative stress results in irreparable cell damage, then apoptosis (programmed cell death) are activated but if cells are

exposed to extremely high levels of ROS, they also become necrotic (Krysko et al., 2008).

In the present study, HEK 293 cells were treated with *t*-BHP (500 µM) for 6 h to induce oxidative stress, to evaluate the ability of pretreatment with pure tea catechins and tea anthocyanin extract to attenuate the *t*-BHP-mediated cytotoxicity and GSH depletion.

MATERIALS AND METHODS

Anthocyanin extraction and purification

Five grams of ground steamed tea leaf from tea clone TRFK 306/1 samples were weighed into 250 ml conical flasks covered with foil and mixed with 50 ml methanol-1.5 M hydrochloric acid MeOH/HCl; (99/1v/v) and magnetically stirred at 900 rpm for 4 h at room temperature. The resultant solution was filtered and evaporated to dryness using a Rotavapour (Buchi Rotavapour R-300, Switzerland) under reduced pressure at 35°C. The extract was dissolved in 10 mL distilled water and passed through a 0.45 µm membrane filter and kept in an ice bath until the next phase of isolation.

The extracts were passed through reverse phase (RP) C_{18} solid phase extraction (SUPELCO, SPE) cartridges (Sigma-Aldrich, USA) previously activated with 10% MeOH/HCl and washed with 0.01% HCl in distilled water. Anthocyanins were adsorbed onto the column while sugars, acids and other water-soluble compounds were eluted from the column with 0.01% HCl in distilled water. Anthocyanins were then recovered using methanol acidified with 10% v/v formic acid. The cartridges were subsequently eluted with ethyl acetate (Fischer Scientific, UK) to remove phenolic compounds other than anthocyanins. The purified extracts were stored at -20°C until further analysis. The extracted anthocyanins crystallized upon solvent removal.

Catechins

The catechins (-)-epicatechin and (-)-epigallocatechin gallate were purchased from Sigma Aldrich, USA.

Incubation of HEK 293 cells

HEK 293 cells (Eton Bioscience, USA) were cultured in 75 cm^3 flasks in minimum essential medium MEM (Invitrogen, Canada) and 10% FBS containing 0.5% gentamycin until confluent. Then 4×10^5 cells/mL were seeded in 60 mm Petri dishes for treatment prior to assay for GSH/GSSG or Western Blot or 4×10^5 cells/ml were seeded in 96 well plates for treatment prior to assay for lactate dehydrogenase release. The cells were incubated overnight at 37°C in a humidified atmosphere containing 95% air and 5% CO_2 prior to treatment.

LDH leakage

Overall cell damage was determined by LDH leakage into the extracellular medium according to the method of Korzeniewski and Callewaert, (1983). Cells were first pre-treated with varying concentrations of either the catechins (10-50µM) or the tea anthocyanin extract (10-100 µM) or vehicle (blank) for 30 min prior to incubation with 500 µM *t*-BHP (Sigma-Aldrich, Canada) for 6 h. Released LDH was analyzed at 490 nm using a Titertek Multiscan Microplate Reader (Phoenix AZ, USA). Cell damage was expressed as the fraction of LDH released into the culture medium relative to the total cellular LDH activity determined after lysis of cells with 0.1% v/v Triton X-100 (Sigma-Aldrich, USA).

Analysis of intracellular GSH

The intracellular concentrations of GSH were determined according to the method of Habig et al. (1974). Cells were plated overnight in 2 ml of minimum essential medium (MEM) (Invitrogen, Canada) and treated with the antioxidants (catechins or anthocyanin) for 30 min after adding fresh media. After incubation with 500 µM *t*-BHP for 6 h, the cells were harvested from the wells by gentle scraping, placed in ice, and centrifuged at 500 x g for 5 min. Cell pellets were re-suspended in 250 µl lysis buffer (10 mM Tris, 130 mM NaCl, 10 mM NaF, 10 mM NaH_2PO_4, 10 mM $Na_4P_2O_7$, and 2 mM EDTA) and quickly frozen in liquid nitrogen. The remaining aliquots were centrifuged for 2 min at 14,000 x g, and the supernatants were used for GSH content measurement based on a GST enzymatic method of Habig et al., (1974). Sample supernatant (50 µl) was added to wells of a 96-well plate and made up to 190 µl with 0.1 M potassium phosphate buffer (pH 7.6). To each sample reaction, 5 µl of 40 mM DNCB, and 0.2 unit of glutathione S-transferase were added, followed by incubation in the dark at room temperature for 20 min. The formation of S-2, 4-dinitrophenyl glutathione was measured by monitoring the absorbance at wavelength 340 nm with a Titertek Multiskan Microplate Reader (Phoenix AZ, USA). GSH content was expressed as nmol GSH/1000 cells.

Statistical analysis

The LDH assays were carried out in triplicate with three different batches of cultured cells and the data were subjected to one way analysis of variance. Student's *t*-test was used to determine significance between treatments. Differences between treatments were considered to be statistically significant if $p<0.05$. The GSH assays were carried out twice with different batches of cells and the means of the two experiments are reported here.

RESULTS

Effect of tea catechins and anthocyanins on LDH leakage from HEK 293 cells

Tea catechins and anthocyanins were compared for their ability to attenuate *t*-BHP induced oxidative stress in HEK 293 cells. Epicatechin (EC) and epigallocatechin gallate (EGCG) were used as representative tea catechins in the assays while the tea anthocyanin extract was obtained from steamed leaves of the tea clone TRFK 306/1, as described above. The molarity of the tea anthocyanin extract solution was obtained from the Relative Molecular Mass (RMM) of anthocyanins (449.2 g) as Cyanidin-3-glucoside equivalents. HEK 293 cells were subjected to oxidative stress by treatment with 500µM *t*-BHP, a cell-permeant organic oxidant that generates ROS and increased the amount of LDH released from these cells by disrupting cell membrane integrity. The effects of catechins and tea anthocyanin extract in attenuating the *t*-BHP induced oxidative stress was evaluated by determining by comparing the release of LDH to the

medium cells treated with the tea constituents and *t*-BHP vs cells treated only with *t*-BHP.

Lactate dehydrogenase leakage is recognized as a sensitive measure of plasma membrane damage and cytotoxicity caused by reactive oxygen species (ROS) which can eventually lead to cell death. Pre-treatment of the cells for 30 min with the catechins (-)-EC alone or before addition of 500 µM *t*-BHP and incubation for 6 h resulted in lowering of LDH leakage to 4.71% ($p<0.001$), 1.57% ($p<0.001$) and 0.16% ($p<0.001$) for 10, 25 or 50 µM (-)-EC, respectively without t-BHP compared to 500 µM t-BHP while the cells that were oxidatively stressed after pre-treatment showed a reduction of LDH leakage to 14.6% ($p>0.05$), 9.1% ($p>0.05$) and 5.2% ($p<0.05$) compared to 500 µM *t*-BHP (Figure 1). Treatment of cells with 500 µM *t*-BHP for 6 h caused a rapid release of LDH into the cytosol which was attenuated in a dose dependent manner by pre-treatment of cells with increasing Epicatechin levels. One way ANOVA results showed significance ($p<0.001$) between the various concentrations of epicatechin used showing that EC did protect the cells under oxidative stress. To examine the attenuation of LDH leakage by EGCG, a similar treatment was carried out. It was observed that LDH leakage in the controls (without 500 µM *t*-BHP) was lowered in a dose dependent manner from 2.58% to 1.67% ($p<0.01$), 1.11% ($p<0.01$) and 0.11% ($p<0.01$) while for the cells that were treated (with 500 µM t-BHP) LDH leakage was lowered from 15.1% to 12.2% ($p>0.05$), 10.9% ($p>0.05$) and 2.8% ($p<0.01$), respectively compared to t-BHP treatment (Figure 2). One way ANOVA results showed significant cytoprotective effects ($p<0.001$) against oxidative stress between the various concentrations of EGCG tested.

In an assay to evaluate the effect of tea anthocyanin extract on LDH leakage in HEK 293 cells, the first group of cells was treated with anthocyanins only and the second group was pre-treated with increasing anthocyanin concentrations for 30 min before induction of oxidative stress with *t*-BHP (500µM) (Figure 3). LDH leakage in the controls (without 500 µM *t*-BHP) was lowered to 9.35% ($p<0.001$), 3.7% ($p<0.001$), 2.0% ($p<0.000$) and 0.23% ($p<0.0001$) for 10, 25, 50 and 100 µM, respectively. In the oxidatively stressed cells (with 500 µM t-BHP), LDH leakage was dose dependently lowered from 20.63% to 16.79% ($p>0.05$), 13.92% ($p<0.01$), 7.23% ($p<0.001$) and 2.41% ($p<0.0001$) for the same tea anthocyanin extract concentrations, respectively. ANOVA results showed significant differences ($p<0.001$) between the various concentrations of tea anthocyanin extract used. These results demonstrated that the anthocyanins were effective cytoprotectants against oxidative stress.

Effect of catechins and tea anthocyanins on intracellular GSH content

The effect of catechins and tea anthocyanin extract on reduced glutathione (GSH) content of HEK 293 cells was also determined in this study. GSH is an important high capacity cellular antioxidant, widely distributed among living cells. It is also involved in many biological functions such as amelioration of *t*-BHP-induced rat hepatotoxicity (Hwang et al., 2005). though not statistically significant, EGCG marginally increased cellular GSH content in *t*-BHP treated cells from 0.043 GSH nmol/mg protein for t-BHP only to 0.058, 0.067 and 0.074 GSH nmol/mg protein, respectively for 10, 25 and 50 µM EGCG (Figure 4). Tea anthocyanin extract at concentrations of 10, 50 and 100 µM similarly, marginally increased GSH concentration in the *t*-BHP treated cells. (Figure 5) but not significantly. The data presented for EGCG and tea anthocyanin extract were obtained from the mean of two independent experiments that were repeated with similar results.

DISCUSSION

Interest in the protective function of naturally occurring antioxidants in biological systems has risen in the recent past. For a long time, tea has been used as a healthy beverage owing to its pharmacological properties which are largely ascribed to the catechins contained. Catechins are known to quench free radicals, a characteristic associated with the hydroxyl groups in the B-ring. Evidence has shown that increased hydroxylation increases the antioxidant power of the catechins with EGCG in green tea being the most potent of all (Saffari and Sadrzadeh, 2004). Epigallocatechin-3-gallate (EGCG) is the most abundant compound found in green tea and has been associated with various health benefits as nutritional supplements for various ailments. The potential health benefits ascribed to EGCG include cancer chemoprevention (Hsuuw and Chan, 2007), antioxidant (Fu and Koo, 2006), improving cardiovascular health (Hirai et al., 2007) and neuroprotective (Mandel et al., 2004) among others. However, increasing evidence has also shown that EGCG is a pro-oxidant in higher concentrations and enhances production of ROS which subsequently lead to cell death (Bandele and Osheroff, 2008; Lambert et al., 2010; Rohde et al., 2011).

Recently, anthocyanin containing tea cultivars have been developed and characterized (Kerio et al., 2011). The anthocyanins/anthocyanidins identified in the tea cultivars were cyanidin-3-O-galactoside, cyanidin-3-O-glucoside, cyanidin, delphinidin, peonidin, pelargonidinand malvidin (Kerio et al., 2011) but the most predominant in the TRFK 306 cultivars is malvidin. Anthocyanins are pigmented plant polyphenols, whose colour in a plant depends on the pH of the cell sap. One purpose of this preliminary study was to compare the antioxidant potency of a mixed extract of tea anthocyanins with the two pure tea catechins, EC and EGCG. All three treatments attenuated the oxidative stress induced by 500 µM *t*-BHP in HEK 293 cells by

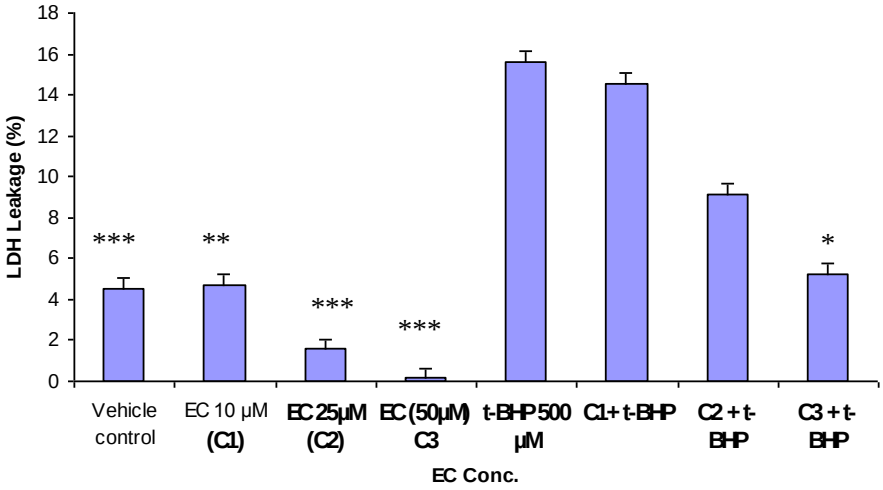

Figure 1. The effect of pre-incubating HEK 293 cells with 0-50 µM epicatechin for 30 min on LDH leakage following incubation with or without t-BHP (500µM) for 6 h. DMSO is the vehicle control (0 µM epicatechin); C1, C2 and C3 contain 10, 25 or 50 µM epicatechin (EC), respectively. Each column represents the mean and SD of three independent experiments. *p<0.05, **p<0.01, ***p<0.001 compared with t-BHP treatment alone (student's t-test).

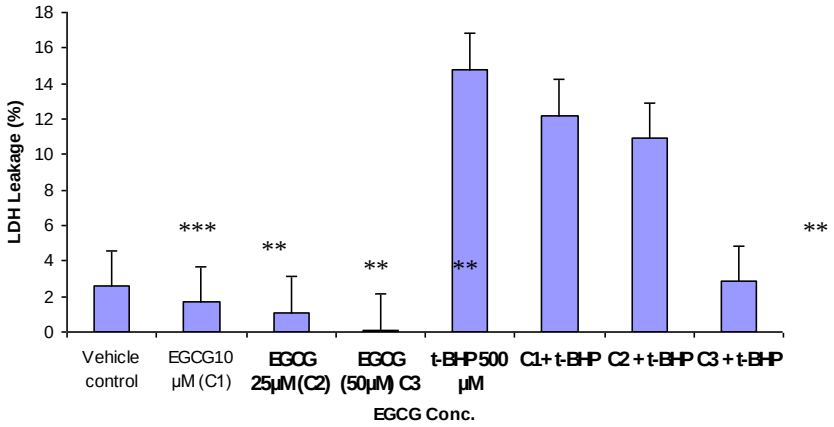

Figure 2. The effect of pre-incubating HEK 293 cells with 0-50 µM Epigallocatechin gallate (EGCG) for 30 min on LDH leakage following incubation with or without *t*-BHP (500 µM) for 6 h. DMSO is the vehicle control (0 µM epicatechin); C1, C2 and C3 contain 10 µM, 25 µM or 50 µM EGCG, respectively. Each column represents the mean and SD of three independent experiments. **p<0.01 and ***p<0.001 compared with *t*-BHP treatment alone.

reducing LDH leakage, an assay for cell damage. The integrity of cellular membranes is critical for normal cell function. Peroxidative decomposition of the membranes leads to impairment of normal cellular functions. Others have shown that *t*-BHP enhances lipid membrane peroxidation that contributes to cytotoxicity of HEK 293 cells (Kim et al., 2011; Hwang et al., 2005; Wang et al., 2000).

The results of this study revealed that catechins (EC and EGCG) as well as tea anthocyanin extract were able to partially protect the cells from the oxidative damage caused by *t*-BHP (500 µM). The effectiveness of the anthocyanidin extract might be attributed to its major constituent, malvidin. Malvidin is a dimethylated compound that is non-polar in nature. It is postulated that this anthocyanidin will have increased partitioning into the lipid phase of the membrane thereby increasing the antioxidant capacity of this tea anthocyanin. In addition, anthocyanin antioxidants are unique in their mode of action which may be attributed to their ability for electron

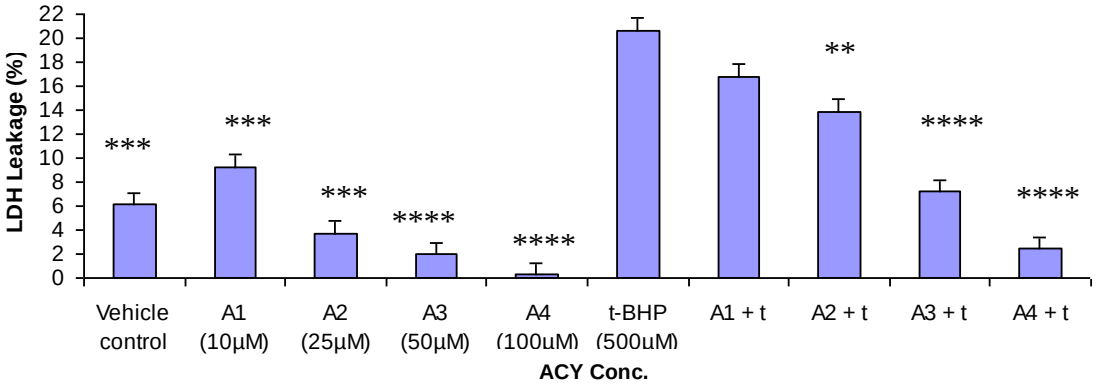

Figure 3. The effect of pre-incubating HEK 293 cells with 0-100 µM tea anthocyanin extract for 30 min on LDH leakage following incubation with or without t-BHP (500 µM) for 6 h. DMSO is the vehicle control (0 µM tea anthocyanin extract); A1, A2 and A3 contain 10, 25, 50 or 100 µM tea anthocyanin extract, respectively. Each column represents the mean and SD of three independent experiments. **p<0.01, ***p<0.001 and ****p<0.0001, compared with t-BHP treatment alone.

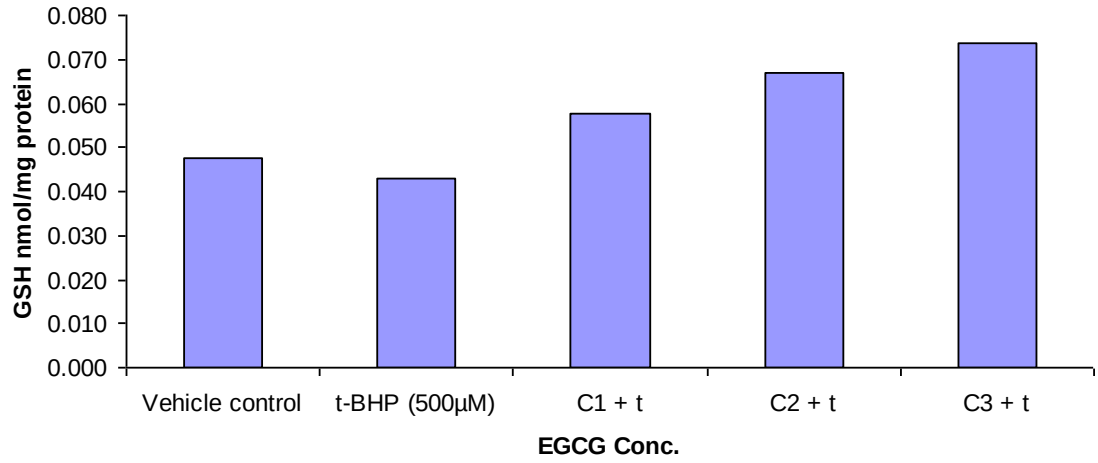

Figure 4. Effect of EGCG on the concentration of GSH in the treated cells and on the depletion of GSH induced by *t*-BHP (500 µM, 6 h) in HEK 293 cells. C1, C2 and C3 contain 10, 25 or 50 µM EGCG, respectively. Each column represents the mean of two independent experiments.

delocalization and to form resonating structures following changes in pH (Bagchi et al., 2006). Anthocyanins have been shown to be novel antioxidants and potent inhibitors of lipid peroxidation in comparison with other classic antioxidants. For example, grape seed proanthocyanidin extract was found to have superior antioxidant efficacy compared to vitamins C, E and β-carotene (Bagchi et al., 2003).

GSH has been established as a very important endogenous molecule for protection against the cytotoxicity of reactive electrophilic metabolites by converting them to covalent S-glutathione conjugates (Habig et al., 1974). GSH is also a very important antioxidant, essential for the maintenance and balance of thiol (-SH) groups on intracellular proteins. Antioxidants (vitamin C, catechins and other polyphenols) have been shown to increase the GSH pool and decrease GSSG content as well as attenuate hydroperoxide induced oxidative stress by stimulating the activity of two enzymes involved in GSH/GSSG balance that is glutathione peroxidase (GPx) and glutathione reductase (GRx) (Wijeratne et al., 2005). Increase in GSH content and decrease in cellular hydroperoxides (oxidants) are related phenomena. Dietary antioxidants for example catechins in tea and lately anthocyanins in the same plant, assist the cells to quench the oxidant evaluated in this study caused by *t*-BHP, complementing cellular antioxidants such as GSH and preventing their depletion. In cells, antioxidants often cause cyclic stimulation of glutathione peroxidase (GPx) and glutathione reductase (GRx), the

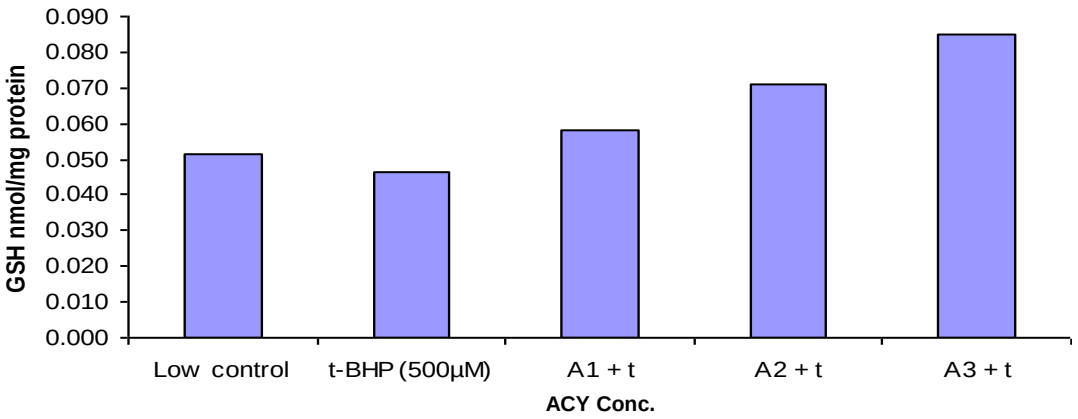

Figure 5. Effect of tea anthocyanin extract on the concentration of GSH in the treated cells and on the depletion of GSH induced by *t*-BHP (500 µM, 6 h) in HEK 293 cells. A1, A2 and A3 contain 10 µM, 50 µM or 100 µM tea anthocyanin extract. Each column represents the mean of two independent experiments.

latter which regenerates GSH from GSSG to be used in other antioxidant processes in the mitochondria or they improve mitochondrial function by detoxifying hydroperoxides (Shih et al., 2007). For Example, EGCG has been shown to modulate GSH metabolism in rat astrocytes (Ahmed et al., 2002). A key finding in this study is that anthocyanins and EGCG not only maintain a good redox status in basal conditions for GSH, but also do so in the *t*-BHP treated cells that have undergone oxidative insult. Our *t*-BHP treatment resulted in depletion of GSH possibly as a result of increased the levels of cellular hydroperoxides and high amounts of ROS responsible for oxidative stress (Alia et al., 2005). The 500 µM *t*-BHP used in this study caused oxidation of cellular GSH and subsequently resulted in an increase in the levels of GSSG, an inhibitor of GPx that can saturate GRx. (Yang et al., 2006). *t*-BHP-induced ROS formation affects GRx activity indirectly by oxidation of the pyridine nucleotide NADPH, a limiting cofactor for GRx. On the positive side, anthocyanins and catechins behave as electron donors to the mitochondrial electron transport chain or as mitochondrial respiring substrates that support the reduction of GSSG formed during oxidative stress (Weissel et al., 2006). The ability of dietary antioxidants including catechins and anthocyanins to function as direct free radical scavengers is related to their electron donating ability which is a direct function of the number of hydroxyl groups in the B-ring and the structural orientation of the compound (Castaneda-Ovando et al., 2009). Studies on the chemistry of EGCG as an antioxidant have shown that the trihydroxyphenyl B-Ring is the more active site of the antioxidant reaction than is the galloyl moiety (Nagle et al., 2006). In anthocyanins, the ring orientation has been found to determine the ease by which a hydrogen atom from a hydroxyl group can be donated to a free radical and its ability to support a free electron by delocalization of its own electrons (Castaneda-Ovando et al., 2009).

A study on *t*-BHP induced hepatotoxicity in rats demonstrated the protective effects of anthocyanins extracted from dried hibiscus flowers, primarily delphinidin-3-glucoside and cyanidin-3-glucoside (Wang et al., 2000) which decreased cytotoxicity (LDH release) and lipid peroxidation at concentrations of 0.1 and 0.2 mg/mL. Similar attenuation of oxidative stress-mediated toxicity was found *in vivo* after the oral administration of 100 or 200 mg/kg anthocyanins to rats for 5 days before a single dose of 0.2 mmol/kg of *t*-BHP. Although the catechin EGCG, also has cytoprotective properties it can also causes oxidative stress, as demonstrated by the effect of ECGC on GSH metabolism in cultured rat astrocytes (Ahmed et al., 2002). Similarly, several other studies have shown that EGCG, at high concentrations has pro-oxidative activities. In pancreatic beta cells, treatment with EGCG reduced cell viability and increased apoptotic cell death, H_2O_2 and ROS production, particularly at 50 and 100 µM although enhanced cell death was found at concentrations as low as 5 µM after 48 h (Suh et al., 2010). Recently, there was a case reported in Denmark of liver toxicity in an individual who drank 4-6 cups of green tea per day for six months (Rohde et al., 2011) which was ascribed to EGCG, the major catechin in green tea. Cell culture studies have revealed that EGCG could inhibit intercellular communication via gap junctions in normal rat liver epithelial cells (Kang et al., 2008). Indeed, ECGC has been shown to poison both isomers of topoisomerase II by a redox-dependent mechanism similar to that for 1, 4-benzoquinone (Bandele and Osherof, 2008).

The lowered levels of LDH leakage and apparent increase in cellular GSH content (due to attenuated depletion) in cells treated with the tea anthocyanin extract in this study reveal potential benefits of these compounds in the normal body cells. The tea anthocyanin extract was able to partially attenuate *t*-BHP-induced oxidative stress in the HEK 293 cells, effectively demonstrating the

cytoprotective properties of these antioxidants in cultured cells.

Conclusion

Oxidative stress results from an excess of intercellular ROS and RNS free radicals which are associated with several degenerative diseases, including cancer and chronic inflammation (Halliwell, 2002; Wang and Stoner, 2008; Tang et al., 2007). Therefore, it seems reasonable that consumption of reasonable amounts of anthocyanin rich teas, containing both anthocyanins and catechins, is likely to attenuate the adverse effects of oxidative stress in the body by decreasing oxidation of essential cellular proteins, lipids and nucleic acids while sparing GSH depletion. In this study, it is shown that the tea anthocyanin extract at the highest concentrations tested (100 µM), did not cause any obvious oxidative stress on the cells. This shows the potential of the tea anthocyanins for cytoprotection against oxidative stressors. It should however be stressed that although regardless of the class of chemical antioxidants selected for this purpose, ingestion of high concentrations over time is likely to be associated with negative effects, as has been reported in experimental animals and humans for EGCG, particularly when taken on an empty stomach which results in enhanced absorption. Additional work is however required to prove that anthocyanin rich tea cultivars have less potential for negative effects because of the presence of both anthocyanin and catechins antioxidants.

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