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In Vitro Effects of Quercetin on Oxidative Stress Mediated in Human Erythrocytes by Benzoic Acid and Citric Acid

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Benzoic acid (BA) and citric acid (CA) are food additives commonly used in many food products. Food additives play an important role in food supply but they can cause various harmful effects. The *in vitro* adverse effects of BA and CA and the protective effect of quercetin on human erythrocytes were investigated by measuring malondialdehyde (MDA) levels and superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione-S-transferase (GST) activities. Erythrocytes were incubated with BA and CA, at three doses of 50, 100 and 200 μg/ml, and quercetin, at a concentration of 10μM. After BA and CA application, a dose-dependent increase in MDA level and decreases in SOD, CAT, GST and GPx activities were found in erythrocytes. Among the two food additives, BA exerted a more harmful influence on human erythrocytes than CA. The protective effects of quercetin against oxidative stress – induction in the human erythrocytes by CA and BA, were found when these two food additives were applied at each of three doses of 50, 100 and 200 μg/ml. However, complete protection of quercetin against CA toxicity was only observed when this agent was applied at a lower dose of 50 μg/ml. Quercetin did not completely protect erythrocytes even at the lowest concentration of BA.

Key words: Benzoic acid, citric acid, quercetin, oxidative stress, erythrocytes.

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Today, food additives have an important role in food supply (MPOUNTOUKAS *et al.* 2008). They have several applications involving coloring, preservation and sweetening (SASAKI *et al.* 2002). Food additives are also used to protect moldering foods over the preparation of factory-made nutrients or to raise nutrient value. Thus, people are unconsciously exposed to food additives in their foods. Because of their toxicity some of these complex mixtures have been prohibited from use (IARC 1983). Food additives can cause various adverse effects like urticaria, hyperactivity, dermatitis, migraine and anaphylaxis (ZENGIN *et al.* 2011).

For the preservation of nutrient substances from the effects of yeast and bacteria, benzoic acid (BA) is frequently used as an antimicrobial substance in many nutrient products such as ketchup, fruit juice, biscuits, margarine, cream and cake (SARIKAYA & SOLAK 2003; YILMAZ *et al.* 2009). However, in experimental animals and in people some unfavourable effects, such as metabolic acidosis, hyperpnoea, and allergic reactions, have been reported (QI *et al.* 2009; OHTSUKI *et al.* 2012).

Citric acid (CA) and sodium citrate, CA's salt form, are the most widely used organic acids in the food industry (SAMMEL & CLAUS 2006). CA is used substantially as a regulator of pH, flavour enhancer and preservative in numerous foods such as beverages, jelly, baked nutrients, jam and candy (GURSOY 2002). CA has widespread usage, but some authors have reported that it causes necrotic changes such as vacuolated and glassy cytoplasm, chromatin wane and increment of collagen fibers among hepatocytes in mouse liver (YILMAZ *et al.* 2008).

Flavonoids are polyphenols present in vegetables and fruits (HEIM *et al.* 2002). Flavonoids include six subclasses: flavonols, flavones, flavanones, catechins, isoflavones and anthocyanidins (BORSKA *et al.* 2012). Because of their antioxidant activities such as free radical scavenging and inhibition of lipid peroxidation (LPO),

they have been assumed to exert advantageous effects on health (HEIM *et al.* 2002; LU *et al.* 2011). There are many studies which have indicated that some polyphenols modulate enzyme activities (BENELLI *et al.* 2002). Flavonoids may interact with enzymes and these interactions display various properties such as synergistic actions or antagonism effects (VIOLI *et al.* 2002; SILBERBERG *et al.* 2005). Quercetin is one of the best described flavonols and is present in large amounts in berries, onions, apples and broccoli (BORSKA *et al.* 2012). In cancer cells, quercetin induces apoptosis and as an antioxidant agent, it protects cells from mutagenesis and oxidative stress (ISHISAKA *et al.* 2011; BORSKA *et al.* 2012). It is a perfect free radical scavenging antioxidant (BOOTS *et al.* 2008) and reduces oxidative stress dependent chronic diseases (SKIBOLA & SMITH 2000; BHUTADA *et al.* 2010).

Free radicals contain one or more unpaired electrons. The most frequent forms of reactive oxygen species (ROS) are oxygen radicals, such as hydroxyl radicals and superoxide radicals, and nonfree radicals such as hydrogen peroxide (H_2O_2). These species originate in many redox processes in the human body. In cells the generation of ROS is arranged by biological antioxidants and antioxidant enzymes. Oxidative damage of carbohydrates, proteins, lipids, and DNA are induced by ROS (SUREDA *et al.* 2011). Many antioxidant compounds hinder oxidative stress dependent disorders by the scavenging of ROS (SLEMMER *et al.* 2008; SUEMATSU *et al.* 2011).

The human body has various enhanced defence mechanisms against oxidative damage, involving enzymatic and nonenzymatic systems. Nonenzymatic systems include exogenous compounds uptaken by the organism. Endogenous compounds found in the body as enzymatic defence systems include antioxidant enzymes (ERASLAN *et al.* 2007). A variety of parameters of erythrocytes are negatively affected by increased oxidative stress. Instability between antioxidant defence systems and ROS production is known to occur in different pathological conditions (DEMIR *et al.* 2011).

SOD, CAT, GPx and GST are important antioxidant enzymes. SOD and CAT are two main enzymes of the antioxidant system that cancel out free radical formation during xenobiotic exposure (TEZCAN *et al.* 2012; UZUN & KALENDER 2013). SOD transforms superoxide radicals to H_2O_2 and O_2 in a reaction that is self-generated and extremely quick, thereby preserving cells from harmful effects induced by superoxide radical reaction products (GAO *et al.* 2012). The antioxidant enzyme CAT converts H_2O_2 into water (MANSOUR & MOSSA 2010). For this reason, the system consisting of SOD and CAT builds up the first defense system against oxidative stress, because these enzymes work together to annihilate oxygen species (KALENDER *et al.* 2013). GPx is a selenoenzyme that is present in the cell cytosol. Its main role is to use glutathione (GSH) as a substrate and decrease the H_2O_2 level by transforming H_2O_2 into water (DEMIR *et al.* 2011; KALENDER *et al.* 2013). GST is an antioxidant enzyme which catalyzes the conjugation of several substrates to the thiol group of GSH, transforming toxic materials into less toxic forms (MANSOUR & MOSSA 2010).

Erythrocyte function is mainly dependent on an intact erythrocyte membrane. The toxic effects of various chemicals are usually due, in large part, to their effects on erythrocyte membranes (BRANDAO *et al.* 2005). There are studies investigating the effects of chemicals on erythrocytes (DURAK *et al.* 2010; DEMIR *et al.* 2011).

However, there are no reports regarding the role of quercetin against BA and CA induced oxidative stress. Therefore, BA, CA and quercetin were examined for their effects on MDA levels and the activities of SOD, CAT, GPx and GST in human erythrocytes.

Material and Methods

Chemicals

Benzoic acid (BA) was obtained from Sigma, and citric acid (CA) was obtained from Merck. Quercetin was provided by Sigma, and dimethyl sulfoxide (DMSO) was procured by Merck. All other chemicals were obtained from Sigma-Aldrich (Germany). Quercetin was dissolved in 0.5% DMSO (DEMIR *et al*. 2011). BA and CA was dissolved in distilled water (YILMAZ *et al*. 2008, 2009), then we added 0.5% DMSO to these mixtures. So, control, BA and CA groups are incubated with 0.5% DMSO just as quercetin, BA+quercetin and CA+quercetin groups. Quercetin was given quickly after BA and CA application.

Erythrocyte preparation

Blood (20 ml) was collected from six healthy male volunteers for each group. Heparin was used as an anticoagulant. Erythrocytes were seperated from plasma by centrifugation and washed with 0.9% NaCl. Then supernatant and white blood cells were removed. After this step, erythrocytes were suspended in phosphate buffer at pH 7.4; thus, a 50% cellular suspension was attained. Erythrocytes that were incubated in buffer without BA, CA and quercetin were used as the control group. The control group was also incubated with 0.5% DMSO. The concentration of hemoglobin was specified by the method of DRABKIN (1946).

Treatment of erythrocytes

The doses of BA and CA (50, 100 and 200 μg/ml), the amount used in foods, were selected based on previous studies (YILMAZ *et al.* 2008, 2009). The concentration of quercetin (10μM) was determined according to KOOK *et al.* (2008).

First, erythrocytes were divided into groups of control, quercetin, BA, BA+quercetin, CA, CA+quercetin. The control group was incubated with 0.9% NaCl at 37°C for 1h. Erythrocytes were treated with BA and CA, at three doses of 50, 100 and 200 μg/ml, and quercetin was applied at a concentration of 10μM. After incubation, mixtures were kept at -20°C for one day. After 24h, the mixtures were thawed, then fractured by osmotic pressure and centrifuged. Afterwards, supernatants were segregated and antioxidant enzyme (SOD, CAT, GPx and GST) activities, and MDA levels were quantified by a spectrophotometer (Shimadzu UV-1800).

Measurement of MDA levels

MDA is a main product of lipid peroxidation (LPO), so it is frequently used as an indicator of LPO. Cells were incubated with thiobarbituric acid (TBA) at 95 °C (pH 3.4); MDA levels were specified at 532 nm by the method of OHKAWA *et al.* (1979) and were presented as nmol/mg hemoglobin.

Antioxidant enzyme assays

Measurement of SOD activity

SOD activity was measured by assaying the autooxidation and illumination of pyrogallol (MARKLUND & MARKLUND 1974). This reaction was monitored at 440 nm. One unit of enzyme activity was calculated as the quantity of protein inducing 50% inhibition of pyrogallol autooxidation. SOD activity is expressed as U/mg hemoglobin.

Measurement of CAT activity

Samples were diluted with Triton X-100 before the determination of CAT activity. CAT activity was measured as the changing rate of H_2O_2 decomposition at 240 nm (AEBI 1984). Data is expressed as U/mg hemoglobin.

Measurement of GPx activity

GPx enzyme activity was evaluated with H_2O_2 as substrate according to the method of PAGLIA & VALENTINE (1967). Reaction mixtures involved reduced glutathione, NADPH, glutathione reductase and Tris-HCl. Enzyme activity was measured mediately as the oxidation rate of NADPH. The reaction was monitored at 340 nm. The activity was presented as U/mg hemoglobin.

Measurement of GST activity

GST activity was analysed by gauging the formation of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB) conjugate (HABIG *et al.* 1974). Absorbance was recorded at 340 nm. The specific enzyme activity of GST was expressed as U/mg hemoglobin.

Statistical analysis

Data was evaluated with SPSS 11.0 for Windows and described as means ± standard deviations. In order to determine the differences between means, one way analysis of variance (ANOVA) was used, followed by a Tukey multiple comparison to calculate significance. A P value of <0.05 was determined to be statistically significant.

Results

There were no statistically significant changes in levels of MDA and in enzyme activities between the quercetin treatment group compared with the values of the control ($P>0.05$, Figs 1-5).

Measurement of SOD activity

A decrease in SOD activity was detected in erythrocytes at all doses used. The activity of SOD increased in BA+quercetin and CA+quercetin treated groups compared to BA and CA groups respectively ($P<0.05$, Fig. 1). The protective effect of quercetin was detected after BA and CA application, at all doses. Quercetin completely protected erythrocytes against toxicity caused by CA at a dose of 50 μg/ml.

Measurement of CAT activity

Decreased CAT activity was detected significantly at 50, 100 and 200 μg/ml compared with the control group ($P<0.05$, Fig. 2). Quercetin application with CA and BA reversed these alterations partially against food additives used at all doses. Quercetin protected against CA induced toxicity completely at a dose of 50 μg/ml. The protective effect of quercetin was found at all doses of BA and CA.

Measurement of GPx activity

Human erythrocytes were treated with BA and CA at doses 50, 100 and 200 μg/ml. A decrease of GPx activity was determined after BA and CA application at all doses of treatment ($P<0.05$, Fig. 3). After combined application of quercetin and BA, and also quercetin and CA, GPx activity was significantly increased, compared to the values ob-

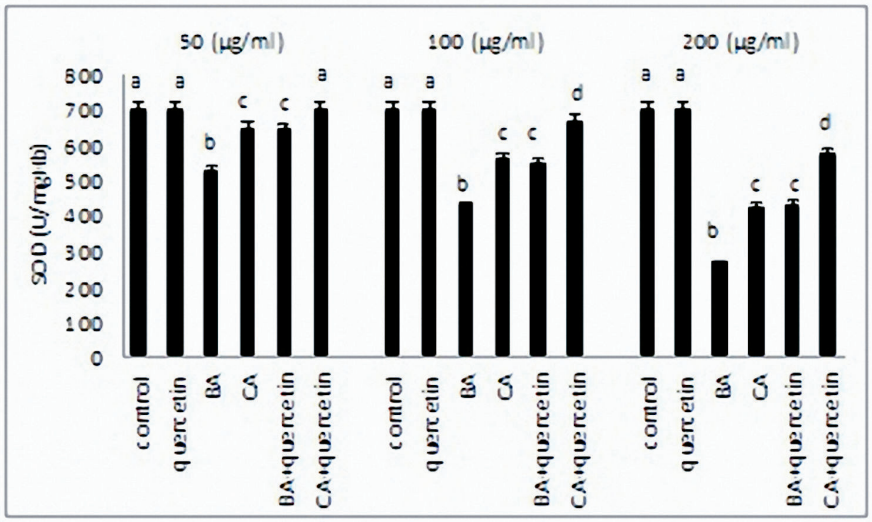

Fig. 1. Effects of BA, CA and quercetin on SOD levels (U/mgHb) in human erythrocytes. Each bar represents mean ±SD in each group. Bars superscripts with different letters are significantly different. Significance at P< 0.05.

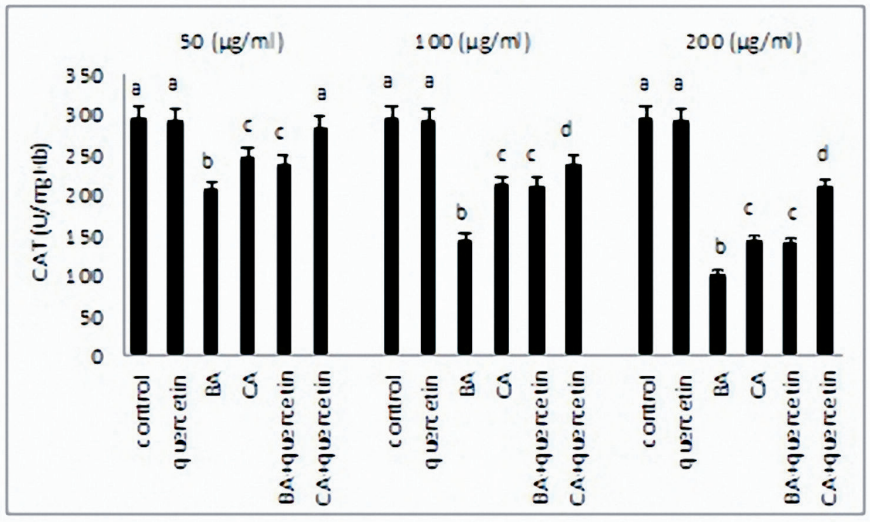

Fig. 2. Effects of BA, CA and quercetin on CAT levels (U/mgHb) in human erythrocytes. Each bar represents mean ±SD in each group. Bar superscripts with different letters are significantly different at P< 0.05.

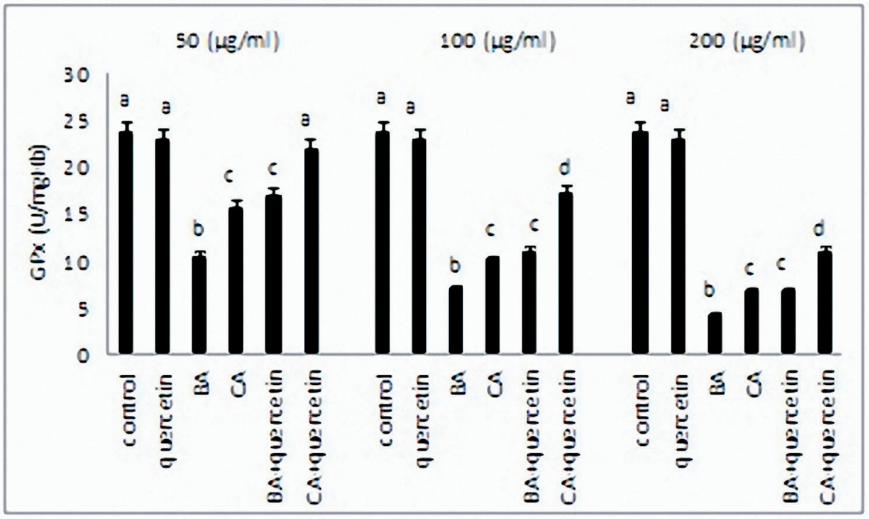

Fig. 3. Effects of BA, CA and quercetin on GPx levels (U/mgHb) in human erythrocytes. Each bar represents mean ±SD in each group. Bars superscripts with different letters are significantly different at P< 0.05.

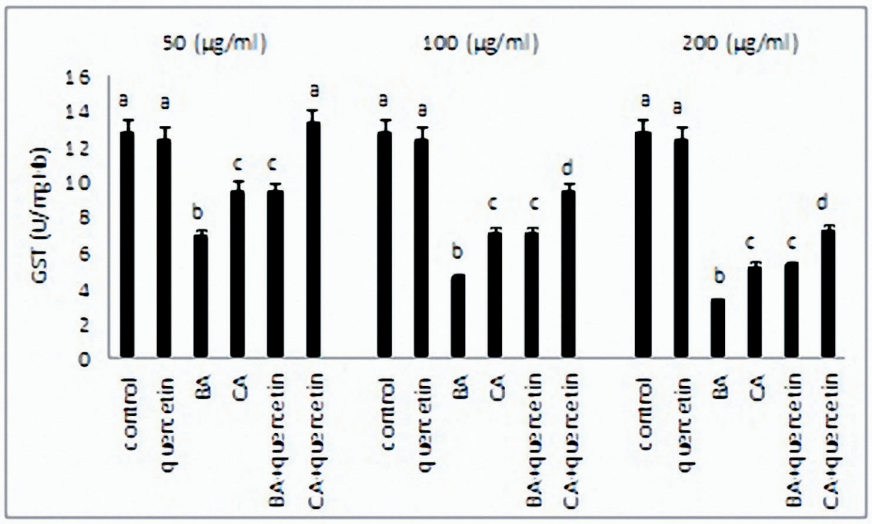

Fig. 4. Effects of BA, CA and quercetin on GST levels (U/mgHb) in human erythrocytes. Each bar represents mean ±SD in each group. Bars superscripts with different letters are significantly different at P <0.05.

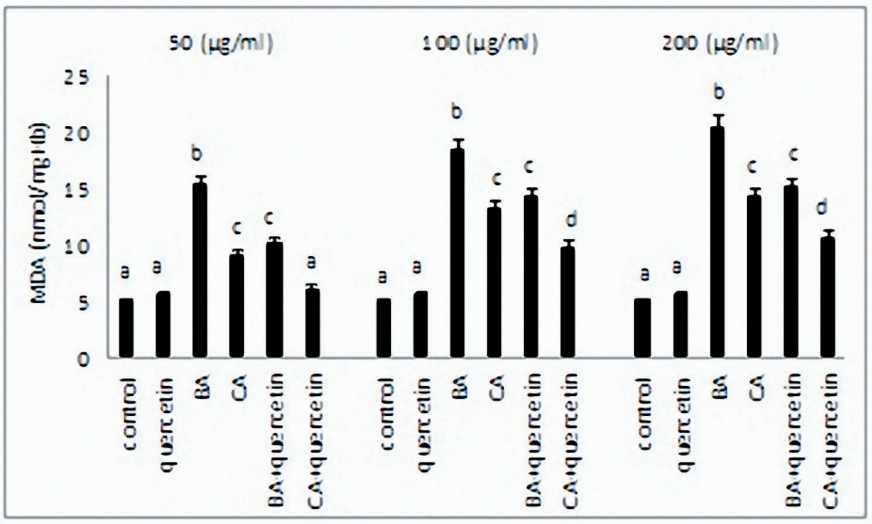

Fig. 5. Effects of BA, CA and quercetin on MDA levels (nmol/mgHb) in human erythrocytes. Each bar represents mean ±SD in each group. Bars superscripts with different letters are significantly different at P <0.05.

tained after treatment of erythrocytes with BA alone, and CA only, respectively. In the CA+quercetin group, the activity of GPx at 50 μg/ml was not significantly different from the value of the control, so quercetin protected erythrocytes completely against toxicity caused by CA applied at a dose of 50 μg/ml.

Measurement of GST activity

BA and CA application caused a significant reduction in GST activity of erythrocytes. On the other hand, the values were significantly increased in combined application of quercetin and BA and quercetin and CA compared with the BA and CA treated erythrocytes, respectively (P<0.05, Fig. 4). There were no significant changes between CA+quercetin and control groups at 50 μg/ml. Thus, we can say that quercetin protects completely against CA mediated changes of GST activity in erythrocytes at 50 μg/ml.

Measurement of MDA level

An increase of MDA level was determined after BA and CA application at all doses of treatment (P<0.05, Fig. 5). The MDA levels decreased significantly when erythrocytes treated with quercetin and BA, and quercetin and CA, were compared to BA and CA treated erythrocytes, respectively. Quercetin has a complete protective effect against CA induced changes in MDA level at a dose of 50 μg/ml.

Discussion

BA (QI *et al.* 2009) and CA (LIU *et al.* 2013) have been widely used in the food industry as an important food preservative. However, they cause some adverse effects on animals (YILMAZ *et al.* 2008; QI *et al.* 2009). BA (YILMAZ *et al.* 2009) and CA (YILMAZ *et al.* 2008) have genotoxic effects in human peripheral lymphocytes. The effects of BA and CA on activity of SOD, CAT, GPx and GST and changes in MDA level in erythrocytes were determined in our work.

In this study, BA and CA caused reduction in activities of SOD, CAT, GPx and GST in human erythrocytes. Additionally, a comparison of erythrocytes treated with BA to those in the group treated with CA has shown that BA caused a greater decrease in enzyme activities, therefore BA has a more toxic effect on erythrocytes than CA. The decrease in enzyme activities following BA and CA treatment may have resulted from increasing generation of free radicals. In the present study, the MDA level results support this probability of the BA and CA treated groups.

The level of MDA is a main oxidation product of peroxidized polyunsaturated fatty acids and it is an important indicator of LPO (MANSOUR & MOSSA 2010). Therefore, MDA is an end product of LPO and an increase in the level of MDA is a major determinant of LPO (COMELEKOGLU *et al.* 2012). For this reason, we measured the MDA level for the determination of oxidative stress caused by LPO in human erythrocytes. LPO is one of the principal processes of oxidative injury that has a critical role in xenobiotic toxicity. It causes a number of unwanted effects, for example increased membrane rigidity and osmotic fragility (UZUN & KALENDER 2013). In this study, increased MDA levels were observed in BA and CA treated erythrocytes. MDA may increase due to the harmful effects of BA and CA on cell membranes. These adverse effects possibly consist of the formation of free radicals causing damage to several cell membrane compounds (ELHALWAGY *et al.* 2008). From the point of MDA level, when BA and CA treated groups compared with each other, there were more increment in BA group. According to the MDA level and antioxidant enzyme activity results, it is clear that BA causes more adverse effects in erythrocytes than CA. A study by YILMAZ *et al.* (2012) supports our findings.

Cells are constantly exposed to endogenously and exogenously formed free radicals. There is a balance between antioxidants and oxidants under normal conditions. On the other hand, at excessive free radical generation, this balance is broken down, leading to oxidative stress formation. Antioxidants have a major role in protection against oxidative stress damage (WILMS *et al.* 2008). Injury caused by free radicals can be inhibited by antioxidants via different mechanisms, e.g. by preventing the formation of radicals and radical scavenging (LI *et al.* 2001). Quercetin is a well-investigated antioxidant and is known to be able to preserve cells against oxidative damages (WILMS *et al.* 2008). It is also able to protect red blood cells from oxidative damage and cellular senescence *in vitro* in a dose-dependent manner (KOOK *et al.* 2008). In this *in vitro* study, we also investigated whether quercetin used at a concentration of 10 μM has protective effects against BA and CA induced toxicity. Quercetin can decrease the MDA level induced by many toxins (RAMESH *et al.* 2009). A combination of quercetin and BA and quercetin and CA treated erythrocytes compared with BA and CA treated erythrocytes, respectively, showed an increment in antioxidant enzyme activities and a decrease in MDA levels. Quercetin becomes protective because of its antioxidant properties, as in other studies (WILMS *et al.* 2008; DEMIR *et al.* 2011). This benefical effect may be due to its elevated diffusion into the cell membranes allowing it to scavenge free radicals (RAMESH *et al.* 2009).

In summary, we determined the protective effects of quercetin on BA and CA oxidative stress in human erythrocytes, *in vitro*. Data in this study showed that BA has more adverse effects on erythrocytes than CA and quercetin has benefical effects on BA and CA mediated toxicity, but does not protect completely.

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