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AIM AND SCOPES

Cell Membranes and Free Radical Research is a print and online journal that publishes original research articles, reviews and short reviews on the molecular basis of biophysical, physiological and pharmacological processes that regulate cellular function, and the control or alteration of these processes by the action of receptors, neurotransmitters, second messengers, cation, anions, drugs or disease.

The journal has been publishing three volumes in a year.

Areas of particular interest are four topics. They are;

A- Ion Channels (Na^+ - K^+ Channels, Cl^- channels, Ca^{2+} channels, ADP-Ribose and metabolism of NAD^+, Patch-Clamp applications)

B- Oxidative Stress (Antioxidant vitamins, antioxidant enzymes, metabolism of nitric oxide, oxidative stress, biophysics, biochemistry and physiology of free oxygen radicals

C- Interaction Between Oxidative Stress and Ion Channels

(Effects of the oxidative stress on the activation of the voltage sensitive cation channels, effect of ADP-Ribose and NAD^+ on activation of the cation channels which are sensitive to voltage, effect of the oxidative stress on activation of the TRP channels)

D- Gene and Oxidative Stress (Gene abnormalities. Interaction between gene and free radicals. Gene anomalies and iron. Role of radiation and cancer on gene polymorphism)

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Keywords

Ion channels, cell biochemistry, biophysics, calcium signaling, cellular function, cellular physiology, metabolism, apoptosis, lipid peroxidation, nitric oxide synthase, ageing, antioxidants, neuropathy.

Protective effect of different layers of onion extracts (*Allium cepa* L.) on markers of oxidative stress in erythrocytes

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List of abbreviations
DPPH - 2, 2-diphenyl-1-picrylhydrazyl
FRSA - free radical scavenging activity
GSH - reduced glutathione
MDA - malondialdehyde
PBS - phosphate buffer saline
QDG - quercetin-3,4'-O-diglucoside
QMG - quercetin-4'-O-monoglucoside
SD - standard deviation
t-BHP - *tert*-butyl hydroperoxide

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Abstract

Onions (*Allium cepa* L.) are the rich source of flavonoids, consisting mainly quercetin-3,4´-O-diglucoside and quercetin-4´-O-monoglucoside, as major flavonoids. In the present study, we investigated the free radical scavenging activity (FRSA) and *in vitro* protective effect of different layers of onion extract on lipid peroxidation, reduced glutathione (GSH) and erythrocyte hemolysis in goat. The outer onion layers showed higher FRSA compared to inner layers. Results show a significant protection of oxidative stress by onion extract on erythrocyte subjected to *tert*-butyl hydroperoxide (t-BHP) treatment, as evidenced by the decrease in malondialdehyde (MDA) and increase in GSH content. Onion extracts also showed significant protection of the erythrocyte from oxidative hemolysis. Quercetin (at micromolar concentration) showed significant antioxidant effect in protecting erythrocytes from *t*-BHP induced oxidative changes. The results are more pronounced for outer layers as compared to inner layers, suggesting that the outermost living layers had higher antioxidant activities compared to innermost layers.

Keywords

Onion, erythrocytes, oxidative stress, hemolysis, quercetin

Introduction

Flavonoids, group of polyphenolic compounds, have recently attracted much attention due to their pleiotropic biological and therapeutic properties (Ross and Kasum, 2002). They play an important role in human health offering protection against cellular damage due to their ability to quench oxygen-derived free radicals either by donating electrons, chelating redox-active metals and inhibition of lipooxygenases (Oteiza et al., 2005).

Onion (*Allium cepa* L.) is a good source of dietary phytochemicals with proven antioxidant properties and ability to modulate the detoxification systems (Desjardins, 2008). Various scientific reports have confirmed its functional properties which include free radical scavenging activities, immune stimulation, cardioprotective effects (by lowering serum cholesterol and blood pressure), anti-cancer, and anti-infectious properties (Corzo-Martinez et al., 2007). Onions have been found to be effective in the prevention and treatment of a number of diseases and have antidiabetic, anti-platelet aggregation and anti-biotic effects (Desjardins, 2008).

The major flavonols found in onion are the quercetin conjugates, mainly quercetin-3,4'-O-diglucoside (QDG) and quercetin-4'-O-monoglucoside (QMG) (Rhodes and Price, 1996; Price and Rhodes, 1997). It has been reported that quercetin metabolites enhance the antioxidant defense system and also evoke various biological functions, pharmacological and medicinal activities (Rizvi and Mishra, 2009; Pandey and Rizvi, 2010a), all these activities are believed to arise from its antioxidant properties.

Many *in vitro* and in vivo studies have demonstrated that several parameters of erythrocyte function and integrity are negatively affected by increased oxidative stress. Because of their high susceptibility to oxidation, erythrocytes have been used as a metabolically simplified model system to investigate oxidative damage in biomembranes. The present study was undertaken to determine the free radical scavenging activity (FRSA) and antioxidant effect of onion extract on lipid peroxidation, reduced glutathione (GSH), and erythrocyte hemolysis in goat, with respect to different layers (the outermost living layers just beneath the dry outer scales of onion and the inner layers), in an effort to categorize the antioxidant efficacy in different parts of the onion.

Material and methods

Plant material and extraction

The red variety onion (Pusa red cultivar) was purchased from local markets of Allahabad, India. The selected plants were collected and the herbarium sheets were sent to the herbarium in Botany Department of Allahabad University, and the voucher specimen number were obtained. They were then subdivided into two different parts: the innermost layers and the outermost layers (the transitional layer with the first living cells). Onion extracts were prepared as described by Stajner et al. (2008), with some modifications. Briefly, 5 g of onion (inner layers and outer layers) were grounded with quartz sand in a cold mortar. The homogenized material was suspended in 10 mL solvent. The solvent used was phosphate buffer saline, PBS (0.9% NaCl, 10 mmol L^{-1} Na_2HPO_4, pH 7.4). After 10 min centrifugation at 4 °C and 15000g, the aliquots of the supernatants were collected. Samples were re-extracted twice and the final volume makes up to 100 mL and stored at 4 °C, before conducting experiments.

Determination of FRSA of onion extracts

FRSA of extracts was measured by slightly modified method of Brand-Williams et al. (1995), 100 µL of methanolic onion extract was added to 3 mL DPPH (2, 2-diphenyl-1-picrylhydrazyl) solution. The mixture was incubated for 15 min and the decrease in absorption was measured at 515 nm. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared. Quercetin of various concentrations (10^{-5} mol L^{-1} and 10^{-6} mol L^{-1}) in methanol were taken as standard (Pandey and Rizvi, 2010a; Rizvi and Mishra, 2009). The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = \frac{\text{A Control} - \text{A Sample}}{\text{A Control}} \times 100$$

Collection of blood and isolation of erythrocytes

Goat erythrocytes were chosen for the study. Freshly obtained heparinized blood samples were immediately centrifuged at 800g for 10 min at 4 °C and plasma and buffy coat were then carefully aspirated. Erythrocytes were washed three times with cold PBS (0.9% NaCl, 10 mmol L^{-1} Na_2HPO_4; pH 7.4). Supernatant and buffy coat were carefully removed after each wash.

Induction of oxidative stress and *in vitro* experiments with onion extracts

Oxidative stress was induced *in vitro* by incubating washed erythrocytes with 10^{-5} mol L^{-1} of tert-

butylhydroperoxide (*t*-BHP) for 30 min at 37 °C. The effect of onion extracts (inner and outer layers) was evaluated by co-incubating erythrocytes with *t*-BHP and onion extracts for 30 min at 37 °C. The concentration of *t*-BHP used in the present study to induce oxidative stress in erythrocytes was in the range of concentration used in other published reports (Pandey et al., 2009; Pandey and Rizvi, 2010b). After 30 min incubation, the suspensions were immediately centrifuged at 1800g, and the erythrocytes were washed two to three times with PBS, pH 7.4 and finally, packed erythrocytes were used for assay. In parallel control experiments, blood was incubated without onion extracts and t-BHP.

In vitro hemolysis with onion extracts

To study the protective effects of the onion extracts against *t*-BHP induced hemolysis, an erythrocyte suspension 40 times diluted was incubated with the onion extracts (dissolved in PBS) at 37 °C, followed by incubation with 10^{-5} mol L^{-1} *t*-BHP. This reaction mixture was shaken gently while being incubated for 2 h at 37 °C. The extent of hemolysis was determined spectrophotometrically as described previously (Ko et al., 1997). Briefly, aliquots of the reaction mixture were taken out after 2 h of incubation and centrifuged at 4000g for 10 min to separate the erythrocytes. The percentage of hemolysis was determined by measuring the absorbance of the supernatant (A) at 545 nm and compared with that of complete hemolysis (B) by treating an aliquot with the same volume of the reaction mixture with distilled water. The hemolysis percentage was calculated using the formula: A/B x 100. The effect of quercetin (10^{-4} mol L^{-1} to 10^{-6} mol L^{-1}), on hemolysis was also evaluated for comparing its effect with onion extracts. The concentration of quercetin used in the present study was in the range of concentration used in other published reports (Pandey and Rizvi, 2010a; Rizvi and Mishra, 2009). Parallel control experiments were also performed in which erythrocyte suspension was incubated without onion extracts and *t*-BHP.

Determination of MDA content

Erythrocyte MDA level was measured according to the method of Esterbauer and Cheeseman (1990) with slight modification. Packed erythrocytes (0.2 mL) were suspended in 3 mL PBS buffer, pH 7.4. The lysate (1 mL) was added to 1mL of 10% trichloroacetic acid and 2 mL of 0.67% thiobarbituric acid and boiled for 20 min at temperature greater than 90 °C. The solution was cooled and the absorbance read at 532 nm (Genesys 10 Vis Visible Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, United States). The concentration of MDA in erythrocytes was determined from a standard plot, and was expressed as nmol mL^{-1} of packed erythrocytes.

Determination of erythrocyte GSH content

Erythrocyte GSH was measured following the method of Beutler (1984). This method was based on the ability of the –SH group to reduce 5,5'-dithiobis, 2-nitrobenzoic acid and form a yellow coloured anionic product whose optical density is measured at 412 nm (Genesys 10 Vis Visible Spectrophotometer, Thermo Scientific, Waltham, Massachusetts, United States). Concentration of GSH is expressed in mg mL^{-1} of packed erythrocytes and was determined from a standard plot.

Statistical Analysis

Values were mean ± standard deviation (SD) of 5-6 separate experiments done in triplicates. Statistical comparisons were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, California, USA). Statistical differences were analyzed with Mann Whitney U test and the differences were considered to be significant when $p < 0.05$.

Results

The onion extracts were studied for their free radical scavenging activity using DPPH radical and compared with quercetin of different concentrations (10^{-5} mol L^{-1} and 10^{-6} mol L^{-1}). The FRSA of the extract showed a wide variation in % DPPH inhibition ranging from 17.28 to 36.73% (Figure 1). Outermost living layers of the onion extracts were found most powerful free radical scavenger compared to the inner layers ($p < 0.01$).

As anucleated cells with intrinsically poor repair mechanisms, erythrocyte makes a good model to test the antioxidant capacity of antioxidant compounds in the presence of an oxidation stimulus (Coimbra et al., 2006). The antioxidant effect of the onion extract was tested on osmotic fragility of erythrocytes which have been oxidatively stressed by incubating with *t*-BHP (with and without onion extract) and quercetin (Figure 2). In the present study, incubation with t-BHP resulted in increase in oxidative hemolysis of erythrocytes. Onion extracts showed significant protection of the erythrocyte from oxidative hemolysis (inner layers 52.3% and outermost layers 43.5%) ($p < 0.01$). Quercetin also protected the erythrocytes from oxidative hemolysis, an effect which was concentration dependent ($p < 0.01$).

Under oxidative stress, the erythrocyte membrane

is prone to lipid peroxidation that involves cleavage of polyunsaturated fatty acids at their double bonds, leading to the formation of MDA (Chiu et al., 1989). Subjecting erythrocytes to increased oxidative stress by incubating them with *t*-BHP caused a significant increase in MDA formation ($p < 0.01$) (Figure 3). Onion extract protected *t*-BHP induced lipid peroxidation, the effect was greater with outer onion layer compared to inner layer extract ($p < 0.01$). Presence of quercetin at different concentrations (10^{-5} mol L^{-1} and 10^{-6} mol L^{-1}) in the incubation medium protected the erythrocytes from *t*-BHP induced oxidative stress as evidenced from the decrease in the level of MDA ($p < 0.01$).

Glutathione, an efficient antioxidant present in almost all living cells, is also considered as a biomarker of redox imbalance at cellular level. The induction of oxidative stress following incubation with *t*-BHP resulted in decrease in intracellular GSH content ($p < 0.01$). Onion extracts protected the erythrocytes against t-BHP induced GSH oxidation (Figure 4), however, this increase was significantly higher in the outermost living layers as compared to the inner layers ($p < 0.01$). Quercetin protected the erythrocytes against *t*-BHP induced oxidative stress at different concentrations (10^{-5} mol L^{-1} and 10^{-6} mol L^{-1}) ($p < 0.01$).

Discussion

Our results show that onion extracts can protect erythrocytes from oxidative stress under *in vitro* conditions. Erythrocytes are highly susceptible to oxidative damage due to the high cellular concentration of oxygen and haemoglobin: a potentially powerful promoter for the oxidative processes. Oxidative damage of erythrocytes membrane (lipid and protein peroxidation) compromise cell integrity, which may be implicated in hemolysis associated with some hemoglobinopathies, certain drugs, transition metal toxicity, radiation, and in conditions of deficiency in some erythrocyte antioxidant systems (Ko et al., 1997). Increased erythrocyte MDA level is known to affect erythrocyte membrane lipid bilayer fluidity (Bryszewska et al., 1995). A high concentration of MDA in erythrocyte is a marker of cellular oxidative damage observed in stress or pathological conditions including aging (Rizvi and Maurya, 2007). In addition, reduced glutathione is a major intracellular nonprotein sulfhydryl compound, having many biological functions, including maintenance of membrane protein -SH groups in the reduced form, the oxidation of which can otherwise cause altered cellular structure and function (Pandey and Rizvi, 2010b).

The strong biological antioxidant activity of the onion extracts against oxidative stress might be due the presence of polyphenolic flavonoids in onion (Shon et al., 2004; Prakash et al., 2007), which protects the cell not only by buffering free radicals but also by altering cell membrane properties (Arora et al., 2000; Pawlikowska-Pawlega et al., 2003). In the present study, the FRSA of onion extracts indicates that the flavonoid content is significantly higher in outer layers compared to inner layers. Our values are

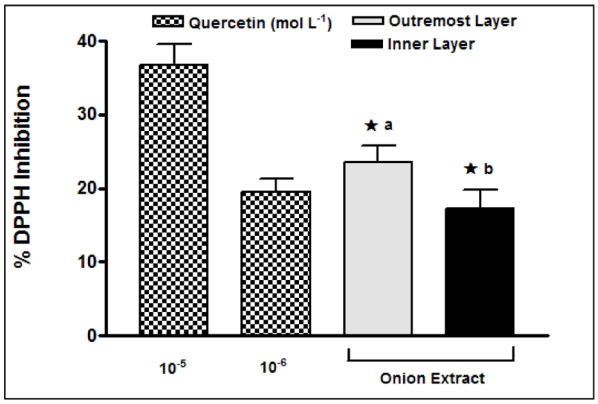

Figure 1. Comparison of free radical scavenging activity (FRSA) of onion extracts (outermost and inner layers) and quercetin.
*a ($p < 0.01$) as compared to inner layers and quercetin of various concentrations. *b ($p < 0.01$) as compared to quercetin (10-5 mol L-1). Values are expressed as mean ± SD of the 5-6 independent experiments.

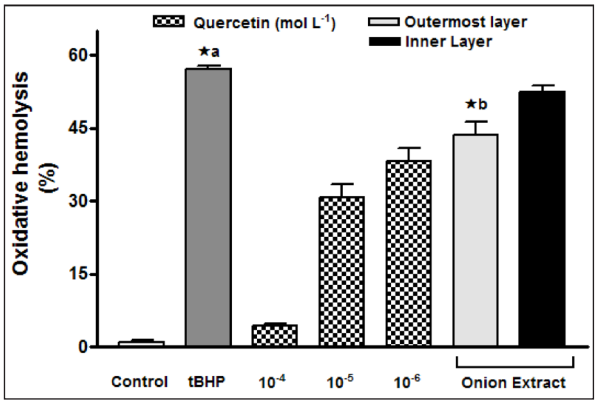

Figure 2. Effect of onion extracts (outermost and inner layers) and quercetin on % oxidative hemolysis in t-BHP induced oxidative stressed goat erythrocytes.
Incubation with *t*-BHP caused significant increase in oxidative hemolysis as compared to control. Treatment with onion outermost layers and inner layers shows significant protection *a ($p < 0.01$) of erythrocytes from *t*-BHP induced hemolysis. Significant difference *b ($p < 0.01$) between outermost layers and inner layers. Treatment with quercetin shows significant protection *a ($p < 0.01$) at different concentrations against *t*-BHP induced oxidative hemolysis. Values are expressed as mean ± SD of the 5-6 independent experiments.

within the range of FRSA reported by other workers in whole bulb extracts of some Italian Allium species (Nencini et al., 2011). The ability of quercetin to incorporate into the hydrophobic core of the membrane bilayer improves the antioxidative effectiveness of flavonoid by causing a reduction in membrane fluidity and membrane stability, which further limit diffusion of free radicals (Arora et al., 2000). Our results corroborate the recent report in which quercetin was found to inhibit both neutrophil oxidative burst activity and protect erythrocytes against hemolysis by free radicals (Hapner et al., 2010). Besides phenolic compounds, organosulfur compounds such as S-propenylcysteine sulfoxide (major component), S-propylcysteine sulfoxide and S-methylcysteine sulfoxide and non-flavonoid compounds have been reported in onion to show alkylperoxyl radical scavenging activity and also responsible for most of its biological properties (Sawa et al., 1999; Corzo-Martinez et al., 2007).

The variation in protective effect with respect to layers of onion extracts against oxidative stress might be due to variation in the quantities of quercetin present in various layers of onion. It has been reported recently that the outer layers consists of QDG and QMG in equal amount while in the inner layers, QDG is the major flavonoid (Beesk et al., 2010). Difference in the distribution of the total flavonoid content in the different parts of the onion bulb has been previously reported by various investigators (Bilyk et al., 1984; Prakash et al., 2007; Beesk et al., 2010), which has been explained due to the increased activity of light-induced enzyme phenylalanine ammonia lyase. This enzyme catalyses the biosynthesis of flavonoids in the outermost localizing living cells of the whole onion bulb due to more exposure to sunlight (Friedman, 1997; Hirota et al., 1999). The cells of outermost dead dried peel of onion are not affected by sunlight, resulting in lesser biosynthesis of flavonoids, thus having flavonoid content less than the outermost living cells layer (Beesk et al., 2010).

Knowledge of the metabolism, bioavailability, and potential health effects of quercetin in onion is important before any conclusions may be drawn regarding their potential to exert biological activity *in vivo,* as suggested by in vitro studies. Onion is considered to be one of the richest dietary sources of quercetin (200-600 mg kg^{-1}) (Hertog et al., 1992). With regard to bioavailability of quercetin, Hollman et al. (1995), showed that quercetin was indeed absorbed in humans moderately rapidly, the glycosides of quercetin being more efficiently absorbed than quercetin itself (Manach et al., 2005). The nature of the sugar residues in the glycosides influences the extent of absorption. For instance, quercetin glycosides from onions are more bioavailable than quercetin glycosides from apples (Manach et al., 2005). Also, quercetin metabolites are eliminated slowly throughout the day, with reported half-lives ranging from 11 to 28 h, which is longer than many other flavonoids such as anthocyanidins and catechins (Graefe et al., 2001). It has been reported that quercetin is significantly absorbed following an onion

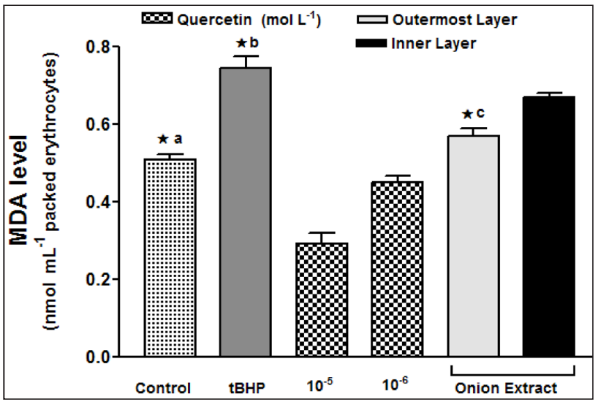

Figure 3. Effect of onion extracts (outermost and inner layers) and quercetin on the malondialdehyde (MDA) level in *t*-BHP induced oxidative stressed goat erythrocytes.
Incubation with *t*-BHP caused an increase in MDA level. Treatment with onion outermost layers and inner layers shows significant protection *b ($p < 0.01$) of erythrocytes from *t*-BHP induced oxidative stress. Significant difference *c ($p < 0.01$) between outermost layers and inner layers. Treatment with quercetin shows significant protection *b ($p < 0.01$) at concentrations 10^{-5} mol L^{-1} and 10^{-6} mol L^{-1} compared with *t*-BHP. *a ($p < 0.01$) compared with quercetin (10^{-5} mol L^{-1} and 10^{-6} mol L^{-1}) and onion extract (outermost and inner layers). MDA content is reported in terms of nmol L^{-1} of packed erythrocytes. Values are expressed as mean ± SD of the 5-6 independent experiments.

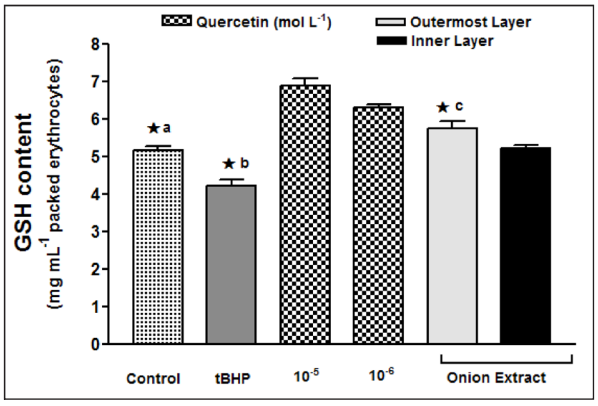

Figure 4. Effect of onion extracts (outermost and inner layers) on reduced glutathione (GSH) content in *t*-BHP induced oxidative stressed goat erythrocytes.
Incubation with *t*-BHP caused significant decrease *a ($p < 0.01$) in GSH content as compared to control. Treatment with onion extracts shows significant protection of erythrocytes *a ($p < 0.01$) for outermost layer as compared to control. The effect of inner layer extract was not significant as compared to control. Treatment with quercetin shows significant protection *a ($p < 0.01$) compared to control and *b ($p < 0.01$) compared with *t*-BHP at concentrations 10^{-5} mol L^{-1} and 10^{-6} mol L^{-1}. Significant difference *c ($p < 0.01$) in GSH content in between both the layers. GSH content is reported in terms of mg mL^{-1} of packed erythrocytes. Values are expressed as mean ± SD of the 5-6 independent experiments.

meal and its peak plasma concentration is achieved after 2–2.7 hours of administration (McAnlis et al., 1999). Thus, plasma quercetin levels in subjects who regularly eat onions may approach those of β-carotene (Hollman et al., 1996), and contribute significantly to the antioxidant defences present in blood plasma.

Conclusion

We provide enough evidence for the strong biological antioxidant activity of the onion extracts against oxidative stress. In conclusion, it can be stated that the outer living layer (the transitional layer with the first living cells below the dry onion peel) is a better resource for food ingredients and easily accessible source for nutraceutical compounds. We consider that this fraction deserves more intensive study, including its *in vivo* antioxidant activity in order to understand its potential as nutraceutical and for better extraction of flavonoids.

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