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Protective Effect of Myricetin on Proteins and Lipids of Erythrocytes Membranes

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ABSTRACT

Myricetin (3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone), a naturally occurring flavonol, is a potent scavenger of Reactive Oxygen Species (ROS) and effectively prevent erythrocyte oxidation. The protective effect of myricetin on proteins and lipids of erythrocytes membranes was investigated. Erythrocytes membranes were subjected to oxidative stress by incubating them with 10^{-5} M tert-butylhydroperoxide; this caused a significant increase in membrane protein carbonyl group content and membrane lipid peroxides while caused a significant decrease of membrane total thiol group and Na^+, K^+-ATPase activity. The presence of myricetin in micromolar concentration in the incubation medium decreased significantly protein carbonylation, lipid peroxidation and caused an increase in total thiol group and Na^+, K^+ ATP-ase activity.

Key words: Erythrocytes membranes, flavonol, lipid peroxides, myricetin, protein, oxidation

INTRODUCTION

Reactive Oxygen Species (ROS) are oxidants usually produced during the course of metabolism by most cells of the body. The intracellular antioxidant systems, either, enzymatic or nonenzymatic react with these oxidant converting them to nonreactive species (Srinivasan and Avadhani, 2012). Oxidative stress occurs as a result of depletion or inadequacy of antioxidant systems causing damage to intracellular biomolecules including proteins, lipids and nucleic acids (Ayer et al., 2010). Oxidative stress may take place under normal conditions but its incidence increases with age and during disease conditions as the efficiency of antioxidant and repair mechanisms decreases (Gil et al., 2006).

As erythrocytes carry oxygen to various organs, they are exposed to ROS. The presence of oxygen, polyunsaturated fatty acids and iron induce lipid peroxidation and protein oxidation causing loss of functions of membrane biomolecules like membrane-bound enzymes and receptors and alter the fluidity of the membranes (Matough et al., 2012).

Erythrocytes possess defense mechanisms against toxic species, but the efficiency of these mechanisms decreases in conditions that cause overproduction of ROS. Therefore, the additional supplementation of antioxidants is important for the protection against oxidative stress and other disease conditions, such as atherosclerosis, ischemia, inflammation, cancers, cardiovascular and neurological diseases (Rice-Evans, 2001).

Flavonoids are polyphenolic compounds found in considerable levels in dietary plants. The flavonoid family is divided into a number of sub-groups; namely flavonols, flavones, flavan-3-ols, isoflavones, flavanones and anthocyanidins (Crozier et al., 2000). The antioxidant activities of polyphenols of dietary origin are well documented and may be responsible for various health profits (Brownson et al., 2002). The penetration of polyphenolic compounds into cell membranes prevents
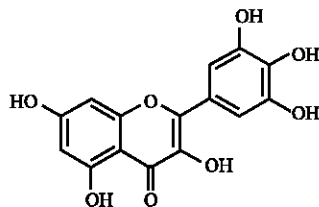
Fig. 1: Chemical structure of myricetin

oxidation and its sequel. The achievement of an efficient protection against membrane oxidation by means of phenolic compounds may be due to their ability to incorporate into biological membranes (Suwalsky *et al.*, 2009).

Myricetin (3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4 chromenone), is a flavonol present in some dietary plants and in walnuts. Myricetin may be occurring as a glycoside (Fig. 1) (Miean and Mohamed, 2001).

In this study, the protective effect of myricetin on proteins and lipids of erythrocytes membranes subjected to oxidative stress (by incubating with 10^{-5} M tert-butylhydroperoxide) was examined, through measuring the membrane protein carbonyl group content, lipid peroxides, total thiol group and the activity of Na^+, K^+- ATPase in presence and absence of myricetin.

MATERIALS AND METHODS

This study was carried out in Biochemistry Department, Sohag Faculty of Medicine in accordance with the guidelines of the ethical committee of Sohag University, during the year 2011.

Chemicals: Myricetin, Guanidine hydrochloride, 5, 5'-Dithio-Bis (2 Nitrobenzoic acid) and tert-butylhydroperoxide were purchased from Sigma (St. Louis, MO, USA). Other chemicals and reagents were purchased from MERK (Darmstadt, Germany).

Blood samples: Venous blood samples (5 mL) were obtained by venipuncture in heparin from 15 healthy volunteers after taking their informed written consent. The blood was centrifuged at 1,800 g for 10 min at 4°C. After the removal of plasma, buffy coat and upper 15% of the packed Red Blood Cells (RBCs), the RBCs were washed twice with cold PBS (0.9% NaCl, 10 mM Na_2HPO_4, pH 7.4) (Dodge *et al.*, 1963).

Erythrocyte ghost preparation: Erythrocyte ghosts from leukocyte-free RBCs were prepared according to the method of Dodge *et al.* (1963). RBCs pellets were hemolyzed on ice with 9 volumes of 5 mM phosphate buffer (pH 7.4) for an hour and centrifuged for 20 min at 15000 g and 4°C. The process was continued until the washing buffer became colorless. The white ghosts finally resolved in equal volumes of PBS. Protein concentrations were determined according to the method of Lowry *et al.* (1951).

Determination of membrane protein carbonyl: Erythrocyte membrane protein carbonyls were measured according to the procedure of Levine *et al.* (1990), as follows, two tubes of 0.25 mL membrane suspension were taken, one as a test and the other as a control. 1.0 mL of 10 mM 2, 4 dinitrophenyl-hydrazine (DNPH) prepared in 1.25 M HCl was added to the test sample and 1.0 mL

of 2.5 M HCl alone was added to the control sample. The contents were mixed and incubated in the dark at room temperature for 1 h and shaken intermittently every 15 min. Then 1. 25 mL of 20% TCA (w/v) was added to both tubes and the mixture left in ice for 10 min. The tubes were then centrifuged at 3,500 rpm for 20 min to obtain the protein pellet. The supernatant was carefully aspirated and discarded. This was followed by a second wash with 10% TCA. Finally, the precipitates were washed three times with 1 mL of ethanol: ethyl acetate (1:1, v/v) to remove unreacted DNPH and lipid remnants. The final protein pellet was dissolved in 1 mL of 6 M guanidine hydrochloride (GuHCL) and incubated at 37°C for 10 min. The insoluble materials were removed by centrifugation. Carbonyl concentrations were determined from the difference in absorbance at 370 nm between the test and the control samples, with $\epsilon 370 = 22000$ M^{-1} cm^{-1}. Carbonyl levels were expressed as nM mg^{-1} protein.

Determination of membrane lipid peroxides: Lipid peroxides were measured using the method described by Draper and Hadley (1990) based on thiobarbituric acid reactivity (TBARs), as following; 200 µL of membrane suspension was mixed with a solution containing 15% trichloroacetic acid (w/v), 0.38% thiobarbituric acid and 0.25 N HCL and heated at 100°C for 10 min in boiling water bath. After centrifugation, the absorbance was taken at 535 nm and the results were expressed as nmol TBARs mg^{-1} protein.

Determination of thiol group: It is determined according to thiol/disulfide reaction of thiol and Ellman's reagent (5,5'-dithiobisnitrobenzoic acid) (Hu, 1994). Fifty microliter of membrane suspension was mixed with 1 mL 0.1 M Tris, 10 mM EDTA pH 8.2, constituting the blank reaction and assessed at 412 nm. After that, we added 40 µL 10 mM DTNB in methanol and the absorption read at 412 nm after stable colour formation (1-3 min). The concentrations of thiol groups were calculated using a molar coefficient of 13.600 $M^1 cm^{-1}$. Thiols were expressed as nM mg^{-1} protein.

Na^+, K^+-ATP-ase activity assay: The activity of Na^+, K^+-ATPase was assayed according to the method of Bartosz *et al.* (1994), as follows, 200 µL of membrane suspension was incubated for 1 h at 37°C in 1 mL of a reaction mixture consisting of 3 mM ATP, 5 mM $MgCl_2$, 140 mM NaCl, 14 mM KCl, 1 mM EDTA and 10 mM tris-HCl. The reaction was stopped by addition of 1 mL of 15% trichloroacetic acid and shaken vigorously, then centrifuged at 3000 g for 15 min. The released Pi in the supernatant was measured by the method of Fiske and Subbarow (1925). Total ATPase activity was expressed as µM of Pi released per hour per mg protein. This assay was repeated in the presence of 200 µM methyldigoxin, an inhibitor of Na^+K^+-ATPase activity. The activity of Na^+K^+-ATPase was subsequently determined by subtracting total ATPase activity in the presence of methyldigoxin from enzyme activity in the absence of the inhibitor drug.

Induction of oxidative stress: Erythrocyte ghosts were incubated with t-BHP, 100 µM mL^{-1} alone or with t-BHP and myricetin, 50 µM mL^{-1} (myricetin is added first followed by t-BHP after 10 min) for 1 h and protein carbonyls, lipid peroxides, total thiol group and Na^+, K^+ ATP-ase activity were measured as described previously.

Statistical analysis: Results were expressed as Mean±SD. Statistical analysis of the data were performed using GraphPad Prism (Graph Pad Software, San Diego, CA, USA).

RESULTS

Obtained results revealed that incubation of erythrocytes membranes with 10^{-5} M t-BHP caused an increase in the level of lipid peroxide and protein carbonyl, meaning that the antioxidant systems present in the membrane cannot overcome the oxidative stress induced by t-BHP. Decrease in Na^+, K^+ ATP-ase activity meant that the oxidative stress caused malfunction of membrane enzymes. Myricetin, a naturally occuring flavonol possessed a powerful antioxidant capacity. Myricetin in concentration of (50 μM mL^{-1}) protected the membrane proteins and lipids and restored the Na^+, K^+ ATP-ase activity. The incubation of erythrocytes membranes with t-BHP caused a significant increase of membrane protein carbonyl from 1.5±0.4-6.2±0.7 0.4 nM mg^{-1} protein (the increase was about 4.1 folds), the presence of myricetin in concentration of (50 μM mL^{-1}) caused a significant decrease in membrane protein carbonyl reached to 2.7±0.4 nM mg^{-1} protein (the decrease was about 2 folds) (Fig. 2). Lipid peroxides increased from 0.43±0.01-5.8±0.68 5 nM mg^{-1} protein, (the increase was about 13.5 folds) in absence of myricetin, the presence of myricetin decreased significantly lipid peroxides to 2.1±0.42 nM mg^{-1} protein (the decrease was about 2.8 folds) (Fig. 3). Total thiol group concentrations decreased significantly from 28.7±5.4-13.4±3.2 (nM mg^{-1} protein) in absence of myricetin (the decrease was about
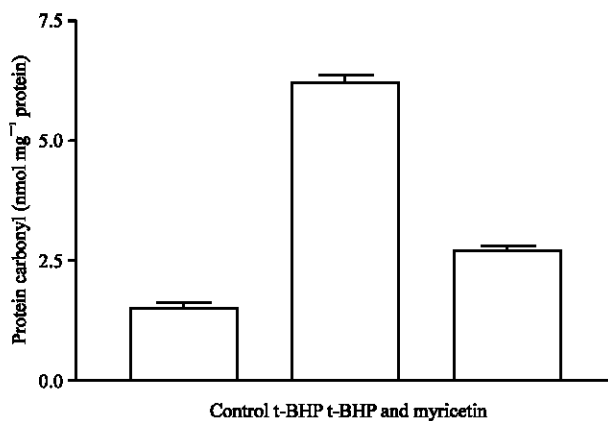

Fig. 2: Effect of myricetin on erythrocyte membrane carbonyl group content of oxidatively stressed RBCs
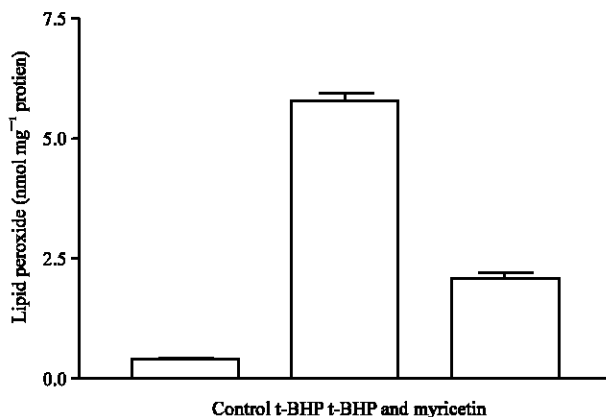

Fig. 3: Effect of myricetin on erythrocyte membrane lipid peroxides of oxidatively stressed RBCs

2.14 folds) and reached 20.4 ± 4.1 nM mg^{-1} protein in presence of myricetin (the increase was about 1.4 folds) (Fig. 4). Na$^+$, K$^+$ ATP-ase activity decreased from 0.59 ± 0.07-0.17 ± 0.05 (μM Pi mg^{-1} protein h^{-1}) in absence of myricetin (the decrease was about 3.4 folds) and decreased to 0.39 ± 0.06 μM Pi mg^{-1} protein h^{-1} in presence of myricetin (the increase was about 1.5 folds) (Fig. 5, 6). The
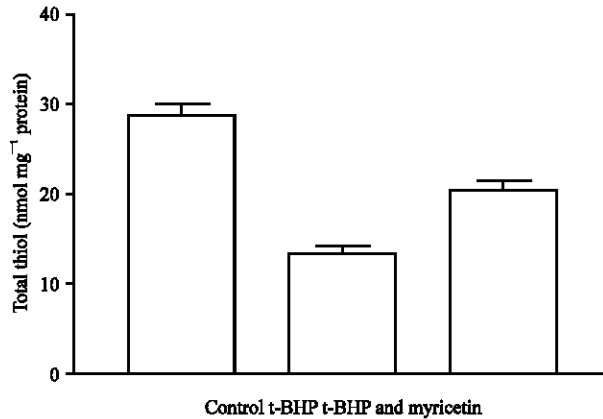

Fig. 4: Effect of myricetin on erythrocyte membrane thiol group of oxidatively stressed RBCs
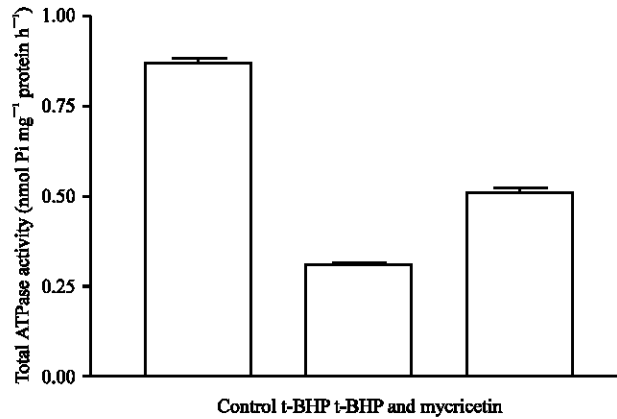

Fig. 5: Effect of myricetin on erythrocyte membrane total ATPase of oxidatively stressed RBCs
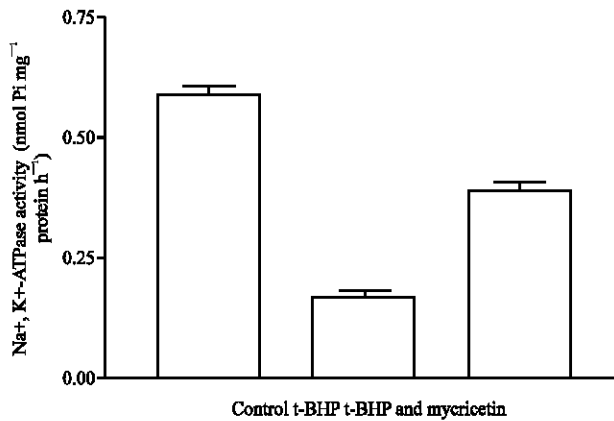

Fig. 6: Effect of myricetin on erythrocyte membrane Na-K ATPase of oxidatively stressed RBCs

Table 1: Effect of incubation with t-BHP alone and t-BHP and myricetin on the variables (*******p<0.05)

Variables	Before incubation with t-BHP	After incubation with t-BHP only	After incubation with t-BHP and myricetin
Protein carbonyl (nM mg^{-1} protein)	1.50±0.40	6.20±0.70**	2.70± 0.40*
Lipid peroxide (nM mg^{-1} protein)	0.43± 0.01	5.80±0.68*,**	2.10± 0.42*
Total thiol (nM mg^{-1} protein)	28.7±5.40	13.4±3.20*,*	20.4±4.10*
Total ATP-ase (μM Pi mg^{-1} protein h^{-1})	0.87±0.05	0.31±0.02*,*	0.51±0.05*
Na$^+$, K$^+$ ATP-ase activity (μM Pi mg^{-1} protein h^{-1})	0.59±0.07	0.17±0.05*,*	0.39±0.06*

*, ** Significant at p<0.05 and <0.001

most affected parameter by oxidative stress was lipid peroxides which increased by about 13.5 folds followed by protein carbonyl; the increase was about 4.1 folds, then Na$^+$, K$^+$ ATP-ase activity; the decrease was about 3.4 folds and finally total thiol group the decrease was about 2.14 folds (Table 1).

DISCUSSION

This investigation revealed an antioxidant protective effect of myricetin (3,5,7-Trihydroxy-2-(3, 4, 5-trihydroxyphenyl)-4-chromenone) on proteins and lipids of erythrocytes membranes subjected to oxidative stress, as it decreased the formation of protein carbonyl, lipid peroxides and caused an increase in total thiol group and Na$^+$, K$^+$ - ATPase activity.

Plant flavonoids including myricetin are powerful therapeutic agents, effective against free radical mediated diseases. Flavonoids can bind to tryptophan residues in erythrocytes membranes ghosts causing antioxidant and antihemolytic effects (Chaudhuri *et al.*, 2007). In accordance with the results of this study, several reports showed the protective effect of flavonoids on erythrocyte membrane lipids, proteins and thiols (Coskun *et al.*, 2005; Rizvi and Mishra, 2009; Pandey and Rizvi, 2010).

Flavonoids intake was inversely related to the mortality from congestive heart disease and the incidence of myocardial infarction. The protection offered by flavonoids was found to be due to their antioxidant activity. The aromatic rings of the flavonoid molecule allow the donation and acceptance of electrons from free radical species (Suwalsky *et al.*, 2009). In addition to reacting with free radicals, flavonoids are able to regenerate the traditional antioxidant vitamins, C and E (Vinson *et al.*, 1995).

Myricetin may act as a hydrogen donor and can suppress free radical processes at three stages, the formation of superoxide ion, the generation of hydroxyl radicals in the fenton reaction and the formation of lipid radicals (Moridani *et al.*, 2003; Rice-Evans *et al.*, 1996). It may also suppress lipid peroxidation by regenerating other antioxidants, such as α-tocopherol, through reduction of α-tocopheroxyl radicals (Rice-Evans *et al.*, 1996). The activity of myricetin against free radicals is due to the presence of O-dihydroxy (catechol) structure of the B ring, the 2,3-double bond in conjugation with a 4-oxo-function in ring C and both of 7- and 5-additional hydroxyl groups in ring A (Fig. 1) (Khanduja and Bhardwaj, 2003) Erythrocytes and erythrocytes membranes are more susceptible to oxidation due to their continuous exposure to high oxygen tension. Erythrocyte membranes are an excellent model for membrane studies because of the simplicity, availability and ease of isolation of them (Kolanjiappan *et al.*, 2002).

Protein oxidation occurs upon attack of ROS forming carbonyl groups. The amino acids; lysine, arginine, proline and histidine are the most liable to modification. (Lemarechal *et al.*, 2006). The accumulation of protein carbonyls w as found to be associated with a number of diseases, such as lateral sclerosis, Alzheimer's disease, respiratory distress syndrome, muscular dystrophy and

rheumatoid arthritis (Dalle-Donne *et al.*, 2003) Lipid peroxidation have been shown to cause profound alterations in the structural organization and functions of the cell membrane-bound enzymes and loss of essential fatty acids (Lam *et al.*, 2007).

Thiols act as antioxidants reacting with ROS, so protect cells against the damage induced by them. The intracellular and extracellular redox states of thiols play a an important role in keeping protein structure and function, regulation of enzymatic activity of transcription factors and antioxidant protection (Wloodek, 2002).

Na^+, K^+-ATPase (EC 3.6.1.3), is a member of P-type family of active cation transport proteins. It transports excessive Na^+ ions out from the cells as it transports three Na^+ ions out of the cell and two K^+ ions into the cell using the energy derived from hydrolysis of one molecule of ATP (Vlkovicova *et al.*, 2008).. The stable ion content is needed for normal physiological activity. ATPases are among the enzymes that can be attacked by ROS leading to decreased enzyme activity (Rodrigo *et al.*, 2007). In accordance with the protective effect of myricetin on Na^+, K^+-ATPase found in this study, (Lam *et al.*, 2007), showed a protective effect of polyphenolic compounds present in dietary plants on Na^+, K^+-ATPase of erythrocytes membranes (Lam *et al.*, 2007). From the previous study, we concluded that myricetin (3,5,7-Trihydroxy-2-(3,4,5-trihydroxyphenyl)-4-chromenone) can protect proteins and lipids of erythrocyte membranes, so can be used as an additional supplementation for protection against oxidative stress and other pathologies, such as atherosclerosis, ischemia, inflammation, cancers, cardiovascular and neurological diseases.

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