Indian Journal of Clinical Biochemistry, 2004, 19 (2) 103-110

EVALUATION OF ANTIOXIDANT PROPERTIES OF BERRIES

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ABSTRACT

The present paper focuses on assessing the levels of various enzymatic and non-enzymatic antioxidants in selected berries of Indian sub-continent viz., gooseberry, grapes, orange and tomato. This study has revealed that orange, tomato and grapes possess predominant quantities of enzymatic antioxidants namely SOD, catalase and glutathione peroxidase respectively. The levels of antioxidants analyzed namely reduced glutathione, vitamin C and vitamin A were maximal in grapes, orange and tomato respectively. All the extracts of berries included in the present study inhibited iron induced *in vitro* lipid peroxidation in erythrocytes, the extend of inhibition being highest for gooseberry and orange compared to tomato and grapes which could be attributed to their in-built antioxidant system.

KEY WORDS

Free radicals, oxidative stress, naturally occurring antioxidants, berries, lipid peroxidation.

INTRODUCTION

Reactive Oxygen Species (ROS) denote a collection of oxygen radicals ($O_2^{\cdot-}$, $OH^{\cdot}$), and some derivatives of oxygen like H_2O_2 and singlet oxygen. ROS are generated during the normal metabolism of eukaryotic cells, which involve mitochondrial electron transport, microsomal P_{450} and other systems. Controlled liberation of ROS has been advantageous to the systems like blastocyst implantation (1), iodination of tyrosine in the thyroxine biosynthesis (2), and mucous secretion in goblet cells (3). Detrimental effects caused by ROS also occur as a consequence of imbalance between the formation and inactivation of these species (4). ROS mediated oxidative damage to macromolecules namely lipids, proteins and DNA have been implicated in the pathogenecity of major diseases such as cancer, rheumatoid arthritis, post-ischemic reperfusion injury, degeneration process of aging, myocardial infarction, cardiovascular disease etc. (5, 6).

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The antioxidant enzymes and free radical scavengers may provide a defensive mechanism against the deleterious actions of ROS. Some of the antioxidant enzymes that are found to provide a protection against the ROS are superoxide dismutase, catalase, peroxidase, glutathione peroxidase (GPx), glucose-6-phosphate dehydrogenase and ascorbate oxidase (7). The non-enzymatic antioxidants which act as scavengers are glutathione, vitamin A, vitamin E, and vitamin C (8). The antioxidants may be of either the natural ones or the synthetic ones. Commonly used synthetic antioxidants are: butylated hydroxy anisole (BHA), butylated hydroxy toluene (BHT), propyl gallate and tertiary butyl hydroquinone (9). While the naturally occurring antioxidants like vitamins are a balanced mixture of redox with reduced and oxidized form, the synthetic antioxidants are unbalanced in this respect and they themselves produces harmful free radicals in some cases, emphasizing the importance of the naturally occurring antioxidants over the synthetic ones (10).

Polyhydroxy flavones, flavanones, flavanols, iso-flavones, chalcones and many members of these groups of natural substances proved to have a high degree of antioxidant activity and they are found to be wide spread in plant material (11). *Proanthocyanidins* are a special class of flavanoids exhibiting potent antioxidant properties. Some of the important and well-established antioxidants from plant sources are curcumin from turmeric (12),

eugenol from clove (13), and thymol from thyme (13). Among the plant materials, fruits, vegetables and spices are reported to be rich in compounds with antioxidants (14, 15).

The antioxidant potential of the berries has not been evaluated so far. Thus, the main objective of the study is :

- to evaluate the enzymatic and non-enzymatic antioxidants of selected berries namely gooseberry (*Emblicus officialis*), grapes (*Vitis vinifera*), orange (*Citrus sinensis*) and tomato (*Lycopersicon esculentum*).
- to study the efficacy of antioxidant potential of these berries using erythrocyte membrane as a model system

MATERIALS AND METHODS

Plant sample extraction

Each of the four berries (gooseberry, grapes, orange and tomato) taken for the study are purchased freshly from the local vegetable market and stored under refrigerated condition till use. The samples were prepared by grinding one gram each of the berries in 2 ml of 50% ethanol, separately, in a pre-chilled mortar and pestle and the extracts were centrifuged at 10,000 g at 4°C for 10 minutes. The supernatants thus obtained were used within four hours for various enzymatic and non-enzymatic antioxidants assays.

Assay of superoxide dismutase (SOD) activity

The assay of superoxide dismutase was done according to the procedure of Das *et al.* (16). In this method, 1.4ml aliquots of the reaction mixture (comprising 1.11 ml of 50 mM phosphate buffer of pH 7.4, 0.075 ml of 20 mM L-Methionine, 0.04ml of 1% (v/v) Triton X-100, 0.075 ml of 10 mM Hydroxylamine hydrochloride and 0.1ml of 50 mM EDTA) was added to 100 µl of the sample extract and incubated at 30°C for 5 minutes. 80 µl of 50 µM riboflavin was then added and the tubes were exposed for 10 min to 200 W-philips fluorescent lamps. After the exposure time, 1ml of Greiss reagent (mixture of equal volume of 1% sulphanilamide in 5% phosphoric acid) was added and the absorbance of the color formed was measured at 543 nm. One unit of enzyme activity was measured as the amount of SOD capable of inhibiting 50% of nitrite formation under assay conditions.

Assay of catalase activity

Catalase activity was assayed by the method of Sinha (17). The enzyme extract (0.5 ml) was added to the reaction mixture containing 1ml of 0.01 M phosphate buffer (pH 7.0), 0.5 ml of 0.2 M H_2O_2, 0.4 ml H_2O and incubated for different time period. The reaction was terminated by the addition of 2 ml of acid reagent (dichromate/acetic acid mixture) which was prepared by mixing 5% potassium dichromate with glacial acetic acid (1:3 by volume). To the control, the enzyme was added after the addition of acid reagent. All the tubes were heated for 10 minutes and the absorbance was read at 610 nm. Catalase activity was expressed in terms of µmoles of H_2O_2 consumed/min/mg protein.

Assay of peroxidase activity

The assay was carried out by the method of Addy and Goodman (18). The reaction mixture consisted of 3ml of buffered pyrogallol [0.05 M pyrogallol in 0.1 M phosphate buffer (pH 7.0)] and 0.5 ml of 1% H_2O_2. To this added 0.1 ml enzyme extract and O.D. change was measured at 430 nm for every 30 seconds for 2 minutes. The peroxidase activity was calculated using an extinction coefficient of oxidized pyrogallol (4.5 litres/mol).

Assay of glutathione peroxidase (GPx) activity

Glutathione peroxidase was assayed according to the procedure of Rotruck *et al.* (19) with some modifications. The reaction mixture consisting of 0.4 ml of 0.4 M sodium phosphate buffer (pH 7.0), 0.1 ml of 10mM sodium azide, 0.2 ml of 4 mM reduced glutathione, 0.1 ml of 2.5 mM H_2O_2, 0.2 ml of water and 0.5 ml of enzyme was incubated at 0, 30, 60, 90 seconds respectively. The reaction was terminated with 0.5 ml of 10% TCA and after centrifugation, 2 ml of the supernatant was added to 3 ml of phosphate buffer and 1ml of DTNB reagent (0.04% DTNB in 1% sodium citrate). The color developed was read at 412 nm and the enzyme activity is expressed in terms of µg of glutathione utilized/min/mg protein.

Assay of ascorbate oxidase activity

Assay of ascorbate oxidase activity was carried out according to the procedure of Vines and Oberbacher (20). The sample was homogenized [1 : 5 (w/v)] with phosphate buffer (0.1 M/ pH 6.5) and centrifuged at 3000 g for 15 min at 5°C. The supernatant obtained was used as enzyme source. To 3.0 ml of the substrate solution (8.8 mg ascorbic acid in 300 ml phosphate buffer, pH 5.6), 0.1 ml of the enzyme extract was added and the absorbance change at 265 nm was measured for every 30 seconds for a period of 5 minutes. One enzyme unit is equivalent to 0.01 O.D. change per min.

Assay of glucose-6-phosphate dehydrogenase activity

The enzyme was assayed by the method of Balinsky and Bernstein (21). The solution containing 0.1ml each of 0.1 M Tris-HCl buffer (pH 8.2), 0.2 mM NADP and 0.1M $MgCl_2$ was taken in a cuvette along with 0.5 ml of water and suitable aliquots of enzyme extract. The reaction was initiated by the addition of 0.1 ml of 6 mM glucose-6-phosphate and O.D. increase was measured at 340 nm. The activity of the enzyme is expressed in terms of units/mg protein, in which one unit is equal to the amount of the enzyme that brought about an increase in O.D of 0.01/min.

Estimation of reduced glutathione

The amount of reduced glutathione in the samples was estimated by the method of Boyne and Ellman (22). 1ml of the sample extracts were treated with 4.0 ml of metaphosphoric acid precipitating solution (1.67 g of glacial metaphosphoric acid, 0.2 g EDTA and 30 g NaCl dissolved in 100ml water). After centrifugation, 2.0 ml of the protein-free supernatant was mixed with 0.2 ml of 0.4 M Na_2HPO_4 and 1.0 ml of DTNB reagent (40 mg DTNB in 100 ml of aqueous 1% tri sodium citrate). Absorbance was read at 412 nm within 2 minutes. GSH concentration was expressed as nmol/mg protein.

Quantification of vitamins

The determination of ascorbic acid was carried out by the procedure given by Sadasivam and Manickam (23). The assay mixture for vitamin C consisted of 0.1 ml of brominated sample extract, 2.9 ml of distilled water, 1 ml of 2% DNPH reagent and 1-2 drops of thiourea. After incubation at 37°C for 3 h, the orange-red osazone crystals formed were dissolved by the addition of 7 ml of 80% sulphuric acid and absorbance was read at 540 nm after 30 minutes. Vitamin C concentration was expressed in terms of mg/g tissue. The vitamin E and A were estimated by the methods as given in Varley *et al.* (24).

Isolation of RBC membrane

The erythrocyte membrane was isolated according to the procedure of Dodge *et al.* (25) with a change in buffer according to Quist (26). Blood was collected using EDTA as anticoagulant. It was centrifuged and plasma was aspirated. Blood cells were washed three times with 0.89% saline and the supernatant was removed. To 0.5 ml of cells, added 7 ml of ice-cold distilled water and left overnight in a freezer. Centrifuged in a refrigerated centrifuge for 20 minutes and removed the upper layer, which is the hemolysate. The pellet was washed twice with distilled water and centrifuged each time for 10 minutes. The pellet of the erythrocyte was resuspended in a known volume of tris-HCl buffer (0.1 M, pH 7.4). Aliquots of this reconstituted membrane preparation was used for experiments.

Assay of malondialdehyde (MDA)

Lipid peroxidation was estimated in terms of thiobarbituric acid reactive species (TBARS), using malondialdehyde (MDA) as standard by the method of Buege and Aust (27). 1.0 ml of the sample extract was added with 2.0 ml of the TCA-TBA-HCl reagent (15% (w/v) TCA, 0.375% (w/v) TBA and 0.25N HCl). The contents were boiled for 15 minutes, cooled and centrifuged at 10,000 g to remove the precipitate. The absorbance was read at 535 nm and the malondialdehyde concentration of the sample was calculated using extinction coefficient of 1.56×10^5 M^{-1} Cm^{-1}.

Assay of diene conjugates

Membrane diene conjugates were assayed by the method of Buege and Aust (27). Membrane solution (1.0 ml) was mixed thoroughly with 5.0 ml of chloroform: methanol (2:1) followed by centrifugation at 1000 g for 15 min to separate the phases. The chloroform layer was placed in a test tube and taken to dryness in 45°C water bath under a stream of nitrogen. The lipid residue was dissolved in 1.5 ml of cyclohexane and the absorbance at 233 nm was determined against a cyclohexane blank. The amount of hydroperoxides produced was calculated using molar coefficient of 2.52×10^4 M^{-1}.

The values of all biochemical parameters are expressed as mean ± S.D. and the data were statistically analysed by the method of Snedecor and Cochran (28).

RESULTS AND DISCUSSION

Enzymes of peroxide metabolism

The inevitable generation of ROS in biological system and the oxidative damage is counterpoised by an array of enzymatic defense system. The levels of antioxidant enzymes assessed in different berries are collectively represented in Table 1. The superoxide dismutase activity was observed to be maximum in orange (13.24 units/mg protein) and minimum in grapes (2.62 units/mg protein). The activity decreased in tomato and gooseberry by 2.5 folds compared to orange. Superoxide scavenging effect of alcoholic extracts of *Emilia sonchifolia* (29) and *Hypericum perforatum* (30) were reported earlier.

Table 1. Enzymatic antioxidants in selected berries

Type of berries	SOD		Catalase		Peroxidase		Glutathione peroxide		Ascorbate oxidase		Glu-6-PO_4 DHase	
	Unit/ mg protein	Unit/ g tissue	Unit/ mg protein	Unit/ g tissue	Unit/ mg protein	Unit/ g tissue	Unit/ mg protein	Unit/ g tissue	Unit/ mg protein	Unit/ g tissue	Unit/ mg protein	Unit/ g tissue
Goose berry	4.60b	21.02b	84.27c	385.11d	0.85b	3.88c	443.13b	2025.10c	0.004a	0.018b	4.54c	20.75c
Grapes	2.62a	6.58a	77.13b	193.59b	0.67a	1.68a	1274.02d	3197.79d	0.002a	0.005a	1.64a	4.20a
Orange	13.24d	30.45c	46.32a	106.54a	0.82b	1.89b	424.48a	976.30a	0.020c	0.046c	14.28d	32.94d
Tomato	5.58c	6.91a	278.14d	344.89c	6.40c	7.94d	628.82c	779.74b	0.006b	0.007a	4.38b	5.43b
SED	0.13		0.15		0.06		5.49		0.0009		0.07	
LSD (5%)	0.27		0.32		0.12		11.45		0.002		0.15	
LSD (1%)	0.37		0.43		0.17		15.61		0.003		0.20	
	1 Unit = inhibition of 50% nitrite formation		1 Unit = 1 µmole of H_2O_2 consumed/ min		1 Unit = 1 µmole pyrogallol oxidised/min		1 Unit = µg of GSH utilised/ min		1 Unit = 0.01 O.D. change/ min		1 Unit = increase in O.D. of 0.01/min	

In a column means followed by a common letter are not significantly different at the 5% level by DMRT. Values are three replicates of two repetitive experiments.

Among the four species, the highest activity of catalase was observed in tomato (278.14 mg protein) and lowest in orange (46.32 units/mg protein). The activity decreased by 3.5 times in gooseberry and grapes when compared to tomato. Peroxidase activity was found to be very high in tomato (6.4 units/mg protein/min). However, the activity in other three berries ranges from 0.7 - 0.85 units/mg protein. In plants, antioxidant enzymes namely catalase (31) and peroxidase (32) have been shown to increase when subjected to stress conditions.

The glutathione peroxidase was observed to be low in orange and gooseberry (424.48 - 443.13 units/mg protein), while the activity increased by two fold in tomato and four fold in grapes. It has been well established that, GPx a selenium enzyme, plays a major role in regulating the concentration of H_2O_2 and a wide variety of organic peroxides (33). The presence of very high activity of GPx in grapes coincides with low activity of catalase and peroxidase.

The ascorbate oxidase activity was highest in orange (0.02 units/mg protein), and the activity in other three fruits studied was in the range of 0.002-0.006 units/mg protein. The predominant ascorbate oxidase activity in orange is associated with high SOD activity and low activity of catalase, peroxidase and glutathione peroxidase, emphasizing the importance of ascorbate system in orange species.

The glucose-6-phosphate-dehydrogenase activity was maximum in orange (14.28 units/mg protein) and minimum in grapes, while gooseberry and tomato showed an activity around 4.5 units/mg protein. It is interesting to note that the high activity of glucose-6-phosphate dehydrogenase noted in orange is also associated with very high activity of ascorbate oxidase compared to other berries under study. Since NADPH is an ultimate reducing equivalent utilized by dehydroascorbate reductase to maintain the balance between dehydroascorbate and ascorbate through GSH-GSSG system, the increase in ascorbate oxidase activity in orange has been compensated with increase in glucose-6-phosphate dehydrogenase activity in the same.

Non-enzymatic antioxidants

The concentration of different non-enzymatic antioxidants in berries were also assessed and the results are represented in Table 2. Reduced glutathione was found to be maximum in grapes (628.57nM/g tissue), while in gooseberry and orange, it ranges form 480 - 580 nM/g tissue. GSH levels in tomato decreased about two times when compared to that of grapes. In plants, GSH concentration is reported to be higher in chloroplasts (4 mM) and a significant quantity also accumulates in cytosol (34). In plants, the role of

Table 2. Non-enzymatic antioxidants in selected berries

Type of berries	Reduced Glutathione (nM/g tissue)	Vitamin C (mg/g tissue)	Vitamin E (mg/g tissue)	Vitamin A (µg/g tissue)	
				Retinol	β-carotene
Goose berry	582.78 c	41.62 c	0.019 b	30.35 b	6.05 b
Grapes	628.57 d	1.70 a	0.026 c	18.06 a	2.36 a
Orange	484.95 b	57.31 d	0.009 a	34.68 c	12.0 c
Tomato	383.23 a	26.09 b	0.017 b	38.24 d	20.5 d
SED	5.36	0.20	0.003	0.1	
LSD (5%)	12.01	0.42	0.007	0.42	
LSD (1%)	16.38	0.58	0.009	0.32	

In a column, means followed by a common letter are not significantly different at the 5% level by DMRT.

Values are means of three replicates in two repetitive experiments

Table 3. Effect of Berry Extracts on Lipid peroxidation and reduced GSH levels in the erythrocytes

Experimental group	TBARS* (µM/mg protein)	Conjugated dienes* (mmole/mg protein)	GSH** (mM/mg protein)
Group I RBC membrane	0.124±0.0039	21.67±0.137	28.31±0.0052
Group II RBC membrane + $FeSO_4$	0.220±0.0052	30.31±0.137	6.60±0.0850
Group III RBC membrane + $FeSO_4$ + Extract			
Gooseberry	0.127±0.0039	20.53±0.277	13.21±0.086
Grapes	0.139±0.0042	20.34±0.158	12.55±0.106
Orange	0.130±0.0060	22.39±0.218	13.38±0.128
Tomato	0.141±0.0027	24.63±0.156	12.61±0.107

* The levels of TBARS and conjugated dienes were compared between Group II and Group III

** GSH level was compared between Group I and Group III

The values are means of three replicates in two repetitive experiments.

glutathione as radical scavenger, membrane stabilizer (35) and precursor of heavy metal binding peptides (36) are well documented. In the present study, high levels of glutathione and glutathione peroxidase activity observed in grapes is a notable result keeping in view that GPx is not being reported in many plant species.

Vitamin C content was very low in grapes (1.7 mg/g tissue), whereas in gooseberry and orange its concentration ranges from 40-57 mg/g tissue. Ascorbate has been found in the chloroplast, cytosol, vacuole and extracellular compartments of the plant cells and shown to function as a reductant for many free radicals (37). Gooseberry and tomato showed no significant difference with each other in the vitamin E content (0.020 µg/g tissue) while orange showed the lowest level of 0.009 µg/g tissue. Tocopherol levels have been documented extensively in plant tissue (38), and its concentration shown to vary among plant tissues from 200 ng/g fresh weight (fw) in potato tubers to 5 mg/g (fw) in

oil palm leaflets. The antioxidant properties of tocopherol are the result of its ability to quench both singlet oxygen and peroxides (39). Grapes showed a low vitamin A content of 18.06 µg/g tissue whereas other berries showed a two fold increase (30-38 µg/g tissue). Similar trend was also noticed in β-carotene content. Vitamin A and related retinols have been reported to increase the immunity to tumors, which has been exploited in the treatment of HIV infection (40).

Protective Role Of Berry Extracts Upon Induction Of Lipid Peroxidation In Erythrocytes

Erythrocytes are prone to oxidative damage due to presence of polyunsaturated fatty acids, heme, iron and oxygen (41). Hence, for the present study, to study the effectiveness of antioxidants in berries, human erythrocyte was considered as a model system. The basal lipid peroxidation of RBC membrane was found to be 0.124 µM/mg protein as measured by malondialdehyde method enhanced two fold in presence of inducer. All the extracts of berries used in the present study inhibited lipid peroxidation. While gooseberry and orange were more effective in curtailing lipid peroxidation (41% inhibition), tomato and grapes were found to be less effective in this aspect.

Upon the induction of lipid peroxidation in the RBC membrane, the GSH level decreased significantly by four fold. When RBC membranes were pre-incubated with different berry extracts, the GSH content was lowered by only 18 - 25%, indicating the effectiveness of these extracts in maintaining the level of glutathione, thereby protecting the membrane (Table 3). The extracts of *Capparis deciduas* (42) and *Tinospora cordifolia* (43, 44) were shown to decrease alloxan induced lipid peroxidation in erythrocytes and organs of rat by improving its antioxidant status. *Emblica officinalis* tannoids have recently been reported to enhance ROS scavenging activity in rat brain by enhancing the concentration of the antioxidant enzymes (45).

To summarize, in the present study, among the enzymatic antioxidants studied in berries, SOD in orange, catalase in tomato, peroxidase in grapes were predominant. Among the non-enzymatic antioxidants studied in berries, reduced glutathione in grapes, vitamin C in orange and vitamin E in tomato were predominant antioxidants. The ability of the extracts of these berries in curtailing the *in vitro* lipid peroxidation of RBC membrane with concomitant increase in GSH can be attributed to the presence of various enzymic and non-enzymic antioxidants in these berries. The present study clearly points out the antioxidant potential of these fruits emphasizing the importance of incorporating these fruits as a regular component in diet. These berries could also be exploited for commercial purification of specific antioxidants, since they are available in abundance.

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