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Antioxidant and Radical Scavenging Activities of Uric Acid

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Uric acid is the important plasma antioxidant that can protect cells from damage by reactive oxygen species. It is the major nitrogenous compound in urine, but it is also found in other biological fluids such as serum, blood and saliva. The aim of this research was to examine the antioxidant and radical scavenging activities of uric acid. Several mechanisms of potential antioxidant and radical scavenging activities of uric acid including 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical ($DPPH^{\bullet}$) scavenging, total antioxidant activity, reducing activity, hydrogen peroxide (H_2O_2) scavenging and metal chelating activitiy on Fe^{2+} ions were examined as *in vitro* assays. Total antioxidant activity was measured according to ferric thiocyanate method. α-Tocopherol and trolox, a water-soluble analogue of tocopherol, were used as the reference antioxidant compounds. Uric acid neutralized the activities of radicals and inhibited the peroxidation reactions of linoleic acid emulsion. Uric acid showed 67.7 % inhibition on lipid peroxidation of linoleic acid emulsion, at the 20 μg/mL concentration. However, α-tocopherol and trolox exhibited 54.7 and 20.1 % inhibition on peroxidation of linoleic acid emulsion, respectively, at the above mentioned concentration. Uric acid had also effective $DPPH^{\bullet}$, $ABTS^{\bullet+}$ scavenging, H_2O_2 scavenging, total reducing power and metal chelating on Fe^{2+} ions activities.

Key Words: Uric acid, Antioxidant activity, Radical scavenging, Lipid peroxidation.

INTRODUCTION

Uric acid, an end product of purine metabolism, produced within peroxisomes and excreted in the urine. It seems to be a potent antioxidant that can protect cells from damage by reactive oxygen species[1]. In addition, it is the important plasma antioxidant[2] and free radical scavenger[3-5] and peroxynitrite scavenger[6]. It is also a paradoxical redox-active molecule, in

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that it is an antioxidant and free radical scavenger, but when it is formed by xanthine oxidaze, superoxide anion radicals are formed in the process[7]. Furthermore, uric acid is the major nitrogenous compound in urine, but it is also found in other biological fluids such as serum, blood and saliva. It is the main final product of purine metabolism and its determination in urine is a powerful indicator of metabolic alterations or disease appearance[8].

Biological free radical reactions are involved in the reduction of molecular oxygen to yield reactive oxygen species (ROS)[9-11]. The uncontrolled production of ROS and the unbalanced mechanism of antioxidant protection result in the onset of many diseases and accelerate ageing. ROS include free radicals such as superoxide anion radicals ($O_2^{\bullet -}$), hydroxyl radicals ($OH^{\bullet}$) and non free-radical species such as H_2O_2 and singlet oxygen ($^1O_2$). They are various forms of activated oxygen[12-14]. There is a balance between the generation of ROS and inactivation of ROS by the antioxidant system in organisms. In case of the imbalance between ROS and antioxidant defence mechanisms, ROS leads to oxidative modification in cellular membrane or intracellular molecules[15-17]. Under oxidative stress or pathological conditions, ROS are overproduced and result peroxidation of membrane lipids, leading to the accumulation of lipid peroxides[18,19]. However, they are removed by antioxidant defence mechanisms. Antioxidants are considered as possible protection agents reducing oxidative damage of human body from ROS and retard the progress of many chronic diseases as well as lipid peroxidation[20-23].

There is currently great interests in free radical mediated damage associated with many diseases and much effort has gone into the study of antioxidant substances[24]. The main objectives of the present study were to assess the *in vitro* antioxidant potential of uric acid in different antioxidant such as 1,1-diphenyl-2-picryl-hydrazyl (DPPH) free radical scavenging, 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging, total antioxidant activity by ferric thiocyanate method, reducing power, hydrogen peroxide scavenging and metal chelating activities.

EXPERIMENTAL

Uric acid, 2,2-azino-*bis*(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl ($DPPH^{\bullet}$), linoleic acid, 3-(2-pyridyl)-5,6-*bis*(4-phenyl-sulfonic acid)-1,2,4-triazine (ferrozine), 6-hydroxy-2,5,7,8-tetramethylchro-man-2-carboxylic acid (trolox), α-tocopherol, polyoxyethylenesorbitan monolaurate (Tween-20) and trichloroacetic acid (TCA) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate was purchased from Merck. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Ferric thiocyanate method-total antioxidant activity: The antioxidant activity of uric acid was determined according to the ferric thiocyanate method in linoleic acid emulsion[25]. The solution, which contains different concentration of stock uric acid solution samples (10-20 μg/mL) in 2.5 mL of potassium phosphate buffer (0.04 M, pH 7.0), was added to 2.5 mL of linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution (5 mL) was incubated at 37 ºC in glass flask. At regular intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 3.7 mL of ethanol, followed by the addition of 0.1 mL of 30 % ammonium thiocyanate and 0.1 mL of 20 mM ferrous chloride in 3.5 % hydrochloric acid. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer (CHEBIOS s.r.l. UV-Vis spectrophotometer). During the linoleic acid oxidation, peroxides are formed, which oxidize Fe^{2+} to Fe^{3+}. The latter ions form a complex with thiocyanate and this complex has a maximum absorbance at 500 nm. These steps were repeated every 12 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid emulsion oxidation. Solutions without added samples were used as blanks. All data on total antioxidant activity are the average of duplicate experiments. The percentage inhibition of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

Inhibition of lipid peroxidation (%) = $100-[(A_{Sample}/A_{Control}) \times 100$

where $A_{Control}$ is the absorbance of the control reaction and A_{Sample} is the absorbance in the presence of the sample of uric acid or standard compounds[26,27].

Ferric ions (Fe^{3+}) reducing antioxidant power assay (FRAP): The samples prepared for ferric thiocyanate method was used for this and the other antioxidant assays. The reducing activity of uric acid was determined by the method of Oyaizu[28]. The capacity of uric acid to reduce the ferric-ferricyanide complex to the ferrous-ferricyande complex of Prussian blue was determined by recording the absorbance at 700 nm after incubation. For this purpose, different concentrations of uric acid (10-20 μg/mL) in 1 mL of distilled water were mixed with phosphate buffer (2.5 mL, 0.2 M, pH 6.6) and potassium ferricyanide [$K_3Fe(CN)_6$] (2.5 mL, 1 %). The mixture was incubated at 50 ºC for 20 min. Aliquots (2.5 mL) of trichloroacetic acid (10 %) were added to the mixture. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1 %) and the absorbance was measured at 700 nm in a spectrophotometer.

Metal chelating activity: Ferrous ion (Fe^{2+}) chelation by uric acid was estimated by the ferrozine assay[29]. For this purpose, uric acid (10 μg/mL) in 0.4 mL was added to a solution of 2 mM $FeCl_2$ (0.05 mL). The

reaction was initiated by the addition of 5 mM ferrozine (0.2 mL) and total volume was adjusted to 4 mL ethanol. Then, the mixture was shaken vigorously and left standing at room temperature for 10 min. After the mixture had reached equilibrium, the absorbance of the solution was then measured spectrophotometrically at 562 nm. The results were expressed as percentage of inhibition of the ferrozine-Fe^{2+} complex formation. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was calculated using the formula given bellow:

$$\text{Bound } Fe^{2+} (\%) = [(A_{Control}-A_{Sample})/A_{Control}] \times 100$$

where $A_{Control}$ is the absorbance of the ferrozine-Fe^{2+} complex and A_{Sample} is the absorbance in the presence of the sample of uric acid or standard compounds[30,31].

Hydrogen peroxide (H_2O_2) scavenging activity: The hydrogen peroxide scavenging ability of uric acid was determined according to the method of Ruch and co-workers[32]. A solution of H_2O_2 (40 mM) was prepared in phosphate buffer (pH 7.4). Uric acid at the 20 µg/mL concentration in 3.4 mL phosphate buffer was added to a H_2O_2 solution (0.6 mL, 40 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution contained the phosphate buffer without H_2O_2. The percentage of H_2O_2 scavenging of uric acid and standard compounds was calculated as:

$$\% \text{ Scavenged } [H_2O_2] = [(A_{Control}-A_{Sample})/A_{Control}] \times 100$$

where $A_{Control}$ is the absorbance of the control and A_{Sample} is the absorbance in the presence of the sample of uric acid or standards[33,34].

ABTS Radical cation decolorization assay: $ABTS^{\bullet+}$ scavenging activity was determined according to method of Re and co-workers[35]. The $ABTS^{\bullet+}$ cation radical was produced by the reaction between 7 mM ABTS in H_2O and 2.45 mM potassium persulfate, stored in the dark at room temperature for 12 h. Before usage, the $ABTS^{\bullet+}$ solution was diluted to get an absorbance of 0.700 ± 0.025 at 734 nm with phosphate buffer (0.1 M, pH 7.4).

Then, 1 mL of $ABTS^{\bullet+}$ solution was added 3 mL of uric acid solution in ethanol at different concentrations (10-20 µg/mL). After 0.5 h, the percentage inhibition of absorbance at 734 nm was calculated for each concentration relative to a blank absorbance (ethanol). All determinations were carried out at least three times. The $ABTS^{\bullet+}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression ($r^2 = 0.9922$):

$$\text{Absorbance} = 0.0116 \times [ABTS^{\bullet+}] + 0.0479$$

The capability to scavenge the $ABTS^{\bullet+}$ radical was calculated using the following equation:

$ABTS^{\bullet+}$ scavenging effect (%) = $[(A_{Control} - A_{Sample}/A_{Control}) \times 100]$

where in $A_{Control}$ is the initial concentration of the $ABTS^{\bullet+}$ and A_{Sample} is absorbence of the remaining concentration of $ABTS^{\bullet+}$ in the presence of uric acid or standards[36].

DPPH Free radical scavenging activity: The hydrogen atoms or electrons donation ability of uric acid was measured from the bleaching of purple coloured methanol solution of DPPH, following the methodology described by Blois[37]. The capacity of uric acid to scavenge the lipid-soluble DPPH radical, which results in the bleaching of the purple colour exhibited by the stable DPPH radical, is monitored at an absorbance of 517 nm. Basically, a 0.1 mM ethanolic solution of $DPPH^{\bullet}$ was prepared daily. Then, 1 mL of this solution was added 3 mL of uric acid solution in ethanol at different concentrations (10-20 μg/mL). After 0.5 h, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The $DPPH^{\bullet}$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression ($r^2 = 0.9974$):

$$\text{Absorbance} = 0.5869 \times 10^{-4} [DPPH^{\bullet}] + 0.0134$$

The capability to scavenge the $DPPH^{\bullet}$ radical was calculated using the following equation:

$DPPH^{\bullet}$ Scavenging effect (%) = $[(A_{Control}-A_{Sample}/A_{Control}) \times 100]$

where in $A_{Control}$ is the initial concentration of the stable DPPH radical without the test compound and A_{Sample} is ansorbence of the remaining concentration of $DPPH^{\bullet}$ in the presence of uric acid or standards[38,39].

Statistical analysis: All the analyses on total antioxidant activity were done in duplicate sets. The other analyses were performed in triplicate. The data were recorded as mean ± standard deviation and analyzed by SPSS (version 11.5 for Windows 98, SPSS Inc.). One-way analysis of variance was performed by Anova procedures. Significant differences between means were determined by LSD tests. P values < 0.05 were regarded as significant and p values < 0.01 very significant.

RESULTS AND DISCUSSION

Total antioxidant activity by ferric thiocyanate method (FTC): The effects of 20 mg/mL concentrations of uric acid on lipid peroxidation of linoleic acid emulsion are shown in Fig. 1 and were found to be 67.7 %. On the other hand, α-tocopherol and trolox, exhibited 54.7 and 20.1 % inhibition, on peroxidation of linoleic acid emulsion, respectively at the same concentration. The results clearly showed that uric acid had higher total antioxidant activity than α-tocopherol and trolox.

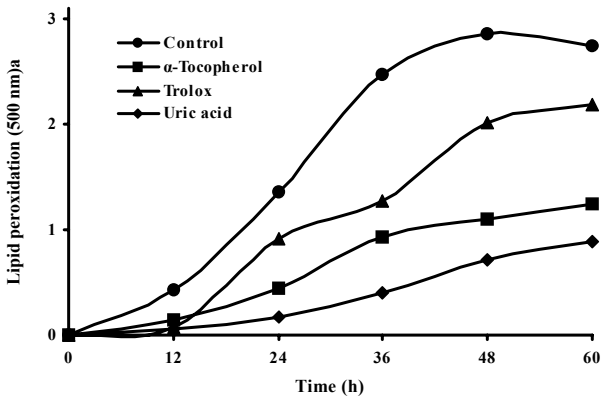

Fig. 1. Total antioxidant activities of uric acid and standards (α-tocopherol and trolox) at the same concentration (20 μg/mL)

Total reductive capability using the potassium ferricyanide reduction method: Fig. 2 depicts the reducing activity of the uric acid and standards (α-tocopherol and trolox) using the potassium ferricyanide reduction method. For the measurements of the reductive activity, the Fe^{3+}-Fe^{2+} transformation was investigated in the presence of uric acid or standards using the method of Oyaizu[28]. The reducing activity of uric acid, α-tocopherol and trolox increased with increasing concentration of samples. As can be seen Fig. 2, uric acid showed an effective reducing activity than control, at different concentrations (r^2 = 9998). These differences were statistically significant ($p < 0.01$). Reducing power of uric acid and standard compounds exhibited the following order: α-tocopherol > trolox ≅ uric acid.

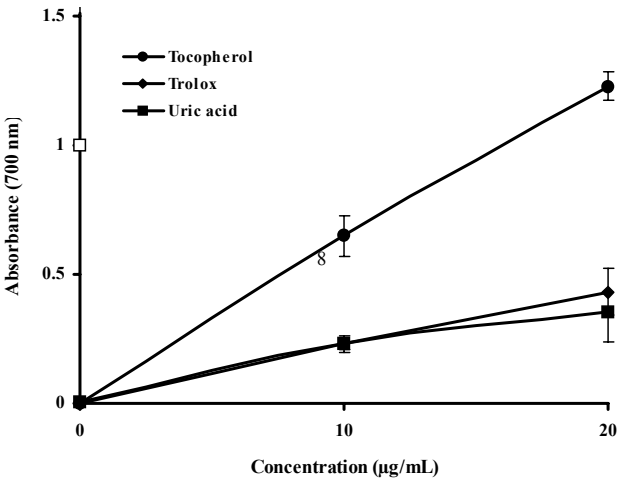

Fig. 2. Total reductive potential of different concentrations (10-20 μg/mL) of uric acid, α-tocopherol and trolox

Metal chelating capacity on ferrous ions (Fe^{2+}): Fe^{2+} ion chelating activities of uric acid, α-tocopherol and trolox are shown in Table-1. The chelating effect of Fe^{2+} ions by the uric acid was determined according to the method of Dinis *et al.*[29]. Iron, is known as the most important lipid oxidation pro-oxidant due to its high reactivity. The ferrous state of iron accelerates lipid oxidation by breaking down hydrogen and lipid peroxides to reactive free radicals *via* the Fenton reaction ($Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + OH^{\bullet}$). Fe^{3+} ion also produces radicals from peroxides, although the rate is tenfold less than that of Fe^{2+} ion. Fe^{2+} ion is the most powerful pro-oxidant among various species of metal ions[40].

TABLE-1
HYDROGEN PEROXIDE SCAVENGING AND Fe^{2+} CHELATING ACTIVITY OF URIC ACID, TROLOX AND α-TOCOPHEROL AT THE SAME CONCENTRATION (10 μg/mL)

	H_2O_2 scavenging (%)	Metal chelating (%)
Trolox	51.1 ± 6.9	48.5 ± 6.3
α-Tocopherol	69.8 ± 6.1	21.6 ± 8.7
Uric acid	41.1 ± 2.9	48.9 ± 2.4

The chelation of Fe^{2+} ion by uric acid was estimated by the ferrozine assay. Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is inhibited and the red colour of the complex fades. Measuring of the colour reduction, therefore, it is possible to estimate of the chelating activity of the co-existing chelator[41]. In this assay, the natural compound interfered with the formation of the ferrozine-Fe^{2+} complex, suggesting that it has chelating activity and captures Fe^{2+} ions before ferrozine.

In fact, as shown in Table-1, uric acid disrupted the Fe^{2+}-ferrozine complex at 10 μg/mL concentration. The difference amoung all uric acid concentration and the control was statistically significant ($p < 0.01$). In additon, uric acid exhibited 48.9 % chelation of Fe^{2+} ion at the 10 μg/mL concentration. On the other hand, the percentages of metal chelating capacity of same concentration of α-tocopherol and trolox were found 21.6 and 48.5 %, respectively. The metal scavenging effect of those samples decreased in the order of uric acid > trolox > α-tocopherol.

Hydrogen peroxide scavenging activity: The ability of uric acid to scavenge hydrogen peroxide was shown in Table-1 and compared with that of α-tocopherol and trolox as standards. Uric acid exhibited 41.1 % scavenging effect of hydrogen peroxide, at the 10 μg/mL concentration. On the other hand, α-tocopherol and trolox exhibited 69.8 and 51.1 %

hydrogen peroxide scavenging activity at the same concentration. At the above concentration, the hydrogen peroxide scavenging effect of uric acid and standards decreased in the order of α-tocopherol > trolox > uric acid.

$ABTS^{\bullet+}$ Radical scavenging activity: As seen in Fig. 3, uric acid had $ABTS^{\bullet+}$ radical scavenging activity in a concentration-dependent manner (10-20 μg/mL, $r^2 = 9998$). There is a significant decrease ($p < 0.01$) in the concentration of $ABTS^{\bullet+}$ due to the scavenging capacity of uric acid and standards. In addition, the scavenging effect of uric acid and standards on the $ABTS^{\bullet+}$ decreased in that order: trolox > α-tocopherol > uric acid, which were 94.8, 89.7 and 42.8 %, at the concentration of 50 μg/mL, respectively.

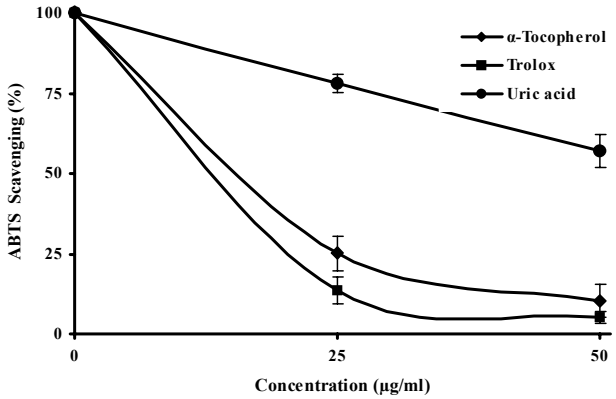

Fig. 3. Scavenging effect of uric acid, α-tocopherol and trolox on the stable $ABTS^{\bullet+}$ at different concentrations (10-20 μg/mL) [$ABTS^{\bullet+}$ = 2,2'-azino-*bis*(3-ethylbenzothiazoline-6-sulfonate) radicals]

$DPPH^{\bullet}$ Radical scavenging activity: Uric acid exhibited marked DPPH free radical scavenging activity in a concentration-dependent manner. Fig. 4 illustrates a significant decrease ($p < 0.05$) in the concentration of DPPH radical due to the scavenging ability of uric acid and standards. α-Tocopherol and trolox were used as positive radical scavengers. The scavenging effect of uric acid and standards on the DPPH radical decreased in that order: α-tocopherol > uric acid > trolox, which were 85.2, 56.0 and 14.3 %, at the concentration of 20 μg/mL, respectively.

Uric acid, a major nitrogenous compound in urine, is the product of purine metabolism in human body and is related to many clinical disorders[42]. However there is no information about *in vitro* antioxidant activity of uric acid. Antioxidant activity is widely used as a parameter for medicinal bioactive components. A number of assays have been introduced for the measurement of the total antioxidant activity of pure compounds[43]. In this study, the antioxidant activity of the uric acid was compared to α-tocopherol and

its water-soluble analogue trolox. The antioxidant activity of the uric acid, α-tocopherol and trolox has been evaluated in a series of *in vitro* tests: DPPH free radical, ABTS radical scavenging, total antioxidant activity by ferric thiocyanate method, reducing activity, hydrogen peroxide scavenging activity and metal chelating activitiy. Total antioxidant activity of uric acid was determined by the ferric thiocyanate method in the linoleic acid emulsion. The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which are the primary products of oxidation. Uric acid had effective antioxidant activity.

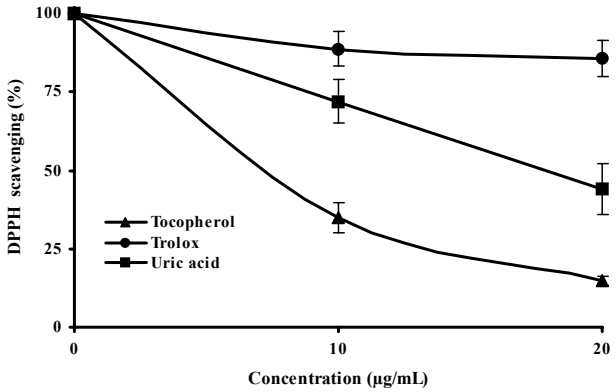

Fig. 4. Scavenging effect of uric acid, α-tocopherol and trolox on the stable DPPH$^{\bullet}$ at different concentrations (10-20 µg/mL) (DPPH$^{\bullet}$ = 1,1-Diphenyl-2-picrylhydrazyl radicals)

In the potassium ferricyanide reduction method, the yellow colour of the test solution changes to various shades of green and blue depending on the reducing power of antioxidant samples. The presence of reductants such as antioxidant substances in the antioxidant samples causes the reduction of the Fe^{3+}/ferricyanide complex to the Fe^{2+} form. Therefore, Fe^{2+} can be monitored by measuring the formation of Perl's Prussian blue at 700 nm[42].

Transition metals have a major role in the generation of oxygen free radicals in living organisms. Iron exists in two distinct oxidation states; Fe^{2+} or Fe^{3+}. The Fe^{3+} ion is the relatively biologically inactive form of iron. However, it can be reduced to the active Fe^{2+}, depending on the conditions, particularly pH and oxidized back through Fenton type reactions, with production of hydroxyl radicals or Haber-Weiss reactions with superoxide anions[44,45].

Also, the production of highly ROS such as superoxide anion radicals, hydrogen peroxide and hydroxyl radicals is also catalyzed by free iron through Haber-Weiss reaction ($O_2^{\bullet-} + H_2O_2 \rightarrow O_2 + OH^- + OH^{\bullet}$)[46]. The

production of these radicals can lead to lipid peroxidation, protein modification and DNA damage. Chelating agents may inactivate metal ions and potentially inhibit the metal-dependent processes[47].

Metal chelating capacity was significant, since it reduced the concentration of the catalyzing transition metal in lipid peroxidation. It was reported that chelating agents are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion.

Hydrogen peroxide can be formed *in vivo* by many oxidizing enzymes such as superoxide dismutase. It can cross membranes and may slowly oxidize a number of compounds. Hydrogen peroxide itself is not very reactive; however it can sometimes be toxic to cell because it may give rise to hydroxyl radical in the cells. Addition of hydrogen peroxide to cells in culture can lead to transition metal ion-dependent $OH^{\bullet}$ radicals mediated oxidative DNA damage.

Excessive formation of free radicals accelerates the oxidation of lipids in foods and decreases food quality and consumer acceptance. Hence, radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems[48].

Generation of the ABTS radical cation forms the basis of one of the spectrophotometric methods that have been applied to the measurement of the total antioxidant activity of solutions of pure substances, aqueous mixtures and beverages[48,49]. The method for the screening of antioxidant activity is reported as a decolorization assay applicable to both lipophilic and hydrophilic antioxidants, including flavonoids, hydroxycinnamates, carotenoids and plasma antioxidants. A more appropriate format for the assay is a decolorization technique in that the radical is generated directly in a stable form prior to reaction with putative antioxidants. The improved technique for the generation of $ABTS^{\bullet+}$ described here involves the direct production of the blue/green $ABTS^{\bullet+}$ chromophore through the reaction between ABTS and potassium persulfate.

Antioxidants react with $DPPH^{\bullet}$, which is a stable free radical and convert it to 1,1-diphenyl-2-picryl hydrazine. The degree of discolouration indicates the radical-scavenging potential of the antioxidant[50]. In this study, antioxidant activities of uric acid was determined using a $DPPH^{\bullet}$ method. Since the $DPPH^{\bullet}$ assay can accommodate a large number of samples in a short period and is sensitive enough to detect natural compounds at low concentrations, it was used in the present study for a primary screening of the uric acid free radical-scavenging activity. This assay provides information on the reactivity of test compounds with a stable free radical. $DPPH^{\bullet}$ gives a strong absorption band at 517 nm in visible spectroscopy. As this electron becomes paired off in the presence of a free radical scavenger, the

absorption vanishes and the resulting decolorization is stoichiometric with respect to the number of electrons taken up.

Conclusion

Uric acid is the one of important plasma antioxidant and can protect cells from damage by reactive oxygen species and reactive nitrogen species. According to data obtained from the present study, uric acid were also found to be an effective antioxidant in different *in vitro* assay including linoleic acid system-ferric thiocyanate method, reducing power, $DPPH^{\bullet}$ scavenging, $ABTS^{\bullet+}$ scavenging, hydrogen peroxide scavenging and metal chelating activities when compared to standard antioxidant compounds such as α-tocopherol, a natural antioxidant and trolox which water-soluble analogue of tocopherol.

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