informa
healthcare

Measurement of antioxidant ability of melatonin and serotonin by the DMPD and CUPRAC methods as trolox equivalent

İLHAMI GÜLÇİN

Department of Chemistry, Faculty of Science and Arts, Atatürk University, 25240 Erzurum, Turkey

Abstract
Melatonin (*N*-acetyl-5-methoxytryptamine) is the chief secretory product of the pineal gland and synthesized enzymatically from serotonin (5-hydroxytryptamine). These indoleamine derivatives play an important role in the prevention of oxidative damage. In the present study, DMPD radical scavenging and cupric ion (Cu^{2+}) reducing ability of melatonin and serotonin as trolox equivalent antioxidant activity (TEAC) was investigated. Melatonin and serotonin demonstrated 73.5 and 127.4 µg/mL trolox equivalent $DMPD^{\cdot+}$ scavenging activity at the concentration of 100 µg/mL. Also, at the same concentration, melatonin and serotonin showed 14.41 and 116.09 µg/mL trolox equivalent cupric ion (Cu^{2+}) reducing ability. These results showed that melatonin and serotonin had marked $DMPD^{\cdot+}$ radical scavenging and cupric ions (Cu^{2+}) reducing ability. Especially, serotonin had higher DMPD radical scavenging and cupric ions (Cu^{2+}) reducing activity than melatonin because of its phenolic group.

Keywords: *Melatonin, serotonin, radical scavenging, $DMPD^{\cdot+}$, Cuprac method*

Introduction

The pineal hormone melatonin (*N*-acetyl-5-methoxytryptamine), an indoleamine, is the chief secretory product of the pineal gland. It is synthesized enzymatically from serotonin (5-hydroxytryptamine) by the sequential action of serotonin *N*-acetyltransferase and hydroxyindole-*O*-methyltransferase [1]. The chemical structures of melatonin and serotonin are given in Figure 1. It is also produced in other organs and found in all body fluids after its release from the pineal. It is known that melatonin influences a variety of biological processes including circadian rhythms, neuroendocrine, cardiovascular and immune functions as well as thermoregulation [2–4]. Additionally, this molecule functions in protecting cell components such as nuclear DNA, membrane lipids, and cytosolic proteins from free radical damage [5–7].

Serotonin (5-hydroxytryptamine) has been implicated in the control of many physiological (cardiovascular, respiratory and thermoregulatory) and behavioural (feeding and sexual behaviour, circadian rhythm, sleep-wake cycle, aggression, learning and pain sensitivity) functions that could be disturbed by depression [8]. It is a biogenic amine belonging to the most common neurotransmitters in nature. Serotonin is a well-established neurotransmitter produced and activated in nervous tissues and digestive tract [9]. In humans, serotonin modifies body temperature, blood pressure, sexual behaviour and mood. It is also involved in several interactions of the immune system [10]. It was reported that serotonin has been implicated in many brain functions, including those related to pleasure and the intake of food, depression, suicide, anxiety, and Parkinson's and Alzheimer's diseases [11].

The importance of free radicals and reactive oxygen species (ROS) has attracted increasing attention over the past decade. ROS are various forms of activated oxygen which include free radicals such as superoxide anion radicals (O_2^-), hydroxyl radicals (OH·) and non free-radical species such as

Correspondence: İ. Gülçin, Department of Chemistry, Faculty of Science and Arts, Atatürk University, 25240 Erzurum, Turkey.
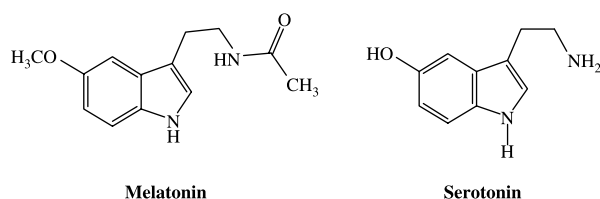Tel: 90 442 2314444. Fax: 90 442 2360948. E-mail: igulcin@atauni.edu.tr; igulcin@yahoo.com

RIGHTSLINK()

Figure 1.   The chemical structures of melatonin and serotonin.

H_2O_2 and singlet oxygen ($^1O_2$). These molecules are exacerbating factors in cellular injury and aging process [12–14]. Antioxidants can protect the human body from free radicals and ROS effects and retard the progress of many chronic diseases as well as lipid oxidative rancidity in foods [15–17].

Antioxidant and radical scavenging activities of melatonin, and its precursor serotonin, have been evaluated by different methodologies previously. In previous studies, we determined the total antioxidant activity, reducing power, DPPH radical scavenging, superoxide anion radical ($O_2^{\cdot-}$) scavenging activity, ferrous ion (Fe^{2+}) chelating and hydrogen peroxide decomposition activities of melatonin [3,4]. In addition, we demonstrated in vitro effects of melatonin on human erythrocyte carbonic anhydrase, in vivo effects on rat erythrocyte carbonic anhydrase [1] and both *in vitro* and *in vivo* effects on rainbow trout (*Oncorhynchus mykiss*) erythrocyte carbonic anhydrase [18]. Recently we determined DPPH radical scavenging activity, ABTS radical scavenging activity, total antioxidant activity by ferric thiocyanate method, total reducing ability by $Fe^{3+} - Fe^{2+}$ transformation method, superoxide anion radical scavenging activity by riboflavin/methionine/illuminate system, hydrogen peroxide scavenging and ferrous ions (Fe^{2+}) chelating activities of serotonin [19]. According to our knowledge, there is no information about $DMPD^{\cdot+}$ radical scavenging and cupric ion (Cu^{2+}) reducing ability of melatonin or serotonin obtained by Cuprac methods. It was aimed in this study to investigate the $DMPD^{\cdot+}$ radical scavenging and cupric ion (Cu^{2+}) reducing ability of melatonin and serotonin as trolox equivalent antioxidant activity.

Material and methods

Chemicals

N,N-Dimethyl-p-phenylenediamine dihydrochloride (DMPD), neocuproine (2,9-dimethyl-1,10-phenanthroline), melatonin (*N*-acetyl-5-methoxytryptamine), serotonin (5-hydroxytryptamine), trolox (($\pm$)-6-Hydroxy-2,5,7,8-tetra-methylchromane-2-carboxylic acid) and $CuCl_2$ were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

Preparation of $DMPD^{\cdot+}$ radical cation

DMPD, 100 mM, was prepared by dissolving 105 mg of DMPD in 5 mL of deionized water; 1 mL of this solution was added to 100 mL of acetate buffer (0.1 M, pH 5.25), and the coloured radical cation ($DMPD^{\cdot+}$) was obtained by adding 0.2 mL of a solution of 0.05 M ferric chloride (final concentration 0.1 mM). One mililiter of $DMPD^{\cdot+}$ radical solution was directly placed in a 1 mL quartz cuvette and then its absorbance was measured at 505 nm in spectrophotometer (CHEBIOS s.r.l. UV-VIS spectrophotometer). An optical density of 0.900 unit of absorbance was obtained and it represents the uninhibited signal. The optical density of this solution, which is daily prepared freshly, is constant up to 12 h at room temperature.

Measurement of $DMPD^{\cdot+}$ radical scavenging

$DMPD^{\cdot+}$ radical scavenging activity of melatonin and serotonin was performed according to the method described by Fogliano and co-workers [20]. A standard solution of trolox was prepared as follows: 1 mg/mL of trolox was prepared by dissolving 25 mg of trolox in 25 mL of deionized water. Different concentrations of melatonin and serotonin samples (5–15 µg/mL) were added in the spectrometric cuvette and total volume of these samples were adjusted to 1 mL with distilled water. Then 1 mL of $DMPD^{\cdot+}$ radical solution was directly transferred to the quartz cuvette. After ten min incubation, absorbances of test samples were measured at 505 nm. The $DMPD^{\cdot+}$ scavenging capacity for each tested sample was expressed as trolox equivalent (µg/mL) and calculated from the calibration curve determined by linear regression (r^2: 0.9831):

$$\text{Trolox equivalent (µg/mL)}$$
$$= \frac{DMPD^{\cdot+} \text{ scavenging (\%)}}{0.7293} - 1.3857 \quad (1)$$

The percentage of $DMPD^{\cdot+}$ scavenging capability of melatonin and serotonin was calculated using the following equation:

$$DMPD^{\cdot+} \text{ scavenging effect (\%)}$$
$$= \left(1 - \frac{A_S}{A_C}\right) \times 100 \quad (2)$$

where A_C is the absorbance of the control which contains 1 mL control reaction (containing $DMPD^{\cdot+}$ solution without the melatonin or serotonin), and A_S is the absorbance in the presence of serotonin and melatonin. $DMPD^{\cdot+}$ decreases significantly upon exposure to radical scavengers [20].

Cupric ion (Cu^{2+}) reducing ability-CUPRAC assay

For determination of reducing ability of melatonin and serotonin, the cupric ions (Cu^{2+}) reducing power method was also used [21] with slight modification. To a test tube were added 0.25 mL $CuCl_2$ solution (0.01 M), 0.25 mL ethanolic neocuproine solution (7.5×10^{-3} M) and 0.25 mL NH_4Ac buffer solution (1 m), followed by mixing; different concentration of melatonin and serotonin (5–15 µg/mL). Then, total volume adjusted 2 mL with distillated water and mixed well. Absorbance against a blank reagent was measured at 450 nm after 30 min. The cupric ions (Cu^{2+}) reducing capacity for either melatonin or serotonin was expressed as trolox equivalent (µg/mL) and calculated from the calibration curve determined by linear regression (r^2: 0.995):

$$\text{Trolox equivalent} \, (\mu g/mL) = \frac{\lambda_{450\,nm}}{0.0165} + 0.0383 \quad (3)$$

Results and discussion

Melatonin is a potent, endogenously produced and diet-derived free radical scavenger and broad-spectrum antioxidant [22–24]. Melatonin is quenching OH· [22,25] and possibly also peroxyl radicals (LOO·) [26] and peroxynitrite anion (ONOO −) [27], a reaction product of O_2^- [3].

It was reported that radical scavenging by indolic compounds is not solely a question of the presence of an indole moiety, but demonstrate that the reactivity of these molecules is profoundly modulated by their functional residues. All indoleamines share a hetero-aromatic ring system with high electroreactivity and they only differ in carrying functional groups in their side chains. These substituents show a great extent reactivity, potency and efficiency of radical scavenging. Variations in redox behaviour of the particular indoles could be of fundamental significance [28]. This was already indicated by initial findings of Tan and co-workers [23] revealing pro-oxidative properties of serotonin. The role of the methoxy group in melatonin might therefore be seen as a means of avoiding formation of *o*-centred indolyl radicals. But it is still unclear if the methoxy residue also contributes to melatonin's reactivity. The importance of the *N*-acetyl group became particularly obvious when Tan and co-workers [25] demonstrated the formation of 3-hydroxymelatonin, a reaction requiring such terminal residue at the side chain. Moreover, it was indicated that 5-methoxytryptamine differed from melatonin in its capacity for scavenging OH·, despite its similar affinity, a finding difficult to interpret at that time [28]. It was expected that serotonin had higher DMPD radical scavenging activity than melatonin because of its phenolic hydroxyl group.

The principle of DMPD assay is that; at an acidic pH and in the presence of a suitable oxidant solution, DMPD can form a stable and coloured radical cation ($DMPD^{.+}$). $DMPD^{.+}$ has a maximum absorbance at 505 nm. Antioxidant compounds which are able to transfer a hydrogen atom to $DMPD^{.+}$ quench the colour and produce a decolorization in the solution which is proportional to their amount. This reaction is rapid (less than 10 min) and the end point, which is stable, is taken as a measure of the antioxidative efficiency. Therefore, this assay reflects the ability of radical hydrogen-donor to scavenge the single electron from $DMPD^{.+}$. Preliminary experiments show that the choice of oxidant solution and the ratio between the concentration of DMPD and oxidative compound are crucial for the effectiveness of the method. It should be outlined that having high sensitive measurements and sufficient inhibition range, a starting point between 0.80 and 1.00 of absorbance at 505 nm are necessary.

Inhibition of the absorbance at 505 nm is linear between 20 and 100 µg/mL of trolox. This assay is based on the extent of radical cation reduction at a fixed time point but not on the rate of reduction. This feature ruled out the complications due to the monitoring of the time course of colour inhibition which are present in other methods [29,30] and allows the simultaneous analysis of many samples [31]. On the other hand, in contrast to the ABTS procedure, the DMPD method guarantees a very stable end point of the measure. In this way I get free of the variable "time of the measure" which is crucial for the ABTS decolorization assay. This is particularly important when a large-scale screening is required. The main drawback of the DMPD method is that its sensitivity and reproducibility dramatically decreased when hydrophobic antioxidants such as α-tocopherol or BHT were used. The same behaviour is observed when methanol is used as DMPD solvent.

$DMPD^{.+}$ scavenging efficiency was expressed in trolox equivalent antioxidant activity (TEAC) according to Miller et al. [32], using the curve of standard trolox reported in Figure 2. There is a correlation between the $DMPD^{.+}$ scavenging and trolox concentrations. The correlation between these two parameters is more than 0.983. $DMPD^{.+}$ scavenging activity of melatonin and serotonin were evaluated as trolox equivalent antioxidant activity (TEAC). As can be seen in Figure 3, $DMPD^{.+}$ scavenging activity of melatonin and serotonin was found as concentration dependent (20–100 µg/mL). Melatonin and serotonin were demonstrated 73.5 and 127.4 µg/mL trolox equivalent $DMPD^{.+}$ scavenging activity, at the concentration of 100 µg/mL, respectively. In previous studies, we investigated the antioxidant activities of melatonin and serotonin with other *in vitro* models including metal chelating, hydrogen peroxide scavenging, $Fe^{3+} - Fe^{2+}$ reducing power, DPPH radical
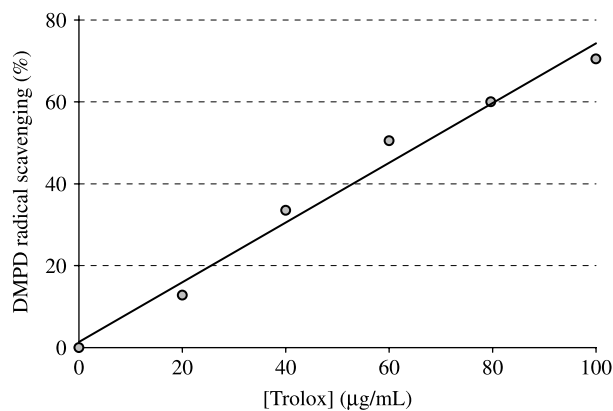
Figure 2. DMPD$^{.+}$ scavenging values of trolox at different concentrations (20–100 µg/mL) for determination of calibration curve (r^2:0.9831).

Figure 4. Cupric ion (Cu^{2+}) reducing ability of the trolox at 450 nm and different concentrations (5–50 µg/mL; r^2:0.9913) for determination of calibration curve.

scavenging, superoxide anion radical scavenging and ABTS$^{.+}$ scavenging activity [3,4,19]. In these methods, it was found that serotonin had higher antioxidant activity than melatonin. These results confirmed the results obtained from present study.

It was indicated that the electron donating capacity, reflecting the reducing power of bioactive compounds, is associated with antioxidant activity [33]. Antioxidants can be reductants, and inactivation of oxidants by reductants can be described as redox reactions in which one reaction species is reduced at the expense of the oxidation of the other. The Cuprac method was developed for an antioxidant capacity assay. This method is simultaneously cost-effective, rapid, stable, selective and suitable for a variety of antioxidants regardless of chemical type or hydrophilicity. Moreover, it was reported that the results obtained from *in vitro* cupric ions (Cu^{2+}) reducing measurements may be more efficiently extended to the possible *in vivo* reactions of antioxidants. Cuprac chromogenic redox reaction is carried out at a pH
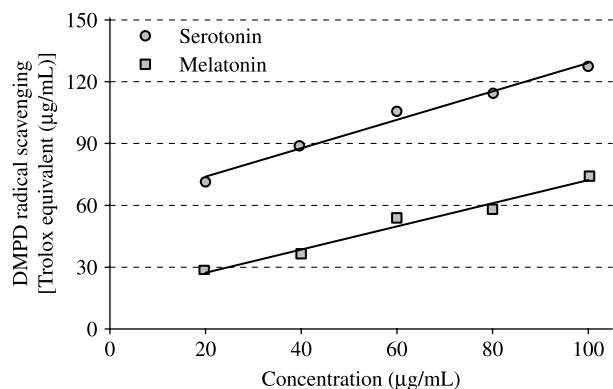
(7.0) close to the physiological pH [34]. These method is capable of measuring thiol-type antioxidants such as glutathione and non-protein thiol unlike the widely applied FRAP test, which is non-responsive to -SH group antioxidants [35]. The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity [36,37]. In the other studies, the reducing capacity of a melatonin and serotonin was measured by the direct reduction of Fe[(CN)$_6$]$_3$ to Fe[(CN)$_6$]$_2$. In this assay we found that serotonin had better ferric ion reducing antioxidant power than melatonin [3,19].

Cupric ion (Cu^{2+}) reducing ability (Cuprac method) of melatonin and serotonin was expressed in TEAC, using the curve of standard trolox reported in Figure 4. It was found a correlation between the cupric ion (Cu^{2+}) reducing ability and trolox concentrations (r^2:0.9913). Cupric ion (Cu^{2+}) reducing capability of melatonin and serotonin by Cuprac method was found as concentration dependent (10–100 µg/mL). At the same concentration (100 µg/mL), melatonin and serotonin showed 14.41 and 116.09 µg/mL trolox equivalent cupric ion (Cu^{2+}) reducing power (Figure 5).

Figure 3. DMPD$^{.+}$ scavenging capacity of different concentrations (10–100 µg/mL) of melatonin (r^2:0.9823) and serotonin (r^2:0.9860) expressed as trolox equivalent antioxidant activity (TEAC).
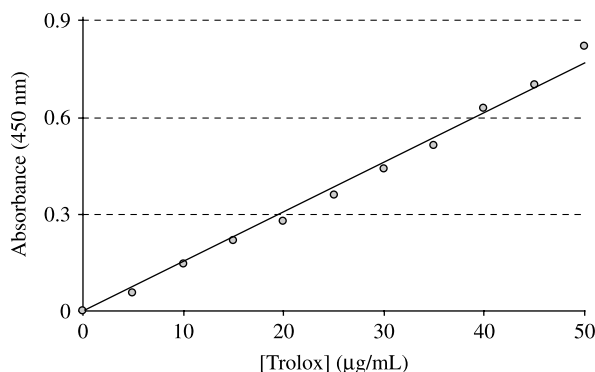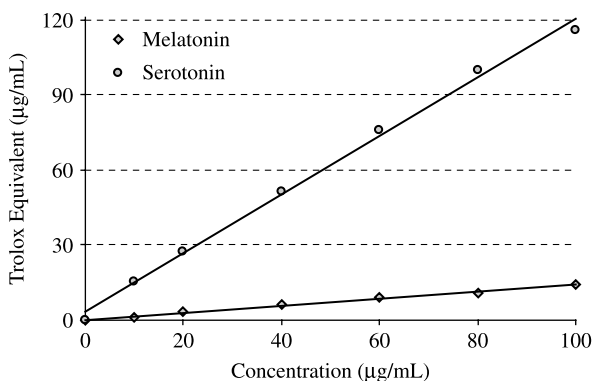

Figure 5. Cupric ion (Cu^{2+}) reducing values of different concentrations (10–100 µg/mL) of melatonin (r^2:0.9951) and serotonin (r^2:0.9961).

As a conclusion, melatonin and serotonin have effective DMPD radical scavenging activity and cupric ions (Cu^{2+}) reducing ability. Serotonin has phenolic hydroxyl group so that serotonin has higher radical scavenging and reducing activity than melatonin. Serotonin can give a hydrogen atom easily and is able to transfer this hydrogen atom to $DMPD^{\cdot+}$ quench the colour and produce a decolorization in the solution which is proportional to their amount. Also, serotonin with the phenolic group can easily reduce cupric ions (Cu^{2+}) to cuprous ions (Cu^{+}).

References

[1] Beydemir Ş, Gülçin İ. Effect of melatonin on carbonic anhydrase from human erythrocyte *in vitro* and from rat erythrocyte *in vivo*. J Enz Inhib Med Chem 2004;19: 193–197.

[2] Saarela S, Reiter RJ. Function of melatonin in thermoregulatory processes. Life Sci 1993;54:295–311.

[3] Gülçin İ, Büyükokuroğlu ME, Oktay M, Küfrevioğlu Öİ. On the *in vitro* antioxidant properties of melatonin. J Pineal Res 2002;33:167–171.

[4] Gülçin İ, Büyükokuroğlu ME, Küfrevioğlu Öİ. Metal chelating and hydrogen peroxide scavenging effects of melatonin. J Pineal Res 2003;34:278–281.

[5] Reiter RJ, Tang L, Garcia JJ, Muñoz-Hoyos A. Pharmacological action of melatonin in oxygen radical pathophysiology. Life Sci 1997;60:2255–2271.

[6] Montilla P, Cruz A, Padillo FJ, Túnez I, Gascon F, Muñoz MC, Gómez M, Pera C. Melatonin versus vitamin E as protective treatment against oxidative stress after extrahepatic bile duct ligation in rats. J Pineal Res 2001;31:138–144.

[7] Kim SJ, Reiter RJ, Qi W, Tan DX, Cabrera J. Melatonin prevents oxidative damage to protein and lipid induced by ascorbate-Fe^{3+}-EDTA: Comparison with glutathione and a-tocopherol. Neuroendocrinol Lett 2000;21: 269–276.

[8] Stahl SM. Mechanism of action of serotonin selective reuptake inhibitors: Serotonin receptors and path ways mediate therapeutic effects and side effects. J Affect Disord 1998;51: 215–235.

[9] Bubenik GA, Ball RO, Pang SF. The effect of rood deprivation on brain and gastrointestinal tissue levels of tryptophan, serotonin, 5-hydroxyindoleacetic acid and melatonin. J Pineal Res 1992;12:7–16.

[10] Hellstrand K, Hermodsson S. Serotonergic 5-HT1A receptors regulate a cell contact-media interaction between natural killer cells and monocytes. Scand J Immunol 1993; 37:7–18.

[11] Hasegawa S, Watanabe A, Nishi K, Nguyen KQ, Diksic M. Selective $5-HT_{1B}$ receptor agonist reduces serotonin synthesis following acute, and not chronic, drug administration: Results of an autoradiographic study. Neurochem Int 2005;46: 261–272.

[12] Gülçin İ, Oktay M, Küfrevioglu Öİ, Aslan A. Determinations of antioxidant activity of lichen *Cetraria islandica* (L) Ach. J Ethnopharmacol 2002;79:325–329.

[13] Gülçin İ, Beydemir Ş, Alici HA, Elmastaş M, Büyükokuroğlu ME. *In vitro* antioxidant properties of morphine. Pharmacol Res 2004;49:59–66.

[14] Gülçin İ, Elmastas M, Aboul-Enein HY. Determination of antioxidant and radical scavenging activity of basil (*Ocimum basilicum*) assayed by different methodologies. Phytother Res 2007;21:354–361.

[15] Gülçin İ. Comparison of *in vitro* antioxidant and antiradical activities of L-tyrosine and L-Dopa. Amino Acids 2007;32: 431–438.

[16] Gülçin İ, Berashvili D, Gepdiremen A. Antiradical and antioxidant activity of total anthocyanins from *Perilla pankinensis* decne. J Ethnopharmacol 2005;101: 287–293.

[17] Gülçin İ, Alici HA, Cesur M. Determination of *in vitro* antioxidant and radical scavenging activities of propofol. Chem Pharm Bull 2005;53:281–285.

[18] Hisar O, Beydemir Ş, Gülçin İ, ArasHisar Ş, Yanık T, Küfrevioğlu Öİ. The effect of melatonin hormone on carbonic anhydrase enzyme activity in rainbow trout (*Oncorhynchus mykiss*) erythrocytes *in vitro* and *in vivo*. Turk J Vet Anim Sci 2005;29:841–845.

[19] Gülçin İ, Reiter RJ. Determination of *in vitro* antioxidant and radical scavenging activity of serotonin (Unpublished data).

[20] Fogliano V, Verde V, Randazzo G, Ritieni A. Method for measuring antioxidant activity and its application to monitoring the antioxidant capacity of wines. J Agric Food Chem 1999;47:1035–1040.

[21] Apak R, Güçlü K, Özyürek M, Karademir SE, Erça E. The cupric ion reducing antioxidant capacity and polyphenolic content of some herbal teas. Int J Food Sci Nut 2006;57: 292–304.

[22] Tan DX, Chen LD, Poeggeler B, Manchester LC, Reiter RJ. Melatonin: A potent, endogenous hydroxyl radical scavenger. Endocr J 1993;1:57–60.

[23] Suzen S, Bozkaya P, Coban T, Nebioğlu D. Investigation of the *in vitro* antioxidant behaviour of some 2-phenylindole derivatives: Discussion on possible antioxidant mechanisms and comparison with melatonin. J Enz Inhib Med Chem 2006; 21:405–411.

[24] Tan DX, Manchester LC, Terron MP, Flores LJ, Reiter RJ. One molecule, many derivatives: A never-ending interaction of melatonin with reactive oxygen and nitrogen species? J Pineal Res 2007;42:28–42.

[25] Tan DX, Manchester LC, Reiter RJ, Plummer BF, Hardies LJ, Weintraub ST, Shepherd AMM. A novel melatonin metabolite, cyclic 3-hydroxymelatonin: A biomarker of *in vivo* hydroxyl radical generation. Biochem Biophys Res Commun 1998;253:614–620.

[26] Pieri CM, Marra F, Moroni R, Recchioni R, Marcheselli F. Melatonin: A peroxyl radical scavenger more effective than vitamin E. Life Sci 1994;55:271–276.

[27] Cuzzocrea SB, Zingarelli E, Gilad P, Hake P, Salzman AL, Szabó C. Protective effect of melatonin in carrageenan-induced models of local inflammation: Relationship to its inhibitory effect on nitric oxide production and its peroxynitrite scavenging activity. J Pineal Res 1997;23: 106–116.

[28] Poeggeler B, Thuermann S, Dose A, Schoenke M, Burkhardt S, Hardeland R. Melatonin's unique radical scavenging properties-roles of its functional substituents as revealed by a comparison with its structural analogs. J Pineal Res 2002;33: 20–30.

[29] Pryor WA, Cornicelli JA, Devall LJ, Tait B, Trivedi BK, Witiak DT, Wut M. A rapid screening test to determine the antioxidant potencies of natural and synthetic antioxidants. J Org Chem 1993;58:3521–3522.

[30] Tubaro F, Micossi E, Ursini F. The antioxidant capacity of complex mixtures by kinetic analysis of crocin bleaching inhibition. JAOCS 1996;73:173–179.

[31] Miller JN, Rice-Evans CA, Davies MJ. A novel method for measuring antioxidant capacity and its application to monitoring the antioxidant status in premature neonates. Clin Sci 1993;84:407–412.

[32] Miller JN, Sampson J, Candeias LP. Antioxidant activities of carotenes and xanthophylls. FEBS Lett 1996;384: 240–242.

[33] Arabshahi-Delouee S, Urooj A. Antioxidant properties of various solvent extracts of mulberry (*Morus indica* L.) leaves. Food Chem 2007;102:1233–1240.

[34] Apak R, Güçlü K, Özyürek M, Karademir SE. A novel total antioxidant capacity index for dietary polyphenols, vitamin C and E, using their cupric ion reducing capability in the presence of neocuproine: The CUPRAC method. J Agric Food Chem 2004;52:7970–7981.

[35] Huang D, Ou B, Prior RL. The chemistry behind antioxidant capacity assays. J Agric Food Chem 2005;53:1841–1856.

[36] Gülçin İ. Antioxidant activity of caffeic acid (3,4-dihydroxy-cinnamic acid). Toxicology 2006;217:213–220.

[37] Gülçin İ. Antioxidant and antiradical activities of L-carnitine. Life Sci 2006;78:803–811.