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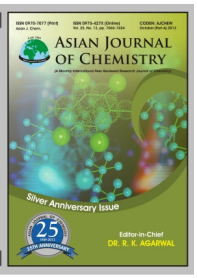

# Evaluation of Antioxidant Activities of Turmeric Extracts from Turkey

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The aim of this study was to assess the *in vitro* potential of ethanolic and methanolic extracts of turmeric as a natural antioxidant. The iron chelation activity of the extracts was increased in a dose-dependent manner. The methanolic extract was found to be richer in antioxidant phytochemicals, such as phenolics (502.6 ± 11.3 mg GAE/g DW) and flavonoids (303.2 ± 4.1 mg PE/g DW). The total antioxidant activity was found to vary in the order: ethanolic extract > methanolic extract > ascorbic acid > α-tocopherol > BHT > BHA. Moreover, extracts had effective reducing power, free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and ABTS scavenging activity. The results obtained in this study clearly indicate that turmeric has a significant potential to use as a natural antioxidant agent.

**Key Words: Turmeric, Antioxidant activity, Scavenging activity, Phenolics, Flavonoids.**

## INTRODUCTION

Antioxidants are used as food additives in order to prevent the oxidative deterioration of fats and oils in processed foods. However, due to limitation on the use of synthetic antioxidants and enhanced public awareness of health issues, there is an increasing need of health-promoting natural antioxidants in foods, such as in bakery products[1].

Free radicals can usually be generated by several biological reactions in the body and these are capable of damaging crucial bio-molecules; if they are not scavenged effectively by cellular constituents, they lead to disease conditions. The harmful action of free radicals can be blocked by antioxidant substances, which scavenge the free radicals and detoxify the organisms. Current researches have confirmed that foods rich in antioxidants play an essential role in the prevention of cardiovascular diseases, cancers and neurodegenerative diseases, as well as inflammation and problems caused by cell and cutaneous aging[2,3]. Antioxidants are important in the control of degenerative diseases in which oxidative damage has been implicated. There are different plant sources which contain important antioxidants capable of free radical scavenging and they are preferred over synthetic antioxidants such as butylated hydroxy toluene (BHT) and butylated hydroxy anisole (BHA) which are reported to be toxic to human health[4].

Turmeric (*Curcuma longa* L.) is one of the most popular spices containing natural antioxidants and is reported to possess numerous medicinal properties including antioxidant, antiprotozoal, antitumour, antiinflammatory and antivenom activities[5]. The major biologically active components of turmeric are curcuminoids which include curcumin, demethoxycurcumin and *bis*-demethoxy curcumin. Curcumin is a yellow-coloured phenolic pigment and is an effective antioxidant that can scavenges superoxide radicals, hydrogen peroxide and nitric oxide from activated macrophages.

The aim of the present study was to determine the antioxidant activity by using different antioxidant tests including total antioxidant activity by ferric thiocyanate method, reducing power, free radical scavenging, ABTS radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging, total flavonoid content, total phenolic compound and metal chelating activities.

## EXPERIMENTAL

Linoleic acid, α-tocopherol, ethylenediaminetetraacetic acid (EDTA), nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulfate (PMS), 1,1-diphenyl-2-picrylhydrazyl (DPPH), 3-(2-pyridyl)-5,6-*bis*(4-phenyl-sulfonic acid)-1,2,4-triazine ferrozine) were obtained from Sigma (Sigma-Aldrich GmbH, Sternheim, Germany). Ammonium thiocyanate, ferrous chloride, polyoxyethylenesorbitan monolaurate (Tween-20), trichloracetic acid were purchased from Merck. All other chemicals used were in analytical grade and obtained from either Sigma-Aldrich or Merck.

**Preparation of extracts:** Turmeric seed was purchased from a spice seller at Tekirdag City (Tekirdag, Turkey). Seeds were kept at ambient temperature (26 ± 2 ºC) overnight before the analyses were performed. For solvent extraction, 25 g sample was put into a fine powder in a mill and was mixed with 500 mL solvent (methanol or ethanol) at the room temperature. The extract was incubated at 300 rpm for 3 h in the water bath. The residue was re-extracted until extraction solvents became colourless. The obtained extracts were filtered over Whatman No. 1 paper and the filtrate was collected and then solvent was removed using a rotary evaporator (Buchi R-200, England) at 40 ºC to obtain dry extract. The both extracts were placed of 0.05 g in a plastic bottle.

**Determination of total phenolics:** Total soluble phenolics of turmeric extracts were determined with Folin-Ciocalteu reagent using gallic acid as a standard phenolic compound[6]. Briefly, 1 mL of extract solution (containing 1 mg exracts) was placed in a volumetric flask diluted with distilled water (46 mL), 1 mL of Folin-Ciocalteu reagent was added and the content of the flask was mixed thoroughly. After 3 min, 3 mL of $Na_2CO_3$ (2 %) was added, then the mixture was allowed to stand for 2 h with intermittent shaking. The absorbance was measured at 760 nm in a spectrophotometer. Results were expressed as milligrams of total phenolics (gallic acid equivalents) per gram of dry weight (mg GAE/g DW). The calibration equation for gallic acid was $y = 0.0011x - 0.0022$ ($R^2 = 0.9992$).

**Determination of total flavonoids:** Total flavonoid content was determined by a colorimetric method described by Wang *et al.*[7] with minor modification. An aliquot of 10 mL of appropriate dilution of each extract was added to volumetric flask containing 1 mL of 5 % (w/v) sodium nitrite and placed for 6 min, followed by reaction with 1 mL of 10 % (w/v) aluminum nitrate to form a flavonoid-aluminum complex. After 6 min, 10 mL of 4.3 % (w/v) NaOH was added and the total was made up to 25 mL with distilled water. After 15 min at room temperature, the final solution was mixed well again and the absorbance was measured against a blank at 510 nm with a UV-1601 UV/VIS Recording Spectrophotometer (Shimadzu, Japan). The total flavonoids were expressed as milligrams of pyrocatechol equivalents (PE) per gram of dry weight (mg PE/g DW). The calibration equation for pyrocatechol was $y = 0.0024x + 0.0244$ ($R^2 = 0.9985$).

**Total antioxidant activity assay:** The total antioxidant activity of the turmeric extracts was determined according to the thiocyanate method described by Mitsuda *et al.*[8]. For stock solutions, 10 mg of turmeric extracts was dissolved in 10 mL deionized water. The solution, which contains the same concentration of turmeric extracts or standard samples (150 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). Fifty millilitres linoleic acid emulsion contained 175 µg Tween-20, 155 µL linoleic acid and 0.04 M potassium phosphate buffer (pH 7.0). On the other hand, 5 mL control was composed of 2.5 mL linoleic acid emulsion and 2.5 mL, 0.04 M potassium phosphate buffer (pH 7.0). The mixed solution (5 mL) was incubated at 37 ºC in a glass flask. At regular intervals during incubation, a 0.1 mL aliquot of the mixture was diluted with 3.7 mL of solvent (ethanol or methanol), 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. This step was repeated every 10 h until the control reached its maximum absorbance value. Therefore, high absorbance indicates high linoleic acid oxidation. All data on total antioxidant activity are the average of triplicate experiments. The per cent inhibition of lipid peroxidation in linoleic acid emulsion was calculated by the following equation:

Inhibition of lipid peroxidation (%)

$$= \left[\frac{(A_0 - A_1)}{A_0}\right] \times 100$$

$A_0$ is the absorbance of the control, $A_1$ is the absorbance of the sample.

**DPPH radical scavenging activity assay:** The free radical scavenging activity of turmeric extracts was determined by the 1,1-diphenyl-2-picryl-hydrazil (DPPH). The activity was measured by following the methodology described by Shimada *et al.*[9]. Where in the bleaching rate of a stable free radical, $DPPH^{\bullet}$ is monitored at a characterictic wavelenght in the presence of the sample. Briefly, 0.1 mM solution of $DPPH^{\bullet}$ in ethanol (or methanol) was prepared and 1 mL of this solution was added 3 mL of turmeric extracts solution in water at different concentrations (50-250 µg/mL). 0.5 h later, the absorbance was measured at 517 nm. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity.

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

$$DPPH^{\cdot} \text{ scavenging effect (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

$A_0$ is the absorbance of the control, $A_1$ is the absorbance of the sample.

**$ABTS^{\bullet+}$ scavenging activity:** The $ABTS^{\bullet+}$ scavenging activity of the turmeric extracts was measured according to procedure described by Re *et al.*[10]. $ABTS^{\bullet+}$ was produced by the reaction between 7 mM $ABTS^{\bullet}$ 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 to 3 mL of turmeric extracts at different concentrations (50-250 µg/mL). After 0.5 h, the absorbance was taken at 734 nm using the spectrophotometer. The $ABTS^{\bullet+}$ scavenging activity was calculated using the following equation:

$$\text{ABTS radical scavenging activity (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

$A_0$ is the absorbance of the control, $A_1$ is the absorbance of the sample.

**Reducing power assay:** The reducing power of turmeric extracts was determined according to the method of Oyaizu[11]. Different concentrations of extracts (50-250 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 (2.5 mL, 1 %). The mixture was incubated at 50 ºC for 20 min. A portion (2.5

mL) of trichloroacetic acid (10 %) was added to the mixture, which was then centrifugated at 1500 × g (Hettich, Rotina 38 R, Germany) for 10 min. 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 against a blank sample. Increased absorbance of the reaction mixture indicated increasing reducing power. All data are an average of triplicate analyses.

**Chelating activity:** Ferrous ions ($Fe^{2+}$) chelation by turmeric extracts and standards was estimated by the Ferrozine assay[12]. Briefly, turmeric extracts (50-250 µg/mL) in 0.4 mL were 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 the mixture was shaken vigorously and left standing at room temperature for 10 min. Absorbance of the solution was then measured spectrophotometrically at 562 nm. The percentage of inhibition of ferrozine-$Fe^{2+}$ complex formation was calculated by using the formula given bellow:

$$\text{Metal chelating effect (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

$A_0$ is the absorbance of the control, $A_1$ is the absorbance of the sample.

**Hydrogen peroxide scavenging activity:** The ability of the turmeric extracts to scavenge hyrogen peroxide was determined according to the method of Ruch *et al.*[13]. A solution of hyrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Extracts (50-250 µg/mL) in distilled water were added to a hydrogen peroxide 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 hydrogen peroxide scavenging of both turmeric extracts and standard compounds was calculated as follows:

$$H_2O_2 \text{ scavenging activity (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

$A_0$ is the absorbance of the control, $A_1$ is the absorbance of the sample.

**Superoxide anion radical scavenging assay:** Measurement of superoxide anion scavenging activity of turmeric extracts was done based on the method described by Liu *et al.*[14] with slight modification. About 1 mL of nitroblue tetrazolium (NBT) solution (156 µM NBT in 100 mM phosphate buffer, pH 7.4), 1 mL NADH solution (468 µM in 100 mM phosphate buffer, pH 7.4) and 0.1 mL of sample solution of turmeric extracts (50-250 µg/mL) in water were mixed. The reaction started by adding 100 µL of phenazine methosulphate (PMS) solution (60 µM PMS in 100 mM phosphate buffer, pH 7.4 ) to the mixture. The reaction mixture was incubated at 25 ºC for 5 min and the absorbance at 560 nm was measured against blank samples. Decreased absorbance of the reaction mixture indicated increased superoxide anion scavenging activity. All data are an average of triplicate analyses. The percentage of inhibition of superoxide anion scavenging formation was calculated by using the formula given below:

$$\text{Inhibition (\%)} = \left(\frac{A_0 - A_1}{A_0}\right) \times 100$$

$A_0$ is the absorbance of the control, $A_1$ is the absorbance of the sample.

**Statistical analysis:** The statistical processing of the data obtained from all studies was implemented by means of dispersion analysis with the Sigma Plot 7.0 software. Data are expressed as means standard deviation (SD). Statistical analysis was performed with student's *t*-test.

## RESULTS AND DISCUSSION

**Extraction yield, total phenolics and flavonoid contents:** The percentage yields of turmeric extracts were shown in Table-1. The extraction yield of both extracts varied from 4.5 ± 0.5-12.5 ± 1.45 % with a descending order of ethanolic extract > methanolic extract. So the ethanolic extract resulted in the higher amount of total extractable compounds. Phenolics or polyphenols are plant secondary metabolites and are important by virtue of their antioxidant activity by chelating redox-active metal ions, inactivating lipid free radical chains and preventing hydroperoxide conversions into reactive oxyradicals. The methanolic extract exhibited the higher total phenolics content (502.6 ± 11.3 mg gallic acid equivalent/g extract), whereas the contents obtained with ethanolic extract were smaller (421.8 ± 10.1 mg gallic acid equivalent/g). These values were higher than that reported for turmeric (3.9 ± 0.1 mg/g)[15]. The content of total flavonoids expressed as pyrocatechol equivalents, varied from 281.1 ± 5.3 to 303.2 ± 4.1 mg as pyrocatechol equivalent/g extract. These amounts were comparable with results described in the literature for other extracts of spice products[16]. The rich-flavonoid spices could be a good source of antioxidants that would help to increase the overall antioxidant capacity of an organism and protect it against lipid peroxidation[17]. Phenolic acids and flavonoids have been reported to be the main phytochemicals responsible for the antioxidant capacity of spices, fruits and vegetables. Plant derived polyphenols display characteristic inhibitory patterns toward the oxidative reaction *in vitro* and *in vivo*[18].

TABLE-1
EXTRACTION YIELDS AND CONTENTS OF TOTAL PHENOLS, TOTAL FLAVONOIDS IN TURMERIC EXTRACTS

| Extracts | Extraction yield (%) | Total phenols (mg GAE/g extract) | Total flavonoids (mg PE/g extract) |
|---|---|---|---|
| Methanolic extract | 4.5±0.5 | 502.6±11.3 | 303.2±4.1 |
| Ethanolic extract | 12.5±1.45 | 421.8±10.1 | 281.1±5.3 |

Values were the means of three replicates ± standard deviation. [a]GAE, gallic acid equivalents. [b]pyrocatechol equivalents.

**Total antioxidant activity determination:** Total antioxidant activity of turmeric extracts was determined by the thiocyanate method. Both turmeric extracts exhibited effective antioxidant activity. The effects of same amounts of methanolic and ethanolic extracts of turmeric (150 µg/mL) on peroxidation of linoleic acid emulsion are shown in Fig. 1. The effects on lipid peroxidation of linoleic acid emulsion of extracts and standards decreased in that order: ethanolic extract > methanolic extract > ascorbic acid > α-tocopherol > BHT > BHA. The total antioxidant activity of plant extract may be attributed to their chemical composition and phenolic acid content demonstrated that some bioactive compounds present in citrus possessed high total antioxidant activity, which was due to the presence of phenolics, carotenoids and flavonoids[19].

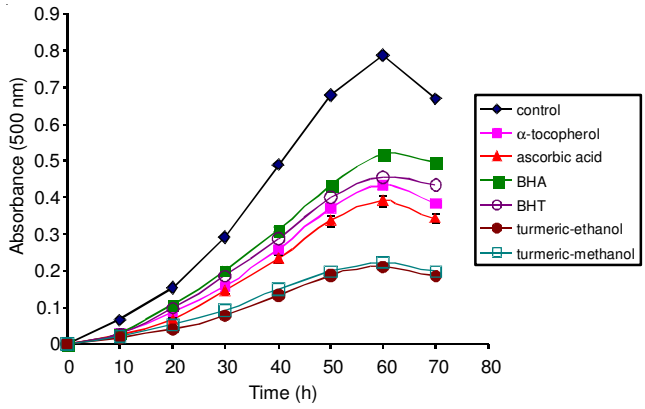


Fig. 1. Inhibitory effect of the extracts from turmeric on lipid peroxidation. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

**DPPH• radical scavenging activity:** DPPH is a commercial oxidizing radical, which can be reduced by antioxidants. The stable DPPH• can be used to study the reaction kinetics of antioxidants, quantify and to compare the free radical scavenging capacities of different antioxidants[20]. It is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule[21]. The reduction capability of DPPH• radicals was determined by the decrease in its absorbance at 517 nm, which is induced by antioxidants. Hence, DPPH is often used as a substrate to evaluate antioxidative activity of antioxidants[21]. On the DPPH·radical, extracts had significant scavenging effects with increasing concentration in the range of 50-250 µg/mL when compared with that of standards, the scavenging effect of methanolic and ethanolic extracts was lower (Fig. 2). The scavenging effect of turmeric extracts and standards on the DPPH• radical decreased in that order: BHA > BHT > α-tocopherol > methanolic extract > ethanolic extract, which were 59.7, 55.7, 43.3, 39.7 and 29.2 %, respectively, at the concentration of 150 µg/mL. These results indicates that both turmeric extracts have a noticeable effect on scavenging free radical. Free radical scavenging activity also increased with increasing concentration. Zaeoung *et al.*[22] reported the strong antioxidant activity of methanol extract of curcuma longa against the DPPH radical with % inhibition in the range of 86-92 %.

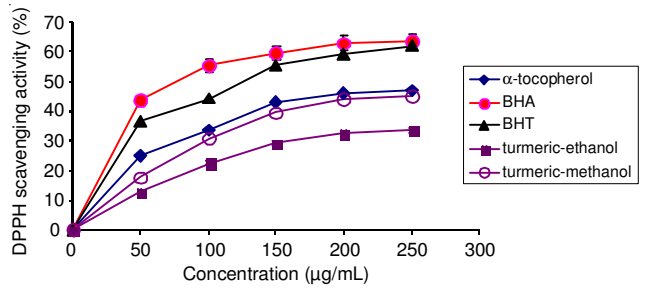


Fig. 2. DPPH radical scavenging activity of the extracts from turmeric. BHA, BHT and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

**ABTS•+ scavenging activity:** The ABTS•+ method is widely employed for measuring the relative radical scavenging activity of hydrogen donating and chain breaking antioxidants in many plants extracts[23]. The ABTS•+ scavenging activity (%) of the methanolic and ethanolic extracts of turmeric, compared to standards are shown in Fig. 3. They increased with increasing concentration, reaching 66 and 64.1 %, respectively at the concentration of 150 µg/mL and these values were comparable to those of the positive controls, ascorbic acid (57.7 %), α-tocopherol (43.8 %), Trolox (35.1 %), BHA (66.5 %) and BHT (47.3 %) at the same concentration, respectively. The scavenging effect of turmeric extracts and standards on the ABTS•+ decreased in the following order: BHA > methanolic extract > ethanolic extract > ascorbic acid > BHT > α-tocopherol > Trolox.

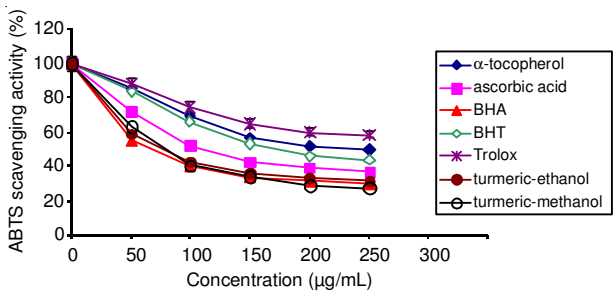


Fig. 3. ABTS radical scavenging activity of the extracts from turmeric. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

**Reducing power:** In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of ferric iron to ferrous iron by electron donation[24]. Fig. 4 shows the extent of the reduction, in terms of absorbance values at 700 nm. The reducing power of methanol (0.091) and ethanol (0.089) extracts was not concentration dependent and found to be above those of α-tocopherol (0.062), ascorbic acid (0.064), BHT (0.067) and BHA (0.074) at 150 µg/mL. Both extracts of turmeric showed higher activities than the standards. Reducing power of extracts and standards decreased in order of methanolic extract > ethanolic extract > BHA > BHT > ascorbic acid > α-tocopherol.

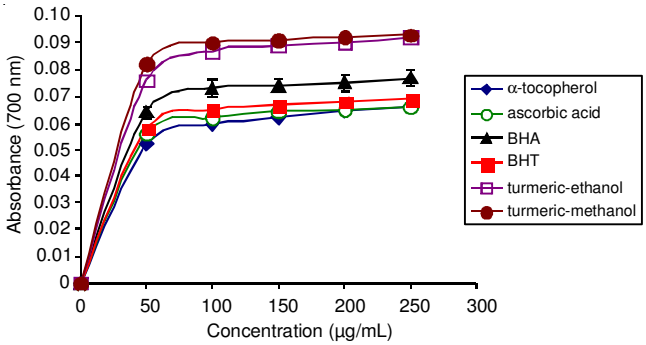


Fig. 4. Reducing power of the extracts from turmeric. BHA, BHT, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

**Iron chelation(II) activity:** Transition metals have a major role in the generation of free oxygen radicals in living organisms. 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. All the extracts demonstrated an ability to chelate iron(II) ions in a dose-dependent manner

(Fig. 5). Methanolic and ethanolic extracts chelated ferrous ions by 28.6 and 30.2 % at 250 µg/mL, respectively. EDTA is a strong metal chelator agent in this study. Metal chelating capacity was significant, since it reduces the concentration of the catalyzing transition metal in lipid peroxidation. The percentages of metal scavenging capacity of 250 µg of EDTA, BHA, BHT, α-tocopherol and ascorbic acid were found as 54.9, 49.1, 42.8, 39.4 and 34 %, respectively. The metal scavenging effect of these standards and extracts decreased in the order of EDTA > BHA > BHT > α-tocopherol > ascorbic acid > ethanolic extract > methanolic extract. Exen at a minimal concentration of 20 µg, turmeric showed a 70 % chelating[25].

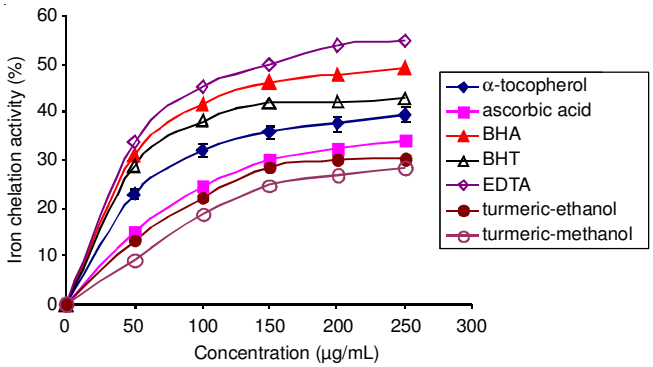


Fig. 5. Iron chelation activity of the extracts from turmeric. BHA, BHT, EDTA, ascorbic acid and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

**Hydrogen peroxide radical scavenging activity:** 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. The scavenging ability of methanolic and ethanolic extracts of turmeric on hydrogen peroxide is shown in Fig. 6 and compared with that of BHA, BHT and α-tocopherol as standards. Turmeric extracts were capable of scavenging hydrogen peroxide in a concentration-dependent manner. Of methanolic and ethanolic extracts 150 µg/mL of turmeric exhibited 56.1 and 59.1 % scavenging activity on hydrogen peroxide, respectively. In the other hand, BHA, BHT and α-tocopherol exhibited 15.1, 19.7 and 12.1 % hydrogen peroxide scavenging activity at the same dose. These results showed that both turmeric extracts had stronger hydrogen peroxide scavenging activity. Those values are higher than that of standards. The hydrogen peroxide scavenging effect of 150 µg/mL concentration of the both extracts of turmeric and standards decreased in the order of ethanolic extract > methanolic extract > BHT > BHA > α-tocopherol. Hydrogen peroxide itself is not very reactive, but it can sometimes be toxic to cells, since it may give rise to hydroxyl radicals inside the cell. Thus, removing hydrogen peroxide is very important throughout food systems.

**Superoxide anion scavenging activity:** Superoxide anions are the most common free radicals *in vivo* and are generated in a variety of biological systems and the concentration of superoxide anions increases under conditions of oxidative stress[26]. In this study, superoxide radicals were generated by auto-oxidation of hydroxylamine in presence of NBT (nitroblue tetrazolium). The reduction of NBT in presence of antioxidants was measured. The decrease of absorbance at 560

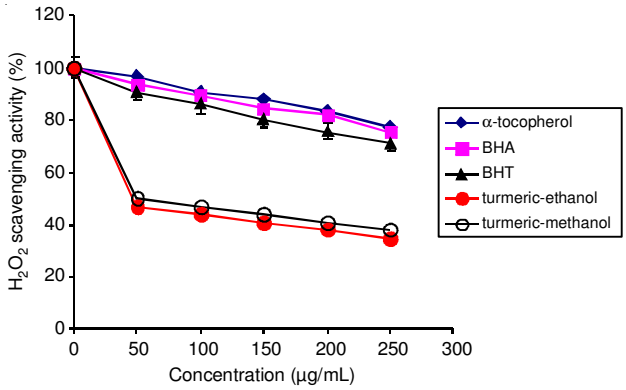


Fig. 6. $H_2O_2$ scavenging activity of the extracts from turmeric. BHA, BHT and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Fig. 7 shows the % inhibition of superoxide radical generation by 50-250 µg/mL of turmeric extracts and comparison with same concentrations of BHA, BHT and α-tocopherol. Both extracts of turmeric had a scavenging activity on the superoxide radicals in a dose dependent manner. Nonetheless, when compared to α-tocopherol, the superoxide scavenging activity of the extracts was found to be high. At 250 µg/mL, the superoxide scavenging activities of methanol extract, ethanol extract, BHA, BHT and α-tocopherol were 32.7, 26.5, 44.9, 30.6 and 24.5 %, respectively. As seen in Fig. 7, superoxide radical scavenging activity of those extracts showed the following order: BHA > methanolic extract > BHT > ethanolic extract > α-tocopherol.

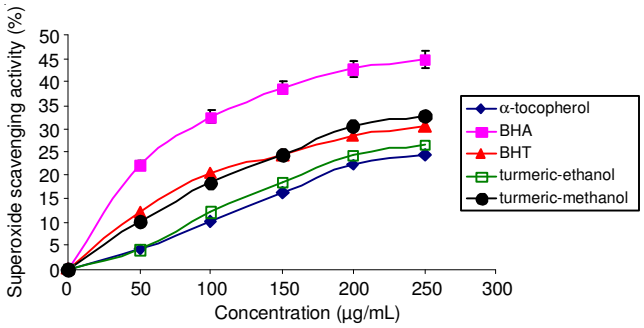


Fig. 7. Superoxide anion scavenging activity of the extracts from turmeric. BHA, BHT and α-tocopherol were used as reference antioxidants. Values are means ± SD (n = 3)

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