Determination of *in Vitro* Antioxidant and Radical Scavenging Activities of Propofol

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Propofol (2,6-diisopropylphenol) is a hypnotic intravenous agent with *in vivo* antioxidant properties. This study was undertaken to examine the *in vitro* antioxidant activity of propofol using different antioxidant tests including by 1,1-diphenyl-2-picryl-hydrazil (DPPH·) radical scavenging, metal chelating, hydrogen peroxide scavenging, superoxide anion radical scavenging, reducing power and total antioxidant activities. At the concentrations of 25, 50, and 75 μg/ml, propofol exhibited 97.7, 98.6 and 100% inhibition on peroxidation of linoleic acid emulsion, respectively. On the other hand, at the 75 μg/ml concentration of standard antioxidants such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol exhibited 88.7, 94.5, and 70.4% inhibition on peroxidation of linoleic acid emulsion, respectively. In addition, at same concentrations, propofol was shown that it had effective reducing power, DPPH· free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities. These various antioxidant activities were compared to standard antioxidants such as BHA, BHT and α-tocopherol. These results indicate that propofol prevents lipid peroxidation and radicalic chain reactions. At the same time, propofol revealed more effective antioxidant capacity than BHA, BHT and α-tocopherol.

Key words propofol; antioxidant activity; radical scavenging; metal chelating; *in vitro*

Propofol (2,6-diisopropylphenol) has structure similar to that of known such as BHA, BHT, and α-tocopherol. In addition, propofol has been shown to attenuate experimental reperfusion injury in the cerebral cortex.[1)] It is commonly used as sedatives for critically ill patients. These patients usually suffer from the pathologic effects of oxidative stress, predominantly caused an imbalance between the generation of reactive oxygen species (ROS) and antioxidant defence system.[2)]

ROS are formed and degraded by all aerobic organisms. ROS can readily react with most biomolecules including protein lipids and lipoproteins and DNA. ROS include a number of reactive molecules derived from oxygen such as hydrogen peroxide (H_2O_2), superoxide ($O_2^{\cdot -}$) and hydroxyl radical (OH·).[3)]

Exogenous chemical and endogenous metabolic processes in the human body might produce highly ROS. Oxidative damages play a significantly pathological role in human diseases. It was found that cancer, emphysema, cirrhosis, arteriosclerosis and arthritis have all been correlated with oxidative damage. Also, excessive generation of ROS induced by various stimuli leads to a variety of pathophysiological processes such as inflammation, diabetes, genotoxicity and cancer.[4)] Antioxidants can interfere with the oxidation process by reacting with free radicals, chelating catalytic metals, and also by acting as oxygen scavengers.[5—7)] However, antioxidant supplement may be used to the help the human body reduce oxidative damage.[8)] The most commonly used antioxidants at the present time are butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), propyl gallate and *tert*-butylhydroquinone.[4,9)] However, they have suspected of being responsible for liver damage and carcinogenesis in laboratory animal. Therefore, the development and utilization of more effective antioxidants are desired.[4,10)]

Some studies have been performed about *in vivo* and *in vitro* antioxidant activity of propofol by using different methods.[1,2,11,12)] Propofol is powerfull inhibitor of neuronal nitric oxide synthase. Furthermore propofol reacts with peroxynitrite and scavenged.[13)] Propofol inhibited *in vitro* lipid peroxidation induced by different free radical generating systems including hydroxyl, ferryl, and oxo-ferryl radicals.[14)] At animals experiments, it is shown that propofol, indeed, reduces the formation of lipid peroxides.[11)] Also, propofol had antioxidant effect in human hepatic microsomes,[12)] protective effect against active oxygen species and inhibited the luminol-enhanced chemiluminescence produced by stimulated human polymorphonuclear leukocytes in a dose dependent manner.[15)]

Numerous procedures are available for the determination of the *in vitro* antioxidant activity of pure compounds. Therefore, the objectives of this study were to investigate the total antioxidant activity, reducing power, DPPH· free radical scavenging, superoxide anion radical scavenging, hydrogen peroxide scavenging and metal chelating activities of propofol.

Experimental

Chemicals Propofol (2,6-diisopropylphenol), linoleic acid, α-tocopherol, nicotinamide adenine dinucleotide (NADH), butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), nitroblue tetrazolium (NBT), phenazine methosulphate (PMS), the stable free radical 1,1-diphenyl-2-picryl-hydrazyl (DPPH·), 3-(2-Pyridyl)-5,6-bis(4-phenyl-sulfonic acid)-1,2,4-triazine (Ferrozine), 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.

1,1-Diphenyl-2-picryl-hydrazil (DPPH) Free Radical Scavenging Activity The free radical scavenging activity of propofol was measured by 1,1-diphenyl-2-picryl-hydrazil (DPPH·) using the method described by Shimada *et al.*[16)] Briefly, 0.1 mM solution of DPPH· in ethanol was prepared. 1 ml of the solution was added to 3 ml of propofol solution in water at different concentrations (25—75 μg/ml). The mixture was shaken vigorously and allowed to stand at room temperature for 30 min. Then the absorbance was measured at 517 nm by using a spectrophotometer (8500 II, Bio-Crom Gmb,

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Zurich, Switzerland). Lower absorbance of the reaction mixture indicated higher free radical scavenging activity. The percent DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH}\cdot \text{ scavenging effect } (\%)=100\times A_1/A_0$$

Where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the standard sample or propofol.[17)]

Ferrous Metal Ions Chelating Activity The ferrous ions chelating by the propofol and standards were estimated by the method of Dinis *et al.*[18)] Briefly, the samples (25—75 μg/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. After the mixture had reached equilibrium, the absorbance of the solution was measured spectrophotometrically at 562 nm by using a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). All test and analyses were run in triplicate and averaged. The percentage of inhibition of ferrozine-Fe^{2+} complex formation was given bellow formula:

$$\% \text{ inhibition}=[(A_0-A_1)/A_0]\times 100$$

where A_0 was the absorbance of the control and A_1 was the absorbance in the presence of the sample of propofol and standards. The control contains $FeCl_2$ and ferrozine, complex formation molecules.[19)]

Scavenging of Hydrogen Peroxide The ability of the propofol to scavenge hydrogen peroxide was determined according to the method of Ruch and colleagues.[20)] A solution of hydrogen peroxide (40 mM) was prepared in phosphate buffer (pH 7.4). Hydrogen peroxide concentration was determined spectrophotometrically from absorption at 230 nm by using a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Propofol (25—75 μg/ml) in distilled water was added to a hydrogen peroxide solution (0.6 ml, 40 mM). Absorbance of hydrogen peroxide at 230 nm was determined after 10 min. against a blank solution containing in phosphate buffer without hydrogen peroxide. The percentage of scavenging of hydrogen peroxide of propofol and standard compounds:

$$\% \text{ scavenged } [H_2O_2]=[(A_0-A_1)/A_0]\times 100$$

where A_0 was the absorbance of the control, and A_1 was the absorbance in the presence of the sample of propofol and standards.[21)]

Superoxide Anion Radicals Scavenging Activity Measurement of superoxide anion radicals scavenging activity of propofol was based on the method described by Liu *et al.*[22)] with slight modification.[10)] Superoxide radicals are generated in PMS-NADH systems by oxidation of NADH and assayed by the reduction of NBT. In these experiments, the superoxide radicals were generated in 3 ml of Tris–HCl buffer (16 mM, pH 8.0) containing 1 ml of NBT (50 μM) solution, 1 ml NADH (78 μM) solution and sample solution of propofol (from 25 to 75 μg/ml) in water. The reaction started by adding 1 ml of PMS solution (10 μM) to the mixture. The reaction mixture was incubated at 25 °C for 5 min, and the absorbance at 560 nm by using in a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland) was measured against blank samples. L-Ascorbic acid was used as a control. Decreased absorbance of the reaction mixture indicated the increasing of superoxide anion scavenging activity. The percentage inhibition of superoxide anion generation was calculated using the following formula

$$\% \text{ inhibition}=[(A_0-A_1)/A_0]\times 100$$

where A_0 was the absorbance of the control (L-Ascorbic acid), and A_1 was the absorbance of propofol or standard compounds.[23)]

Total Reduction Capability Total reduction capability of propofol was estimated by using the method of Oyaizu.[24)] The different concentration of propofol (25, 50, 75 μg/ml) in 1 ml of distilled water was 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 °C for 20 min. A portion (2.5 ml) of trichloroacetic acid (10%) was added to the mixture. Then, it was centrifugated for 10 min at 1000×***g*** (MSE Mistral 2000, U.K.). 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 by using a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland). Higher absorbance of the reaction mixture indicated greater reducing power.

Determination of Total Antioxidant Activity in Linoleic Acid Emulsion The total antioxidant activity of propofol was determined according to the ferric thiocyanate method.[25)] For stock solutions, 10 mg propofol was dissolved in 10 ml ethanol. Then, 25, 50, and 75 μg/ml of propofol or standards samples in 2.5 ml of potassium phosphate buffer (0.04 M, pH 7.0) were added to 2.5 ml linoleic acid emulsion in potassium phosphate buffer (0.04 M, pH 7.0). 5 ml linoleic acid emulsion consists of 17.5 μg Tween-20, 15.5 μl linoleic acid and 0.04 M potassium phosphate buffer (pH 7.0). Tween-20 was used as an emulgator. On the other hand, 5.0 ml control consists of 2.5 ml linoleic acid emulsion and 2.5 ml potassium phosphate buffer (0.04 M, pH 7.0). The mixed solution was incubated at 37 °C in a glass flask and in the dark. After the mixture was stirred for 3 min, the peroxide value was determined by reading the absorbance at 500 nm by using a spectrophotometer (8500 II, Bio-Crom Gmb, Zurich, Switzerland), after reaction with $FeCl_2$ and thiocyanate at intervals during incubation. During the linoleic acid oxidation, peroxides formed and these compounds oxidize Fe^{2+} to Fe^{3+}. The latter Fe^{3+} ions form complex with SCN^- and this complex has maximum absorbance at 500 nm. Therefore, high absorbance indicates high linoleic acid oxidation. The solutions without added propofol or standards used as blank samples. All data about total antioxidant activity are the average of duplicate analyses. The inhibition of lipid peroxidation in percentage was calculated by following equation:

$$\% \text{ inhibition}=[(A_0-A_1)/A_0\times 100$$

where A_0 was the absorbance of the control reaction and A_1 was the absorbance in the presence of the sample of propofol.[26)]

Statistical Analysis Experimental results were mean±S.D. of tree parallel measurements. Analysis of variance was performed by ANOVA procedures (SSPS 9.0 for Windows). Significant differences between means were determined by Duncan's Multiple Range tests. p values <0.05 were regarded as significant and p values <0.01 very significant.

Results and Discussion

Total Antioxidant Activity Determination in Linoleic Acid Emulsion System by Ferric Thiocyanate Method There are numerous antioxidant methods for evaluation of antioxidant activity. For antioxidant methods, total antioxidant activity, reducing power, DPPH assay, metal chelating, active oxygen species such as H_2O_2, $O_2\cdot^-$ and OH· quenching assay are most commonly used.[25)] However, the total antioxidant activities of an antioxdants cannot be evaluated by using one single method, due to oxidative processes. Therefore, at least two methods should be employed in order to evaluate the total antioxidant activity. In this study, we eavaluated the total antioxidant ability of propofol with six antioxidant assays.

Total antioxidant activity of propofol was determined by the thiocyanate method. Propofol exhibited effective and powerful antioxidant activity at all concentrations. The effects of various concentrations of propofol (from 25 to 75 μg/ml) on peroxidation of linoleic acid emulsion are shown in Fig. 1. The antioxidant activity of propofol increased with increasing concentration. The different concen-

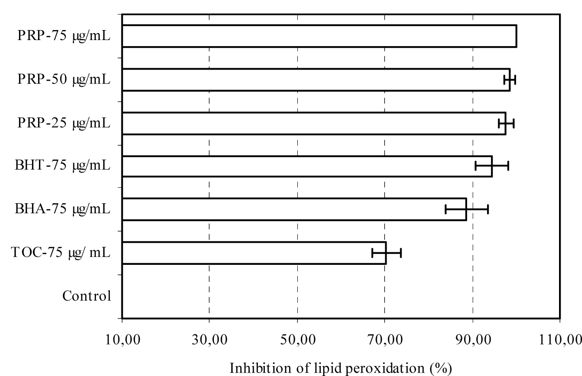

Fig. 1. Determination of Antioxidant Activity of Different Concentrations of Propofol and Standard Antioxidants Like α-Tocopherol, BHA and BHT in the Linoleic Acid Emulsion Determined by the Ferric Thiocyanate Method

TOC: α-tocopherol, PRP: propofol, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene.

tration of propofol (25, 50 and 75 μg/ml) showed higher antioxidant activities than that 75 μg/ml concentration of BHA, BHT, and α-tocopherol. The percentage inhibition of peroxidation of 25, 50 and 75 μg/ml concentrations of propofol in linoleic acid system was 97.7, 98.6 and 100%, respectively. On the other hand, percentage inhibition of 75 μg/ml concentration of BHA, BHT, and α-tocopherol was found 88.7, 94.5 and 70.4%, respectively.

Determination of Total Reductive Capability by Potassium Ferricyanide Reduction Method Figure 2 shows the reductive capabilities of propofol compared to BHA, BHT and α-tocopherol. For the measurements of the reductive ability, the Fe^{3+}–Fe^{2+} transformation in the presence of propofol samples was investigated.[24] The reducing capacity of a compound may serve as a significant indicator of its potential antioxidant activity. The antioxidant activity of an antioxidant compound has been attributed to various mechanisms, among which are prevention of chain initiation, binding of transition metal ion catalysts, decomposition of peroxides, prevention of continued hydrogen abstraction, reductive capacity and radical scavenging. Like the antioxidant activity, the reducing power of propofol increased with increasing concentration. All of the amounts of propofol showed higher activities than BHT and α-tocopherol and these differences were statistically very significant ($p<0.05$). Propofol and BHT statistically have similar reducing power ($p>0.05$). Reducing power of propofol and standard compounds followed the order: Propofol>BHT>BHA>α-tocopherol.

Superoxide Anion Radical Scavenging Activity Superoxide anion radicals are produced endogenously by flavoenzymes, *e.g.*, xanthine oxidase, which converts hypoxanthine to xanthine and subsequently to uric acid in ischemia-reperfusion. Superoxide is generated *in vivo* by several oxidative enzymes,including xanthine oxidase, In the PMS-NADH-NBT system, superoxide anion derived from dissolved oxygen by PMS-NADH coupling reaction reduces NBT. The decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Figure 3 shows the percentage inhibition of superoxide radical generation by 25 μg/ml concentration of propofol and comparison with same doses of BHA, BHT, and α-tocopherol. Propofol had strong superoxide radical scavenging activity and exhibited higher superoxide radical scavenging activity than BHA, but lower than BHT and α-tocopherol. These differences were not found statistically significant ($p>0.05$). The percentage inhibition of superoxide generation by 25 μg/ml concentration of propofol was found as 73.3%. On the other hand, at the same concentration, BHA, BHT and α-tocopherol have 69.6, 82.2 and 75.4% inhibition of superoxide radical generation, respectively. Superoxide radical scavenging activity of these samples followed the order: BHT>α-Tocopherol>Propofol>BHA.

Determination of Free Radical Scavenging Activity by DPPH· Method The model of scavenging the stable DPPH radical is a widely use method to evaluate antioxidant activities in a relatively short time compare with other methods. The effect of antioxidants on DPPH radical scavenging was thought to be due to their hydrogen donating ability. DPPH· is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[26] Propofol is chemically similar to phenol-based free radical

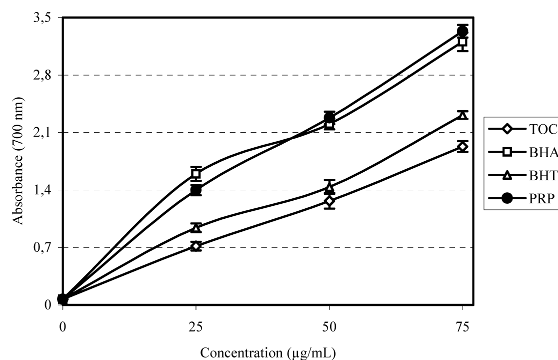

Fig. 2. Reductive Ability of Different Concentrations of Propofol, BHA, BHT and α-Tocopherol by Spectrophotometric Detection of the Fe^{3+}–Fe^{2+} Transformation

TOC: α-tocopherol, PRP: propofol, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene.

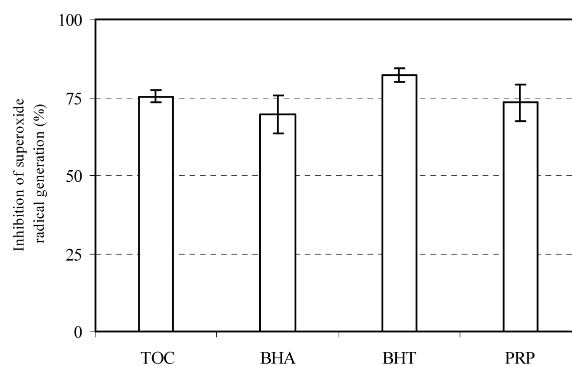

Fig. 3. Comparison of Percent Inhibition of Superoxide Anion Radical Generation of 25 μg/ml Concentration of Propofol, BHA, BHT and α-Tocopherol by the PMS-NADH-NBT Method

TOC: α-tocopherol, PRP: propofol, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene.

scavengers such as BHA, BHT and α-tocopherol. Murphy and colleagues demonstrated that propofol had been using electron spin resonance spectroscopy, to scavenge free radicals by a process of hydrogen abstraction leading to the formation of a phenoxyl radical.[27]

The reduction capability of DPPH radicals was determined by the decrease in its absorbance at 517 nm induced by antioxidants. The decrease in absorbance of DPPH radical caused by antioxidants, because of the reaction between antioxidant molecules and radical progresses, which results in the scavenging of the radical by hydrogen donation. It is visually noticeable as a discoloration from purple to yellow. Hence, DPPH· is usually used as a substrate to evaluate antioxidative activity of antioxidants. Figure 4 illustrates a significant ($p<0.01$) decrease the concentration of DPPH radical due to the scavenging ability of the propofol and standards. We used BHA, BHT and α-tocopherol as standards. The scavenging effect of propofol and standards on the DPPH radical decreased in the order of propofol>BHA>α-tocopherol>BHT and were 77, 67, 69 and 62% at the concentration of 50 μg/ml, respectively. The effects of propofol reached a plateau in the 50 μg/ml propofol in the figure. These results indicated that propofol has a noticeable effect on scavenging free radical. Free radical scavenging activity also increased with increasing concentration.

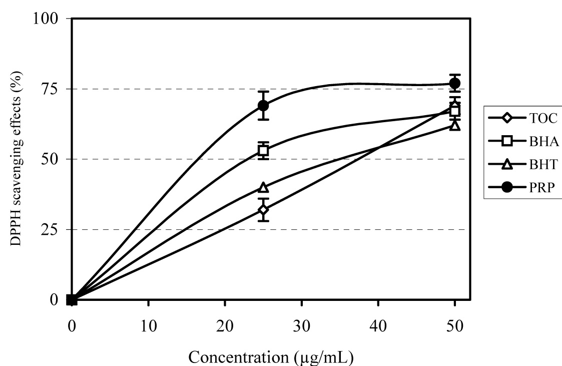

Fig. 4. Free Radical Scavenging Activity of Different Concentrations of Propofol, BHA, BHT and α-Tocopherol by 1,1-Diphenyl-2-picrylhydrazyl Radicals

TOC: α-tocopherol, PRP: propofol, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene.

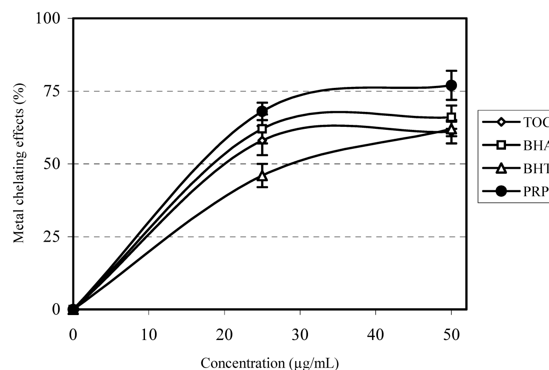

Fig. 5. Ferrous Ions Chelating Effect of Different Concentrations of Propofol, BHA, BHT and α-Tocopherol on Ferrous Ions

TOC: α-tocopherol, PRP: propofol, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene.

It was reported that oxidative stress, which occurs when free radical formation exceeds the body's ability to protect itself, forms the biological basis of chronic condition such as arteriosclerosis.[28] Based on the data obtained from this study, propofol is powerful free radical inhibitor or scavenger, as well as a primary antioxidant that reacts with free radicals, which may limit free radical damage occurring in the human body.

Iron Metal Ions Chelating Activity Iron can stimulate lipid peroxidation by the Fenton reaction, and also accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radicals that can themselves abstract hydrogen and perpetuate the chain reaction of lipid peroxidation.[19,29]

The ferrous ions chelating activity by propofol and standards were estimated by the method of Dinis *et al.*[18] Ferrozine can quantitatively form complexes with Fe^{2+}. In the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Measurement of colour reduction therefore allows estimation of the chelating activity of the coexisting chelator. In this assay, the propofol and standard antioxidant compounds interfered with the formation of ferrous and ferrozine complex, suggesting that they have chelating activity and capture ferrous ion before ferrozine.

As shown in Fig. 5, the formation of the Fe^{2+}-ferrozine complex is not completed in the presence of propofol, indicating that propofol chelate the iron.[30] The absorbance of Fe^{2+}-ferrozine complex was linearly decreased dose dependently (from 25 to 50 μg/ml). The difference between propofol and the control was statistically significant ($p<0.01$). The percentage of metal chelating capacity of 50 μg/ml concentration of propofol, α-tocopherol, BHA, and BHT were found as 77, 61, 66 and 62%, respectively. The effects of propofol reached a plateau in the 50 μg/ml propofol in the figure. The metal scavenging effect of propofol and standards were decreased in the order of propofol$>\alpha$-tocopherol$>$BHA$>$BHT.

Metal chelating capacity was significant since it reduced the concentration of the catalysing transition metal in lipid peroxidation. It was reported that chelating agents, which form σ-bonds with a metal, are effective as secondary antioxidants because they reduce the redox potential thereby stabilizing the oxidized form of the metal ion. The data obtained from Fig. 6 reveal that propofol demonstrates a marked capacity for iron binding, suggesting that their action as peroxidation protector may be related to its iron binding capacity.

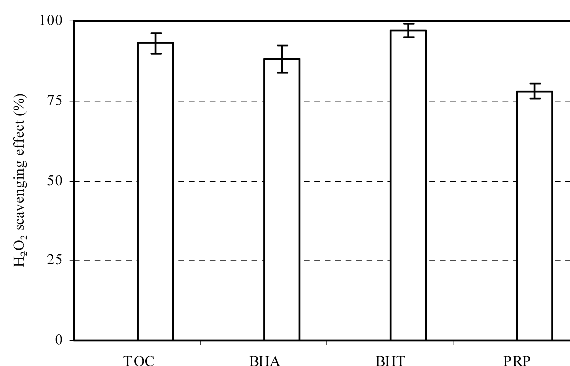

Fig. 6. Comparison of Hydrogen Peroxide Scavenging Activity of 25 μg/ml Concentration of Propofol, BHA, BHT and α-Tocopherol

TOC: α-tocopherol, PRP: propofol, BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene.

Scavenging of Hydrogen Peroxides H_2O_2 is highly important because of its ability of penetrate biological membranes. H_2O_2 itself is not very reactive, but it can sometimes be toxic to cell because of it may give rise to hydroxyl radical in the cells. Thus, removing of H_2O_2 is very important for protection of food systems.

The ability of propofol to scavenge H_2O_2 was determined according to the method of Ruch and colleagues.[20] Figure 6 shows the H_2O_2 scavenging activity by 25 μg/ml of propofol and comparison with same doses of BHA, BHT, and α-tocopherol. Propofol had strong H_2O_2 scavenging activity when compared to control ($p<0.01$). Moreover, this activity was close to BHA, BHT and α-tocopherol. The percentage of H_2O_2 scavenging activity by same concentration (25 μg/ml) of propofol BHA, BHT and α-tocopherol was found as 78% 88, 87 and 93%, respectively. These results showed that propofol had effective H_2O_2 scavenging activity. H_2O_2 scavenging activity of those samples followed the order: α-tocopherol$>$BHA$>$BHT$>$Propofol.

Conclusion

The results obtained from this study are clearly indicate

that propofol had powerful antioxidant activity against various antioxidant systems *in vitro*. The anesthetic agent propofol chemically resembles the chain-breaking antioxidant alfa-tocopherol, because it also has a phenolic hydroxyl group.[27) This phenolic hydroxyl group is responsible for the antioxidant properties of propofol, as found in various *in vitro* experiments.[31,32)]

The various antioxidant mechanisms of propofol also may be attributed to a strong lipid peroxidation inhibitor, reducing agent, metal chelator, hydrogen donating ability and their effectiveness as scavengers of hydrogen peroxide, superoxide, and free radicals. Propofol can be used as easily accessible synthetic and standard antioxidant in medicinal, pharmaceutical and food industries.

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