Radical Scavenging and Antioxidant Activity of *Carthamus tinctorius* Extracts

Rajesh Mandade[1]*, S.A. Sreenivas[2], Avijit Choudhury[3]

[1]Department of Pharmacology, S.N. institute of Pharmacy, Pusad, India. [2]Guru Nanak Institute of Pharmacy, Ibrahimpattnam, Hyderabad. (A.P.) India. [3]Shree Dhanvantary Pharmaceutical analysis and research centre, Kim, Surat, Gujarat India

ABSTRACT

Free radicals induce numerous diseases by lipid peroxidation, protein peroxidation, and DNA damage. It has been reported that numerous plant extracts have antioxidant activities to scavenge free radicals. In the present study, we examines the in vitro radical scavenging and antioxidant capacity of Crude extract of *Carthamus tinctorius* by using different in vitro analytical methodologies such as total antioxidant activity determination by ferric thiocyanate, hydrogen peroxide scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging, 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity and superoxide anion radical scavenging by riboflavin–methionine-illuminate system. Also, butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and α-tocopherol were used as the reference antioxidant radical scavenger compounds.

Extract inhibited 94.50% lipid peroxidation of linoleic acid emulsion at 20 μg/mL concentration. On the other hand, the above mentioned standard antioxidants indicated an inhibition of 93.75%, 96.66% and 83.33% on peroxidation of linoleic acid emulsion at 60 μg/mL concentration, respectively. In addition, hydrogen peroxide scavenging, DPPH scavenging, $ABTS^{+}$ radical scavenging and superoxide anion radical scavenging. Also, those various antioxidant activities were compared to BHA, BHT and α-tocopherol as references antioxidant compounds. The present study shows that Extract is the effective natural antioxidant component.

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Keywords: Antioxidant activity; scavenging activity, free radical, *Carthamus tinctorius*.

***Correspondence: Phone:** +917233249795 Fax: 07233247308

Mob. No. +918888673088; raj_mandade@rediffmail.com

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INTRODUCTION

Antioxidants help organisms deal with oxidative stress, caused by free radical damage. Free radicals are chemical species, which contains one or more unpaired electrons due to which they are highly unstable and cause damage to other molecules by extracting electrons from them in order to attain stability. Reactive oxygen species (ROS) formed in vivo, such as superoxide anion, hydroxyl radical and hydrogen peroxide, are highly reactive and potentially damaging transient chemical species. These are continuously produced in the human body, as they are essential for energy supply, detoxification, chemical signaling and immune function. ROS are regulated by endogenous superoxide dismutase, glutathione peroxidase and catalase but due to over-production of reactive species, induced by exposure to external oxidant substances or a failure in the defense mechanisms, damage to cell structures, DNA, lipids and proteins[1] occur which increases risk of more than 30 different disease processes.[2]

Carthamus tinctorius L. (Safflower) has long been used as Chinese medicine in clinics to treat cardiovascular disease, and has demonstrated anti-myocardial ischemia effects.[3,4] Wagner et al.[5] developed the C. tinctorius monograph and gave it a comprehensive introduction. Safflower also possesses other pharmacological effects, including anti- coagulant[6] and neuroprotective.[7] The chemical constituents in safflower are reported to be Flavonoid,[8] lignans, triterpene alcohols and polysaccharides,[9] among others. Safflower has also been reported to prevent electro- physiological abnormalities induced by hydrogen peroxide in guinea pig ventricular myocytes.[10] It can be used to improve neuropsychological disorders.[11]

The free radical neutralizing property of several plants was reported by previous studies. The extracts from number of medicinal plants which are known to have some biologically active principles are used in ayurvedic preparations and these extracts are prepared in bulk for commercial purpose. In this present study we have measured antioxidant activity of *Carthamus tinctorius* extracts by using different in vitro analytical methodologies such as total antioxidant activity determination by ferric thiocyanate, hydrogen peroxide scavenging, 1,1-diphenyl-2-picryl-hydrazyl free radical (DPPH) scavenging, 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical scavenging activity and superoxide anion radical scavenging by riboflavin–methionine-illuminate system.

MATERIAL AND METHODS

Plant Material

Aerial part of *Carthamus tinctorius* collected in the month of April from the Hingoli district of Maharashtra India. Identification and authentication of the samples was done by using standard botanical monographs. They were further confirmed by Dr. Miss. A. Chaturvedi, Post Graduate Teaching Department of Botany, Rashtra Santa Tukadoji Maharaj Nagpur University, Nagpur (Voucher specimen no. 9715).

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Preparation of crude extract

The plant materials were cleaned, shade dried and coarsely ground. The powdered material was soaked in 70 % aqueous-methanol for 3 days with occasional shaking. It was filtered through a muslin cloth and then through a filter paper. This procedure was repeated thrice and the combined filtrate was evaporated on a rotary evaporator under reduced pressure to a thick, semi-solid mass of dark brown color, i.e. the crude extract (Co. Cr), yielding approximately 6.1%.[12]

Total antioxidant activity determination by ferric thiocyanate method (FTC)

The antioxidant activity of extract and standards was determined according to the ferric thiocyanate method[13] as described by Gulcin. For[14] stock solutions, 10 mg of extract were dissolved in 10 mL distillate water. Then, the solution which contains 20 μg/mL concentration of extract solution in 2.5 mL of sodium phosphate buffer (0.04 M, pH 7.0) was added to 2.5 mL of linoleic acid emulsion in sodium phosphate buffer (0.04 M, pH 7.0). Therefore, 5 mL of the linoleic acid emulsion was prepared by mixing and homogenising 15.5 μL of linoleic acid, 17.5 mg/g of tween-20 as emulsifier, and 5 mL phosphate buffer (pH 7.0).

On the other hand, 5 mL of control was composed of 2.5 mL of linoleic acid emulsion and 2.5 mL, 0.04 M sodium phosphate buffer (pH 7.0). The mixed solution (5 mL) was incubated at 37 °C in polyethylene flask. The peroxide level was determined by reading the absorbance at 500 nm in a spectrophotometer after reaction with $FeCl_2$ (3.5%) and thiocyanate (30%) at intervals during incubation. During the linoleic acid peroxidation, peroxides are formed and that leads to oxidation of Fe^{2+} – Fe^{3+}. The latter ions form a complex with ammonium thiocyanate and this complex has a maximum absorbance at 500 nm. This step was repeated every 5 h. The percentage inhibition values were calculated at this point (30 h). High absorbance indicates high linoleic acid emulsion peroxidation. The solutions without extract were used as blank samples. Total antioxidant activity determination was performed triplicate. The inhibition percentage of lipid peroxidation in linoleic acid emulsion was calculated by following equation:

$$\text{Inhibitation of Lipid peroxidation (\%)} = 100 - \left(\frac{As}{Ac \times 100}\right)$$

Where, A_C is the absorbance of control reaction which contains only linoleic acid emulsion and sodium phosphate buffer and A_S is the absorbance in the presence of sample extract or standard compounds.[15]

Hydrogen peroxide scavenging activity

The hydrogen peroxide scavenging assay was carried out following the procedure of Ruch et al.[16] For this aim, a solution of H_2O_2 (43 mM) was prepared in phosphate buffer (0.1 M, pH 7.4). Extract at the 20 μg/mL concentration in 3.4 mL phosphate buffer was added to 0.6 mL of H_2O_2 solution (0.6 mL, 43 mM). The absorbance value of the reaction mixture was recorded at 230 nm. Blank solution was containing the sodium phosphate buffer without H_2O_2. The concentration of hydrogen peroxide (mM) in the assay medium was determined using a standard curve (r^2: 0.9895):

$$\text{Absorbance} = 0{:}038 \times [H_2O_2] + 0{:}4397$$

The percentage of H_2O_2 scavenging of extract and standard compounds was calculated using the following equation:

$$H_2O_2 \text{ scavenging effect (\%)} = \left(\frac{1 - As}{Ac}\right) \times 100$$

Where, A_C is the absorbance of the control and A_S is the absorbance in the presence of the sample extract or standards.[17]

DPPH free radical scavenging activity

The methodology described by Gulcin[14] was used with slight modifications in order to assess the DPPH free radical scavenging capacity of Extract. Where in the bleaching rate of a stable free radical, DPPH was monitored at a characteristic wavelength in the presence of the sample. In its radical form, DPPH absorbs at 517 nm, but upon reduction by an antioxidant or a radical species its absorption decreases.

Briefly, 0.1 mM solution of DPPH was prepared in ethanol and 0.5 mL of this solution was added 1.5 mL of Extract solution in ethanol at different concentrations (20-60 μg/mL). These solutions were vortexes thoroughly and incubated in dark. A half hour later, the absorbance was measured at 517 nm against blank samples. Lower absorbance of the reaction mixture indicates higher DPPH free radical scavenging activity. A standard curve was prepared using different concentrations of DPPH. The DPPH concentration scavenging capacity was expressed as mM in the reaction medium and calculated from the calibration curve determined by linear regression (r^2: 0.9845):

$$\text{Absorbance} = 9.692 \times [\text{DPPH}] + 0.215$$

The capability to scavenge the DPPH radical was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = \left(\frac{1 - \text{As}}{\text{Ac}}\right) \times 100$$

Where A_C is the absorbance of the control which contains DPPH solution and A_S is the absorbance in the presence of extracts.[18,19]

ABTS radical cation decolorization assay

The spectrophotometric analysis of $ABTS^+$ radical scavenging activity was determined according to method of Re et al.[20] This method is based on the ability of antioxidants to quench the long-lived ABTS radical cation, a blue/green chromophore with characteristic absorption at 734 nm, in comparison to that of BHA, BHT and α-tocopherol a water-soluble α-tocopherol analogue. The $ABTS^+$ was produced by reacting 2 mM ABTS in H_2O with 2.45 mM potassium persulfate ($K_2S_2O_8$), stored in the dark at room temperature for four hours. Before usage, the $ABTS^+$ solution was diluted to get an absorbance of 0.750 ± 0.025 at 734 nm with sodium phosphate buffer (0.1 M, pH 7.4). Then, 1 mL of $ABTS^+$ solution was added 3 mL of Extract solution in ethanol at different concentrations (20-60 μg/mL).

After 30 min, the percentage inhibition of at 734 nm was calculated for each concentration relative to a blank absorbance. Solvent blanks were run in each assay. The extent of decolorization is calculated as percentage reduction of absorbance. For preparation of a standard curve, different concentrations of $ABTS^+$ were used. The $ABTS^+$ concentration (mM) in the reaction medium was calculated from the following calibration curve, determined by linear regression (r^2: 0.9841):

$$\text{Absorbance} = 4.6788 \times [\text{ABTS}] + 0.199$$

The scavenging capability of $ABTS^+$ radical was calculated using the following equation:

$$\text{ABTS scavenging effect (\%)} = \left(\frac{1 - \text{As}}{\text{Ac}}\right) \times 100$$

Where, A_C is the initial concentration of the $ABTS^+$ and A_S is absorbance of the remaining concentration of $ABTS^+$ in the presence of Extract.

Superoxide anion radical scavenging activity

Superoxide radicals were generated by method of Beauchamp and Fridovich[21] described by Zhishen et al.[22] with slight modification. Superoxide radicals are generated in riboflavin, methionine, illuminate and assayed by the reduction of NBT to form blue formazan (NBT^{2+}). All solutions were prepared in 0.05 M phosphate buffer (pH 7.8). The photo-induced reactions were performed using fluorescent lamps (20 W). The concentration of Extract in the reaction mixture was 20 μg/ml. The total volume of the reactant mixture was 3 mL and the concentrations of the riboflavin, methionine and NBT was 1.33×10^{-5}, 4.46×10^{-5} and 8.15×10^{-8} M, respectively. The reactant was illuminated at 25 °C for 40 min. The photochemically reduced riboflavin generated $O_2^{\cdot}$ This reduced NBT to form blue formazan. The unilluminated reaction mixture was used as a blank. The absorbance was measured at 560 nm. Extract was added to the reaction mixture, in which $O_2^{\cdot}$ Was scavenged, thereby inhibiting the NBT reduction. Decreased absorbance of the reaction mixture indicates increased superoxide anion scavenging activity. The inhibition percentage of superoxide anion generation was calculated by using the following formula:

$$O_2.\text{- scavenging effect (\%)} = \left(\frac{1 - \text{As}}{\text{Ac}}\right) \times 100$$

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where, A_C is the absorbance of the l-ascorbic acid and A_S is the absorbance of Extract or standards.[23,24]

RESULT AND DISCUSSION

A wide variety of in vitro methods have been set up to assess radical scavenging ability and antioxidant activity. Antioxidant capacity is widely used as a parameter for medicinal bioactive components. Different artificial species have been used such as 2, 2-azinobis-3-thylbenzothiazoline-6-sulfonic acid (ABTS) and 1, 1-diphenyl-2-picrylhydrazyl (DPPH) and radicals scavenging activity, H_2O_2 scavenging activity. In this study, the antioxidant activity of the extract of *Carthamus tinctorius* was compared to BHA, BHT and α-tocopherol.

Total antioxidant activity determination in linoleic acid emulsion system by ferric thiocyanate method

Lipid peroxidation contains a series of free radical-mediated chain reaction processes and is also associated with several types of biological damage. The role of free radicals and ROS is becoming increasingly recognized in the pathogenesis of many human diseases, including cancer, aging and atherosclerosis.[25] The ferric thiocyanate method measures the amount of peroxide produced during the initial stages of oxidation which is the primary product of lipid oxidation.

Total antioxidant activity of extract, BHA, BHT and α-tocopherol was determined by the ferric thiocyanate method in the linoleic acid system. Extract showed effective antioxidant activity in this system. The effect of different concentration (20-60 μg/mL) of extract on lipid peroxidation of linoleic acid emulsion is shown in Figure 1.

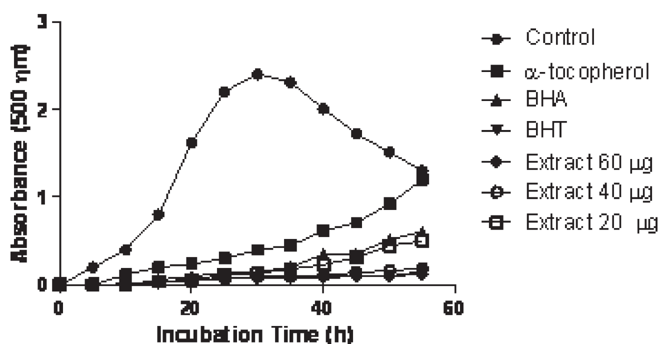

Figure 1. Total antioxidant activities of *Carthamus tinctorius* extract (20 μg/mL) and standard antioxidant compounds such as BHA, BHT and α-tocopherol at the concentration of 60 μg/mL (BHA: butylated hydroxyanisole, BHT: butylated hydroxytoluene). Total antioxidant activity determined by ferric thiocyanate method (FTC).

At these concentrations, extract caused 94.50%, 96.03% and 96.45% lipid peroxidation inhibition of linoleic acid emulsion. Their activities are greater than 60 μg/mL concentration of BHA (93.75%), α-tocopherol (83.33%), but close to BHT (96.66%). Consequently, these results clearly indicate that extract has an effective and powerful antioxidant activity by ferric thiocyanate method.

Hydrogen peroxide scavenging activity

Hydrogen peroxide has strong oxidizing properties. It 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 ability of extract to scavenge hydrogen peroxide was determined according to the method of Ruch et al (1989) as shown in Table 1 and compared with that of BHA, BHT and α-tocopherol as standards. Hydrogen peroxide scavenging activity of extract at the used concentration 20 μg/mL was found to be 63.9 ± 3.2%. On the other hand, BHA, BHT and α-tocopherol exhibited 38.2 ± 2.8, 36.3 ± 3.2 and 41.2 ± 2.7 hydrogen peroxide scavenging activity at the same concentration, respectively.

These results showed that extract had an effective hydrogen peroxide scavenging activity. At the above concentration, the hydrogen peroxide scavenging effect of extract and four standard compounds decreased in the order of extract > α-tocopherol > BHA > BHT. 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.

Radical scavenging activity

Radical scavenging activities are very important due to the deleterious role of free radicals in foods and in biological systems. Diverse methods are currently used to assess the antioxidant activity of plant phenolic compounds. Chemical assays are based on the ability to scavenge synthetic free radicals, using a variety of

Table 1.

	H_2O_2 scavenging activity (%)	Superoxide scavenging activity (%)
BHA	38.2 ± 2.8	76.4 ± 5.3
BHT	36.3 ± 3.2	72.2 ± 6.4
α-Tocopherol	41.2 ± 2.7	24.1 ± 3.2
Extract	63.9 ± 3.2	74.2 ± 3.7

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radical-generating systems and methods for detection of the oxidation end-point. $ABTS^+$ or DPPH radical scavenging methods are common spectrophotometric procedures for determining the antioxidant capacities of components.

These chromogens (the violet DPPH radical and the blue green ABTS radical cation) are easy to use, have a high sensitivity, and allow for rapid analysis of the antioxidant activity of a large number of samples. These assays have been applied to determine the antioxidant activity of pure components.[26,27,28] DPPH has been widely used to evaluate the free radical scavenging effectiveness of various antioxidant substances.[29] In the DPPH assay, the antioxidants were able to reduce the stable radical DPPH to the yellow colored diphenyl-picrylhydrazine. The method is based on the reduction of alcoholic DPPH solution in the presence of a hydrogen-donating antioxidant due to the formation of the non-radical form DPPH–H by the reaction. DPPH is usually used as a reagent to evaluate free radical scavenging activity of antioxidants. DPPH is a stable free radical and accepts an electron or hydrogen radical to become a stable diamagnetic molecule.[30]

With this method it was possible to determine the antiradical power of an antioxidant by measuring of a decrease in the absorbance of DPPH at 517 nm. Results a colour change from purple to yellow, the absorbance decreased when the DPPH was scavenged by an antioxidant through donation of hydrogen to form a stable DPPH molecule. In the radical form, this molecule had an absorbance at 517 nm which disappeared after acceptance of an electron or hydrogen radical from an antioxidant compound to become a stable diamagnetic molecule. Figure 2 illustrates a significant decrease ($p < 0.01$) in the concentration of DPPH radical due to the scavenging ability of Extract and standards. BHA, BHT and α-tocopherol were used as references. The scavenging effect of Extract and standards on the DPPH radical decreased in the order of BHA ≈ Extract > BHT > α-tocopherol which were 86.63%, 86.49%, 71.54% and 59.55%, at the concentration of 60 μg/mL, respectively. DPPH free radical scavenging activity of Extract also increased with an increasing concentration (r^2: 8246).

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 pure substances solutions, aqueous mixtures and beverages.[31] 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.

All the tested compounds exhibited affectual radical cation scavenging activity. As seen in Figure 3, extract had effective $ABTS_{\bullet}^{+}$ radical scavenging activity in a concentration-dependent manner (20-60 μg/mL). There is a significant decrease ($p < 0.01$) in the all of concentration of $ABTS_{\bullet}^{+}$ due to the scavenging capacity of all extract concentrations. Also, the scavenging effect of extract and standards on the $ABTS_{\bullet}^{+}$ decreased in that order: BHA > BHT > extract > α-tocopherol, which were 97.33%, 97.06%, 96.80% and 73.33% at the concentration of 60 μg/mL, respectively.

Superoxide is biologically quite toxic and is deployed by the immune system to kill invading microorganisms. It is an oxygen-centred radical with selective reactivity. It also produced by a number of enzyme systems in

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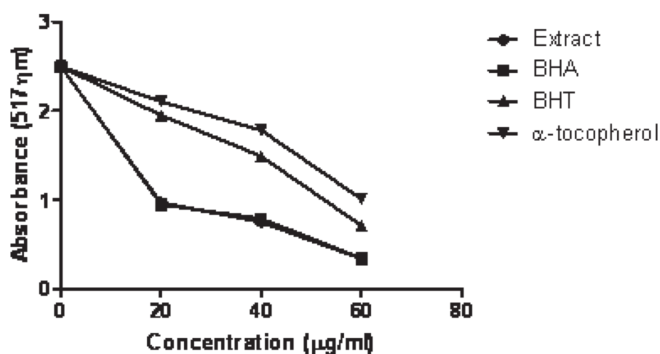

Figure 2. DPPH free radical scavenging activity of different concentrations (20-60 μg/mL) of *Carthamus tinctorius* extract and reference antioxidants; BHA, BHT and α-tocopherol.

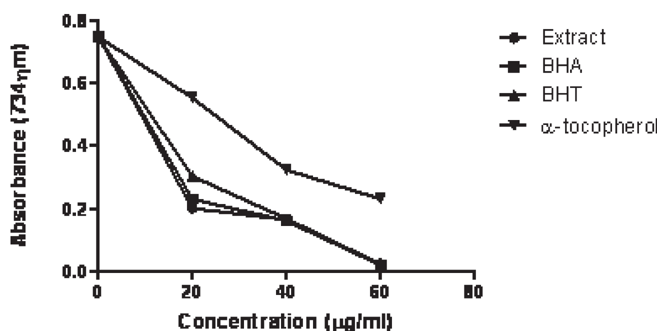

Figure 3. ABTS free radical scavenging activity of different concentrations (20-60 μg/mL) of *Carthamus tinctorius* extract and reference antioxidants; BHA, BHT and α-tocopherol.

auto oxidation reactions and by non-enzymatic electron transfers that univalently reduce molecular oxygen. The biological toxicity of superoxide is due to its capacity to inactivate iron–sulfur cluster containing enzymes, which are critical in a wide variety of metabolic pathways, thereby liberating free iron in the cell, which can undergo Fenton-chemistry and generate the highly reactive hydroxyl radical. It can also reduce certain iron complex such as cytochrome c.

Superoxide anions are a precursor to active free radicals that have potential of reacting with biological macromolecules and thereby inducing tissue damage. It has been implicated in several pathophysiological processes due to its transformation into more reactive species such as hydroxyl radical that initiate lipid peroxidation. Also, superoxide has been observed to directly initiate lipid peroxidation.[32] In addition, it has been reported that antioxidant properties of some flavonoids are effective mainly via scavenging of superoxide anion radical. Superoxide anion plays an important role in the formation of other ROS such as hydrogen peroxide, hydroxyl radical, and singlet oxygen, which induce oxidative damage in lipids, proteins and DNA.[33] Superoxide radical is normally formed first, and its effects can be magnified because it produces other kinds of free radicals and oxidizing agents. Superoxide anion derived from dissolved oxygen by riboflavin/methionine/illuminate system and reduces NBT in this system. In this method, superoxide anion reduces the yellow dye (NBT^{2+}) to produce the blue formazan which is measured spectrophotometrically at 560 nm.

Antioxidants are able to inhibit the blue NBT formation and[34] the decrease of absorbance at 560 nm with antioxidants indicates the consumption of superoxide anion in the reaction mixture. Table 1 shows the inhibition percentage of superoxide radical generation by 20 μg/mL concentration of Extract and standards. The inhibition of superoxide radical generation results of Extract and standards were found to be similar statistically. As shown in Table 1, the percentage inhibition of superoxide anion radical generation by 20 μg/mL concentration of Extract was found as 74.2 ± 3.7 %.

On the other hand, at the same concentration, BHA, BHT and α-tocopherol exhibited 76.4 ± 5.3, 72.2 ± 6.4 and 24.1 ± 3.2% superoxide anion radical scavenging activity, respectively. According to these results, Extract had similar superoxide anion radical scavenging activity to BHA and BHT; however, it had higher superoxide anion radical scavenging activity than α-tocopherol.

CONCLUSION

According to data obtained from the present study, *Carthamus tinctorius* extract was found to be an effective antioxidant in different in vitro assay including reducing power, DPPH radical, ABTS radical, superoxide anion radical scavenging and hydrogen peroxide scavenging when it is compared to standard antioxidant compounds such as BHA, BHT and tocopherol.

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