Antioxidant and radical scavenging properties of *Carthamus caeruleus* L extracts grow wild in Algeria flora

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Abstract
Four sub-fractions of *Carthamus caeruleus* L (which belongs to the family Asteraceae) root extracts, i.e., methanol (CE), chloroform (CHE), ethyl acetate (EAE) and aqueous (AE) extracts were screened for their total phenolics, flavonoids and possible antioxidant activities, using DPPH free radical-scavenging and β-carotene/linoleic acid assays. The results indicated that total polyphenols were highest in EAE followed by CHE. However, the free radical scavenging activity of CHE was higher than the other extracts with an IC_{50} value of 53.26 µg/mL. Determination of quercetin, gallic acid and rutin showed antioxidant activity as positive controls in parallel experiments. Moreover, oxidation of linoleic acid was effectively inhibited by AE, EAE and CE which were too close to each other. Also, the CHE was more effective compared to the synthetic antioxidant BHT. It could be concluded that *Carthamus caeruleus* L growing wild in the Mediterranean regions and especially in Algeria has a potent antioxidant activity and could be evaluated as a starting point for further investigations.

Keywords: *Carthamus caeruleus* L., polyphenol, antioxidant activity, DPPH, β-carotene, linoleic acid.

Propriedades dos radicais antioxidantes extraídos de *Carthamus caeruleus* L nativos da flora da Argélia

Resumo
Quarto sub-frações extraídas da raiz de *Carthamus caeruleus* L (que pertencem à família Asteraceae), i.e., metanol (CE), clorofórmio (CHE), acetato de etil (EAE) e extratos aquosos foram examinados minuciosamente quanto aos seus fenólicos, flanóides e suas atividades antioxidantes possíveis, usando DPPH extração de radicais livres e B-caroteno/ácido linoléico. Os resultados indicaram que os polifenóis totais foram maiores em EAE seguido de CHE. Entretanto, a atividade de extração de radicais livres em CHE foi maior que de outros extratos com um valor de IC_{50} de of 5326 µg/mL. A determinação de quercetina ácido gálico e rutina demonstrou que a atividade de antioxidante funciona como controle positive em experimentos paralelos. Além disso, a oxidação de ácido lenoico foi efetivamente inibida por AE, EAE e CE que estavam muito próximos uns aos outros. Também, o CHE foi mais eficaz se comparado ao antioxidante sintético BHT. Foi possível concluir que *Carthamus caeruleus* L nativos das nas regiões do Mediterrâneo, e, especialmente na Argélia, tem uma potente atividade antioxidante e poderia ser avaliada como o ponto de partida para investigações futuras.

Palavras-chave: *Carthamus caeruleus* L., polifenol, atividade antioxidante, DPPH, B-caroteno/ácido linoleico.

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Introduction

Formation of the free radicals may play an important role in the origin of life. The increased production of the superoxide free radical has come to occupy a remarkable central role in a wide variety of diseases, as documented by thousands of published studies (McCord, 2000). For example, oxygen radicals exert critical actions such as signal transduction, gene transcription and regulation of soluble guanylate cyclase activity in cells (Lander, 1997; Zheng & Storz, 2000). However, free radicals and other relative species cause the oxidation of biomolecules (e.g., protein, amino acids, lipid, and DNA), which leads to cell injury and death (McCord, 2000; Lander, 1997; Fridovich, 1999; Zheng & Storz, 2000). Reactive oxygen species (ROS) which are formed during normal cell aerobic respiration are the main cause of cell damage, mainly related to cancer, cardiovascular diseases, and other cell damages. The cytotoxic effect of free radicals is deleterious to mammalian cells (Koleva et al., 2002; Ou et al., 2002).

Antioxidants have been widely used as food additives to provide protection against oxidative degradation of foods by free radicals. Since ancient times, spices in different types of food to improve flavours are well known for their antioxidant capacities (Madsen & Bertelsen, 1995). In addition to that and to prolong the storage stability of foods, synthetic antioxidants such as butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT) are widely used as potential inhibitors of lipid peroxidation and thereby stabilizing fat-containing food stuffs. Nevertheless, due to their unstable and highly volatile nature, they have frequently brought some questions on their safety and efficiency ever since their first introduction to food industry (Dapkevicius et al., 1998). According to toxicologists and nutritionists (Branen, 1975; Chihoung et al., 1992; Raza et al., 2009), the side effects of the synthetic antioxidants (BHT, BHA) used in food processing have already been documented. For example, these substances can exhibit carcinogenic effects in living organisms (Ames, 1983; Baardseth, 1989). Many edible plant species such as tea, fruits, juices, spices, and vegetables throughout the history of mankind have been attractive to scientists as natural sources of compounds that are safer than the synthetic ones. Many other plants have also been screened for their antioxidant capacities, and attempts led to the introduction of natural antioxidants such as those from rosemary and sage (Schuler, 1991). Antioxidants display different behaviours, and one of these is related to the prevention from the reactive oxygen species (ROS). The genus Carthamus (Safflower) of the family Asteraceae, includes 14 species. It is a rare perennial grasses species that prefers sunny site in the Mediterranean basin, it is from the South - West Asia (Mioulane, 2004), but widespread in Asia, North Africa, Australia, both Americas , and Europe (Boullard, 2001). Safflower oil meal is mainly used as animal feed. Safflower cake has the potential to be used as a human food if the bitter principles are removed (Nagaraj, 1995). Safflower cake in combination with all-purpose flour in 1:3 proportion was found to be highly suitable for manufacturing of protein- enriched biscuits with 22% protein in them (Singh & Abidi, 2005). In Algerian folk medicine, *C. Caeruleus* is widely used for many inflammatory cases. Considering all these points, the aim of the present study was to investigate the antioxidant capacity of *Carthamus caeruleus* L. and its correlation with polyphenolic contents.

Materials and Methods

Plant material

The roots of *Carthamus caeruleus* were collected from Bouandas region, Wilaya (Department) of Setif northeast of Algeria during the summer of 2008, and authenticated by Dr. Daniel Jeanmonod, conservator of Geneva University, Switzerland. A voucher specimen was deposited at the Laboratory of Botany, Department of Biology, Faculty of Sciences, University Ferhat Abbas of Setif, Algeria.

Chemicals

Methanol, hexane, chloroform, ethyl acetate, Tween 40 and hydrochloric acid were obtained from Merck (Darmstadt, Germany). Potassium ferricyanide [$K_3Fe(CN)_6$], ferric chloride ($FeCl_3$), phosphoric acid (H_3PO_4), aluminum chloride ($AlCl_3$), 2,2'-diphenylpicrylhydrazyl (DPPH), gallic acid, butylated hydroxytoluene (BHT), β-carotene and linoleic acid were purchased from Sigma Chemical Co. (Germany). All solvents used were HPLC grade.

Extraction procedure

The extractions of flavonoids were carried out using various polar and non-polar solvents according to the method described earlier (Markham, 1982) with slight modifications. Dried plant material was ground in warring blender, mixed with a 10-20 volume of 85% aqueous methanol. The slurry was placed on shaker for 24 h and the extract was filtered through a Buchner funnel and the methanol was removed on rotary evaporator to give crude extract (fraction labeled CE). The aqueous solution was extracted several times with hexane, in order to eliminate lipids. The water fraction was partitioned against chloroform (labeled CHE). The remaining aqueous phase was exhaustively extracted with ethyl acetate until the final ethyl acetate extract was colorless (fraction labeled EAE) and the remaining aqueous extract was labeled AE. All solvents were removed by evaporation under reduced pressure and the extracts were stored at -20 °C until use.

Determination of total polyphenols content

Total polyphenols were measured using Prussian blue assay method described by Price & Butler (1977) and modified by Graham (1992). Phenolic contents were expressed as gallic acid equivalents. Briefly, 0.1 mL of *Carthamus caeruleus* root extract (CCRE) was dissolved in methanol and 3 mL distilled water were added and mixed up. One mL of $K_3Fe(CN)_6$ (0.016 M) was added to each sample followed by the addition of 1 mL of $FeCl_3$ (0.02 M dissolved in 0.1 M HCl) and immediately mixed up using a vortex. After the addition of the reagents to the sample, 5 ml/ mL stabilizer (30 mL of 1% gum arabic, 30 mL of 85% H_3PO_4 and 90 mL distilled water) were added to the sample and mixed up. The absorbance was measured at 700 nm using a UV/VIS-8500 Techom spectrophotometer. The amount of total polyphenols in different extracts was determined from a standard curve of gallic acid ranging from 0.00 to 200 µg/mL.

Determination of flavonoids

Flavonoids were measured by $AlCl_3$ method described previously (Bahorun et al., 1996) and expressed as quercetin equivalents. One ml of the plant extracts samples was dissolved in methanol, 1 mL of $AlCl_3$ (2 %) in methanol was added. After incubation for 10 min, the absorbance was measured at 430 nm.

Purification of milk xanthine oxidoreductase (XOR)

XOR was routinely purified from mammalian milk in the presence of 10 mM of dithiothreitol by ammonium sulphate fractionation followed by affinity chromatography on heparin-agarose as previously described for human, bovine, sheep and camel (Baghiani et al., 2002; Baghiani et al., 2003). XOR concentration was determined by UV-visible spectrum using an absorption coefficient of 36000 M/cm at 450 nm. The purity of enzyme was assessed on protein/flavin ratio (PFR = A_{280}/ A_{450}) (Bray, 1975) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (10%) (Laemmli, 1970). The activity of XOR was specrophotometrically determined by measuring the production of uric acid from xanthine (100 µM, final concentration) at 295 nm using an absorption coefficient of 9600 M/cm (Avis et al., 1956). Assays were performed at room temperature in air-saturated with 50 mM phosphate buffer and pH 7.4 supplemented with 0.1 mM EDTA.

Determination of anti-radical power of CCRE

Anti-radical activity was determined spectrophotometrically (Robak & Gryglewski, 1988) by monitoring the effect of CCRE on superoxide anion radicals (which are able to reduce cytochrome c) produced by xanthine/ xanthine oxidase (XO) system. The reaction mixture contained xanthine (100 µM) with various amounts of plant extracts and horse heart cytochrome c (25 µM). The reactions were started by the addition of XO. The cytochrome c activity was calculated using an absorption coefficient of 21100 M/cm and the sensibility of the reaction was determined by using bovine erythrocytes superoxide dismutase (SOD) (330 U/ mL final concentration).

Effects of CCRE on XOR activity

The effect of CCRE on the xanthine oxidation was examined spectrophotometrically at 295 nm following the production of uric acid using an absorption coefficient of 9600 M/cm (Avis et al., 1956). Assays were performed at room temperature in the presence of final concentration of 100 µM of xanthine with various amounts of plant extracts. The reaction was started by the addition of BXOR (1176 nmol of urate/min/mg protein). Enzyme activity of the control sample was set to 100% activity.

Antioxidant activity

DPPH assay

The hydrogen atom or electron donation abilities of the corresponding extracts and some pure compounds were measured from the bleaching of the purple-coloured methanol solution of 2, 20-diphenylpicrylhydrazyl (DPPH). This spectrophotometric assay used the stable radical DPPH as a reagent (Cuendet et al., 1997; Burits & Bucar, 2000). Fifty µL of various concentrations of the extracts in methanol were added to 5 mL of a 0.004 % methanol solution of DPPH. After a 30 min incubation period at room temperature, the absorbance was read against a blank at 517 nm. Inhibition percent (I%) of free radical by DPPH was calculated according to the following equation:

$$I\% = (A_{blank} - A_{sample}/A_{blank}) \times 100$$

Where A_{blank} is the absorbance of the control reaction (containing all reagents except the test compound), and A_{sample} is the absorbance of the test compound. Extract concentration providing 50% inhibition (IC_{50}) was calculated form the plot of inhibition percentage against the extract.

Determination of β-caroten-linoleic acid

The antioxidant capacity was determined by measuring the inhibition of the volatile organic compounds and the conjugated diene hydroperoxides arising from linoleic acid oxidation (Dapkevicius et al., 1998). "i.e., the methodology described by Dapkevicius et al. (1998)".

Statistical analysis

All samples were analyzed in triplicate and data were expressed as means ± SE using InStat software (Graphpad, San Diego, California). Analysis of variance was used to test for differences between groups. The Tukey-Kramer multiple

range test was used to determine the significance of differences between the mean values of the treatment groups at the level of $p \leq 0.05$ (Sokal & Rohlf, 1969).

Results and discussion

Total phenolics and flavonoids were estimated at µg/ mg dry weight extract. The data presented in Table 1 indicated that the amount of total phenolics was highest in EAE (75.710 ± 4.878 µg/ mg) followed by CHE (36.899 ± 1.863 µg/ mg), CE (12.966 ± 0.727 µg/ mg) and AE (10.358 ± 0.428 µg/ mg). The inhibition of linoleic acid oxidation and the DPPH radical scavenging activities of CHE were more effective than EAE. Despite the fact that EAE contain more polyphenols than CHE indicating that non-polar phenols may play an important role in preventing the formation of conjugated dienes (Table 1). The DPPH radicals scavenging activities of the extracts and inhibition of linoleic acid oxidation led to the general conclusion that the activity observed in various extracts of *Carthamus caeruleus* is closely related to the content of phenolic compounds (Bull et al., 2002). Moreover, CHE exhibited stronger activity than the others. Similar and moderate activities of non-polar extracts can be attributed to the presence of several types of compounds belonging to different classes (Dapkevicius et al., 1998). Furthermore, a positive and significant correlation existed between antioxidant activity and total phenolic content which revealed that phenolic compounds were the dominant antioxidant (Sánchez-Gonzalez et al., 2005; Maksimović et al., 2005). In the same concern, *Carthamus caeruleus* was reported to have potent singlet oxygen quencher (Kambayashi et al., 2006).

Table 1. Total polyphenol and flavonoid contents of *Carthamus caeruleus* root extracts (CCRE).

Extracts	Total Polyphenols and Flavonoids	
	mg equivalent gallic acid g^{-1} lyophilisat	mg equivalent quercetin g^{-1} lyophilisat
CE	12.966 ± 0.727	2.231 ± 0.146
CHE	36.899 ± 1.863	9.984 ± 0.080
EAE	75.710 ± 4.878	7.065 ± 0.336
AE	10.358 ± 0.428	1.508 ± 0.094

CE: crude (methanol) extract, CHE: chloroform extract, EAE: ethyl acetate extract, AE: aqueous extract.

The results of the freshly purified milk XOR from different species showed an ultraviolet/ visible spectrum with three major peaks (280, 325, 450 nm) with A_{280}/A_{450} (protein to flavin ratio, PFR) of 5.15 indicating a high degree of purity (Bray, 1975). Run on SDS-PAGE, purified enzyme showed quite similar patterns with one major band of approximately 150 KDa. Moreover, traces of degradation bands appeared on storage (Abadeh et al., 1992).

The effect of CCRE on the generation of superoxide anion radicals by the xanthine/ xanthine oxidase system is illustrated in Figure 1. Cytochrome c^{3+} has been extensively used for the detection of $O_2^{\cdot-}$ produced in biological systems due to its fast superoxide-mediated reduction to cytochrome c^{2+} (McCord & Fridovich, 1968). The effect of the three tested *Carthamus caeruleus* root extracts, at different concentrations, were studied for their ability to scavenge superoxide anion radicals ($O_2^{\cdot-}$) generated by the xanthine/ xanthine oxidase system. The amount of generated $O_2^{\cdot-}$ was determined by measuring the reduction of cytochrome c. Under the current experimental conditions, the activity of cytochrome c in the absence of extracts (1706.16 nmols min/ mg protein) were reduced by $O_2^{\cdot-}$ generated by bovine XOR. The reduction of cytochrome c^{3+} was almost totally inhibited by superoxide dismutase (SOD) (330 U/mL). All the extracts were able to inhibit cytochrome c^{3+} in a concentration dependent manner (Figure 1). The most potent scavenger of superoxide anion radical observed was EAE with IC_{50} of 0.503 ± 0.0044 mg/mL followed by CHE and CE which exhibited the same effect on $O_2^{\cdot-}$ generating by bovine XOR (Figure 2).

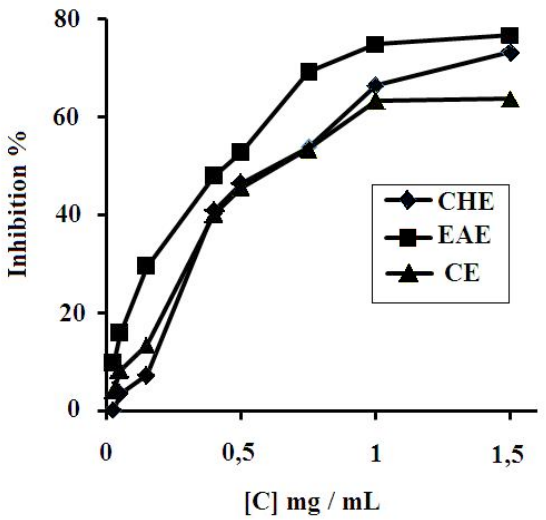

Figure 1. Inhibition of the generation of superoxide anion radicals from xanthine/xanthine oxidase by the plant extracts as measured by the cytochrome c test. Amount of superoxide anion radicals in the control sample without extract was set to 100%.

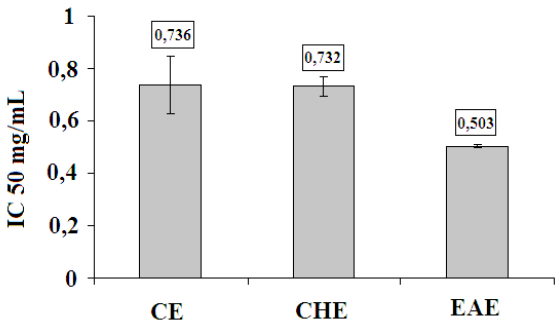

Figure 2. IC50, Inhibitory concentration of CCRE for 50% of Cyt C activity which reduced by O2-. Generate from BXOR (Results are means of three different experiment).

Since an inhibitory effect on the enzyme itself would also lead to decrease in reducing cytochrome c^{3+}, the effect of extracts on XO activity was checked. The effects of these extracts on the metabolic conversion of xanthine to uric acid are presented in Figure 3. In the xanthine/xanthine oxidase system, the extracts were effective in inhibiting the uric acid formation in a concentration dependent manner (Figure 3) which was demonstrated with the respective data. The most efficient one was CHE with IC_{50} of 0.0573 ± 0.0023 mg/mL followed by EAE IC_{50} of 0.105 ± 0.0013 than CE with IC_{50} of 0.706 ± 0.0039 (Figure 4).

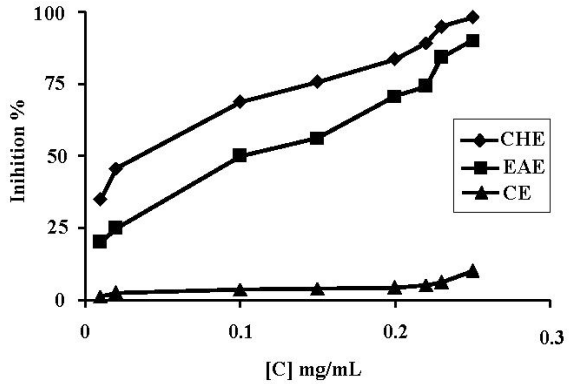

Figure 3. Effect of the extracts on the activity of xanthine oxidase as measured spectrophotometrically at 295 nm by the production of uric acid. Xanthine oxidase was added to xanthine (100 µM). Enzyme activity of the control sample without CCRE was set to 100%.

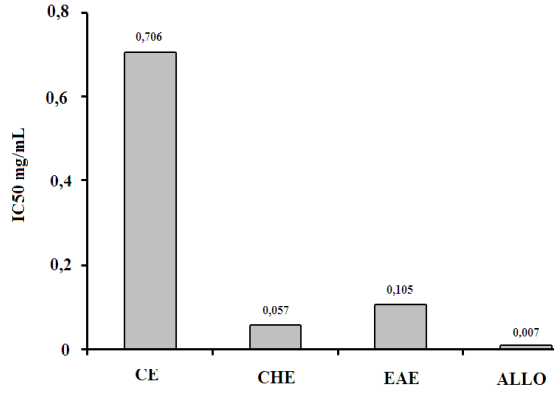

Figure 4. IC50, Inhibitory concentration of CSRE and allopurinol for 50% of BXOR activity. (Results are means of three different experiments).

The current results demonstrated that the extracts have an inhibitory effect on XO. Thereby, the inhibition of cytochrome c reduction is due to dual effects of the extracts. Firstly, these compounds inhibit the xanthine oxidase activity and secondly, some of them scavenge superoxide radical. Taking this fact into account, it was not possible to show a clear-cut scavenging effect on superoxide radicals.

In order to correlate the observed antioxidant activity with the phenolic composition of CCRE, total polyphenols were measured using Prussian blue assay methods. Values of total polyphenols and flavonoids in CCRE are shown in Table 1. There was a wide range of phenol concentration in CCRE. The values varied from 10.35 to 75.71 mg equivalent gallic acid/g lyophilisate and 1.5 to 9.98 mg equivalent quercetin/g lyophilisate for polyphenols and flavonoids, respectively. The highest level of polyphenols and flavonoids were recorded in the chloroform (CHE) and ethyl acetate (EAE) extracts.

Several flavonoids and other phenolic compounds are considered as antioxidants not only because they act as free radical scavengers, but also because of their ability to inhibit XO (Cos et al., 1998). The antioxidant activity of quercetin and its derivatives have been reported in several experimental models such as in the prevention of methyl linoleat hydroperoxide formation (Hopia & Heinonen, 1999). The scavenging of superoxide radical generated either by an enzymatic system or non-enzymatically has been reported (Robak & Gryglewski, 1988). The inhibition of XO was also observed with rutin (Selloum et al., 2001). These results indicated that CHE and EAE were sufficient to cause a noticeable effect on scavenging free radicals. Previously, it was reported that polyphenolics are associated with antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993).

According to Chu et al. (2000), total antioxidant activities of the plant extracts cannot be evaluated with a single method due to the complex nature of phytochemicals. Two or more methods should always be employed in order to evaluate the total antioxidative effects of plant material (Nuutila et al., 2003). Thus, in the current study we applied two complementary test systems 2,2'-diphenylpicrylhydrazyl (DPPH) and β-carotene-linoleic acid to evaluate the antioxidant capacities of *Carthamus caeruleus* root extracts. DPPH was used to elucidate the relationship between free radicals scavenging ability and the extracts. The addition of *Carthamus caeruleus* extracts (50 µL) to the DPPH solution induced a rapid decrease in the optical density at 517 nm, indicating the disappearance of the radical (Figure 5). The 50% inhibition of DPPH radical was obtained with lower concentrations. CHE exhibited notable antioxidative potential (IC_{50}, at 53.26 ± 1.74 µg/mL) flowed by CE (IC_{50}, at 187.38 ± 2.36 µg/mL, whereas AE and EAE showed a weak activity with an IC_{50} of 271.12 ± 3.67 µg/mL and 291.87 ± 4.98 µg/mL, respectively (Figure 5). Moreover, CCRE showed a dose-dependent activity on the inhibition of DPPH (Figure 6) as indicated by the visually noticeable discoloration from purple to yellow . The scavenging effect of CCRE was also dose-dependent ranging from 2 to 40 µg m/L CHE at 40 µg/mL showed 41.14% scavenging effect on

DPPH radical. The decrease in absorbance of the DPPH radical caused by CCRE may be due to the scavenging of the radical by hydrogen donation. Early studies demonstrated that DPPH test could be used to determine antioxidant activity of single compounds as well as different plant extracts (Brand-Williams et al., 1995). Furthermore, it was reported that polyphenolics are associated with the antioxidant activity and play an important role in stabilizing lipid peroxidation (Yen et al., 1993). According to Fessenden & Fessenden (1994), phenolic compounds could easily donate hydroxy hydrogen due to resonance stabilization. In this concern, previous reports (Koyama et al., 2008) indicated that the antioxidant activity of *Carthamus caeruleus* was due to its higher content of serotonin hydroxycinnamic acid amides. Moreover, another report indicated that *Carthamus caeruleus* induces its antioxidant effects via its major constituents of serotonin (Koyama et al., 2007). The current results are highly reproducible and comparable to the other free radical scavenging methods reported in the literature (Gil et al., 2000).

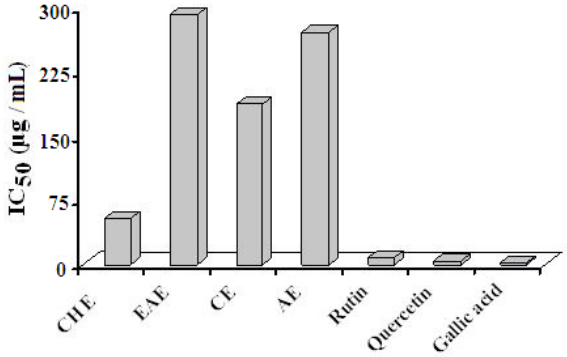

Figure 5. Free radical scavenging capacities of the *Carthamus caeruleus* extracts measured in DPPH assay. CHE: chloroform extract, EAE: ethyl acetate extract, CE: crud extract and AE: aqueous: extract.

In the β-carotene/linoleic acid assay, all extracts showed marked antioxidant activities when compared to the synthetic antioxidant BHT which had 96% at the same concentration (2 mg/ mL). The inhibition ratios of linoleic acid oxidation by AE, EAE and CE extracts were too close to each other (79.75% ± 2.34, 77.13% ± 1.32 and 75.14% ± 2.12, respectively), whereas, CHE was more effective with 81.008% ± 2.03 (Figures 7 and 8). The higher radical-scavenging activity of CHE could be related to the presence of phenolics such as phenolic acids and flavonoids especially aglycone flavonoids which play an important role in the radical scavenging activity (Abdel-Wahhab & Aly, 2003, 2005).

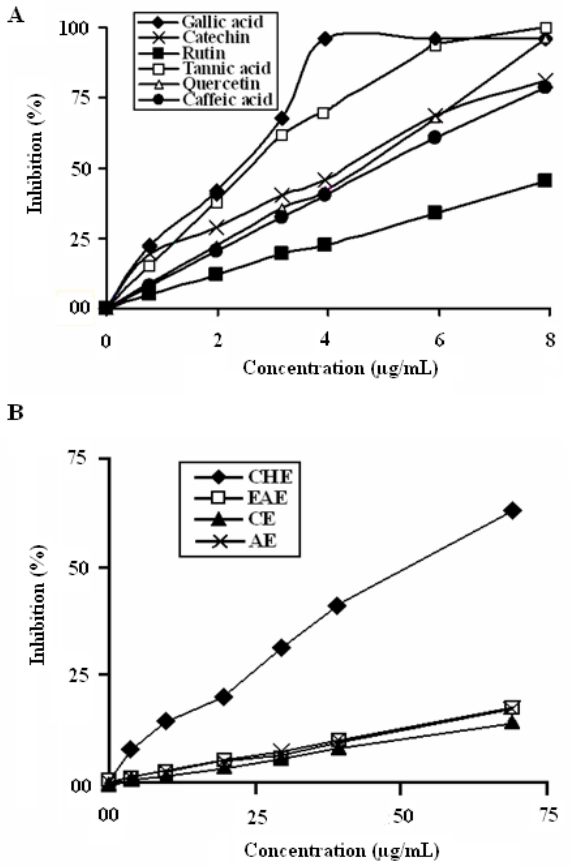

Figure 6. The percentage inhibition of free DPPH radical in the presence of different concentration of (A): antioxidants: gallic acid, quercetin, rutin, catechin, caffeic acid and tannic acid; (B): CCRE: CHE: chloroform extract, EAE: ethyl acetate extract, CE: crude extract and AE: aqueous: extract. Concentrations of antioxidants and plant extracts are expressed as µg ml-1 in reaction mixture.

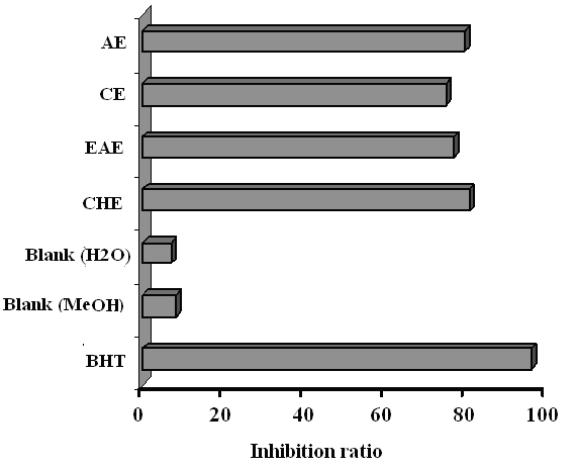

Figure 7. Inhibition ratio of the linoleic acid oxidation by *Carthamus caeruleus* extracts after 24 h.

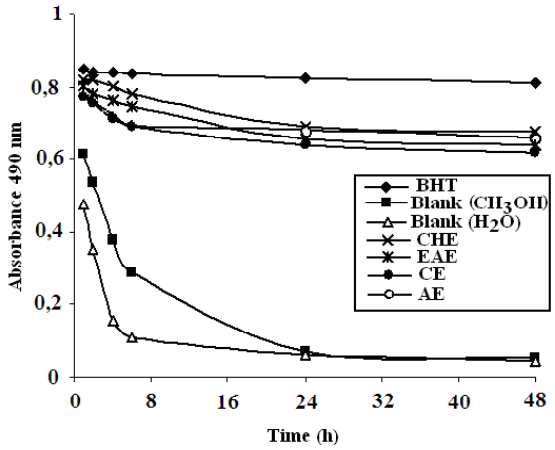

Figure 8. Absorbance change of ß-carotene at 490 nm in the presence of Carthamus caeruleus extracts (CHE: chloroform extract, EAE: ethyl acetate extract, CE: crud extract and AE: aqueous extract) and butylated hydroxytoluene (BHT).

Conclusions

It could be concluded that *Carthamus caeruleus* root extracts is an efficient inhibitor of xanthine oxidase and it has significant antioxidant and free radical scavenging properties due to its higher content of phenolic acids and flavonoids. CCRE has a rapid and strong inhibition of DPPH radical and β-carotene/linoleic acid presents a strong correlation between the values obtained from both assays. The inter-relationship between phenolic compounds and antioxidant activity is a promising to understand and to elucidate possible mechanisms for the functionality of traditional medicinal plants taken for diseases prevention and treatment.

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