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Antioxidant activity of *Kelussia odoratissima* Mozaff. in model and food systems

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Abstract

Kelussia odoratissima Mozaff. is a sweet-smelling, self-growing plant which is traditionally consumed in Iran as a garnish. Little, however, is known about its potential antioxidant activity. In this study, the antioxidant activity of the methanolic extract of the plant was evaluated using β-carotene bleaching assay, reducing power, thiocyanate, accelerated oxidation of sunflower oil, and DPPH radical-scavenging. In DPPH and reducing power models the antioxidant activity of the plant extract was generally found to be less effective than that of ascorbic acid, but it was comparable to and/or greater than the activities of α-tocopherol and BHT. Although the antioxidant activity of BHT in β-carotene bleaching assay was greater than that of the sample, the difference was not significant ($\rho < 0.01$). Ascorbic acid showed low activity in this assay. The activity of the plant extract in the thiocyanate model system was lower than that of BHT but greater than that of α-tocopherol. The methanolic extract inhibited the oxidation of sunflower oil at 60 ± 3 °C more efficiently than did BHT.

Keywords: *Kelussia odoratissima*; Antioxidant activity; DPPH; β-Carotene bleaching; Reducing power; Thiocyanate; Sunflower oil

1. Introduction

Rancidity is considered as a serious problem in food products, reducing their shelf life and nutritional quality. Antioxidants can be effectively used to prevent lipid oxidation (Abdalla & Roozen, 1999). Antioxidants are defined as "substances that, when present in low concentrations compared to those of an oxidizable substrate, significantly delay or inhibit oxidation of that substrate" (Sikorski, 2001). However, some synthetic antioxidants, namely, butylated hydroxyanisole (BHA) and butylated hydroxytoluene (BHT), have been suspected to be responsible for liver damage and carcinogenesis in laboratory animals (Hwang, Shue, & Chang, 2001). Numerous types of natural antioxidants with various activities have been identified but a lot of attention has recently been drawn to the addition of polyphenols to foods and biological systems, due to their known abilities to scavenge free radicals (Kulisic, Radonic, Katalinic, & Milos, 2004; Pinelo, Rubilar, Sineiro, & Núñez, 2004). Phenolic compounds are among the most widely distributed plant secondary products and are found in many plants used as food and feed (Hagerman et al., 1998).

The antioxidant activity of phenolic compounds in plants is mainly due to their redox properties and chemical structure, which can play important roles in neutralizing free radicals, chelating transitional metals, and quenching singlet and triplet oxygen molecules through delocalizing or decomposing peroxides. These properties are linked to beneficial health functionality of phenolic antioxidants due to their inhibitory effects against development of many oxidative-stress related diseases, such as cardiovascular, inflammatory bowel syndrome and Alzheimer's disease. Increased intake of fruits and vegetables has also been associated with reduced risk of coronary heart disease (CHD), stroke, diabetes, cancer, cataracts, atherosclerosis,

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arthritis, immune deficiency diseases, aging, and brain dysfunction (Chun, Vattem, Lin, & Shetty, 2005; Lee, Mitchell, & Shibamoto, 2000; Sikorski & Kołakowska, 2003).

Kelussia odoratissima Mozaff., locally called "Karafskoohi" in Iran, is commonly used in some parts of Iran as a popular garnish. It is also used as a folk medicine to treat hypertension, inflammation, ulcer, and cardiovascular diseases. The United Nations Developing Programme (UNDP) has recently announced Kelussia as an endangered plant and, therefore, investigations aimed at promoting its regular cultivation receive financial support from relevant bodies. The aim of this study was to determine the potential ability of *K. odoratissima* M. to postpone oxidation in model systems (*in vitro*) and in accelerated oxidation systems using sunflower oil.

2. Materials and methods

2.1. Chemicals and reagents

β-Carotene, α-tocopherol, epicatechin, linoleic acid, and DPPH were purchased from Sigma Chemical Co. (St. Louis, MO). BHT, Folin-Ciocalteu's phenol reagent, K-ferricyanide, ammonium thiocyanate, ascorbic acid, trichloroacetic acid, $FeCl_3 \cdot 6H_2O$, ferrous chloride and sodium carbonate were procured from Merck (Darmstadt, Germany). All solvents/chemicals used were of analytical grade and obtained from Merck.

2.2. Plant sample

Fresh plant sample was purchased from a local grocery, dried (at +24 °C in the dark), and stored in the freezer at −18 °C (Kähkönen et al., 1999). The sample was powdered in a coffee grinder before analysis.

2.3. Extraction of phenolics

Ground dry plant material (500 mg) was weighed into a test tube. A total of 10 ml of 80% aqueous methanol was added, and the suspension was stirred slightly. Tubes were sonicated for 5 min and centrifuged (Sigma 2–16) for 10 min at 1500*g*. Plant materials were re-extracted and supernatants were collected (Kähkönen et al., 1999). This solution was concentrated under reduced pressure to remove methanol and part of the water content. It was then freeze-dried to protect the potentially sensitive compounds.

2.4. Total phenolic assay

The total phenolics were assayed colorimetrically using the Folin-Ciocalteu method, as described in Pinelo et al. (2004); 0.5 ml of plant extract was added to a test tube and mixed well with 2.5 ml of 10-fold diluted Folin-Ciocalteu reagent, and 2 ml of 7.5% Na_2CO_3. Absorbance was measured at 765 nm using an M350 Double Beam UV–Visible Comspec spectrophotometer, after heating for 15 min at 45 °C. A standard curve was established for the assay using tannic acid in 80% aqueous methanol. A mixture of water and reagents was used as a blank. The phenolic content was expressed as milligrams of tannic acid equivalents (TAE).

2.5. Total flavonoid contents

The total flavonoids were determined using a colorimetric method as described by Liu et al. (2002). Briefly, 0.25 ml of the phytochemical extract was diluted with 1.25 ml of distilled water. Then, 75 μl of a 5% $NaNO_2$ solution was added to the mixture. After 6 min, 150 μl of a 10% $AlCl_3 \cdot 6H_2O$ solution was added and the mixture was allowed to stand for a further 5 min; 0.5 ml of 1 M NaOH was added and the total mixture was made up to 2.5 ml using distilled water. The solution was mixed well and absorbance was measured immediately against the prepared blank at 510 nm according to the standards prepared similarly with known epicatechin concentrations. The results were expressed as milligrams of epicatechin equivalents.

2.6. Radical-scavenging activity using DPPH

The stable DPPH radical-scavenging effect of the plant extract was measured according to Singh, Chudambara Murthy, and Jayaprakasha (2002). Different concentrations (50, 100, 250 and 500 μl equivalent to 50, 100, 250 and 500 ppm) of plant extract and standards (BHT, ascorbic acid and α-tocopherol) were taken in a series of test tubes. The volumes were adjusted to 100 μl by adding 80% methanol. Five millilitres of a 0.1 mM methanolic solution of DPPH was added to these tubes, which were vigorously shaken. The tubes were allowed to stand at 27 °C for 20 min. The control was prepared as described above without any extract and 80% methanol was used for the baseline correction. Changes in the absorbance of the samples were measured at 517 nm. Radical-scavenging activity was expressed as the percent inhibition and calculated using the following relation:

$$\%\ \text{radical-scavenging activity} = (\text{control OD} - \text{sample OD}/\text{control OD}) \times 100.$$

2.7. β-Carotene bleaching (BCB) assay

The method proposed by Moure et al. (2000) and Peterson et al. (2001), Peterson, Emmons, and Hibbs (2002) and Tepe et al. (2005) was used, based on the ability of the plant extract to decrease oxidative losses of β-carotene in a β-carotene/linoleic acid emulsion. For this purpose, various concentrations of plant extract were used to determine the effect of concentration level on antioxidant activity. A 2.0 mg sample of crystalline β-carotene was dissolved in 10 ml of chloroform. One millilitre of this solution was

then pipetted into a round-bottomed flask which contained 20 mg of purified linoleic acid and 200 mg of Tween-40 emulsifier. After removal of chloroform under a stream of N_2, the resulting mixture was diluted with 10 ml of oxygenated water and was then mixed well. To this emulsion was added 40 ml of oxygenated water; 0.5 ml aliquot of the aqueous emulsion formed was then pipetted into each of a series of tubes, which contained 0.2 ml of methanolic antioxidant solution. BHT, α-tocopherol and ascorbic acid were used for comparison. A control sample with 80% methanol instead of the extract was also analyzed for antioxidant activity. A zero reading was taken at 470 nm on the reaction mixture in each tube immediately after addition of the emulsion to the antioxidant solution ($t = 0$). The tubes were kept at 50 °C in a water bath and measurement of absorbance was continued at intervals of 15 min until the colour of β-carotene disappeared in the control tubes ($t = 180$ min). A mixture, prepared as above but without β-carotene, served as blank. All determinations were carried out in triplicate. The antioxidant activity levels (AA) of the extracts were evaluated as β-carotene bleaching using the following relationship:

$$AA = 100[1 - (A0 - At)/(A°0 - A°t)],$$

where $A0$ and $A°0$ are the absorbance values measured at time zero of the incubation for test sample and control, respectively; and At and $A°t$ are the absorbance values measured in the test sample and control, respectively, after incubation for 180 min.

2.8. Reducing power assay

Reducing power was carried out according to the method described by Hwang et al. (2001). Plant extracts were well mixed with 0.5 ml of 0.2 M phosphate buffer (pH 6.6) and 0.5 ml of 1.0% K-ferricyanide. Then, the mixtures were incubated at 50 ± 1 °C in a water bath for 20 min. Centrifugation (1500*g*, 10 min) followed the addition of 0.5 ml of 10% trichloroacetic acid to the sample solution. The supernatant thus collected (1.0 ml) was well mixed with 1.0 ml of distilled water and 0.2 ml of 0.1% $FeCl_3 \cdot 6H_2O$. Next, it was allowed to stand at room temperature (28 ± 2 °C) for 10 min. Colour changes were monitored at 700 nm by a spectrophotometer. BHT, α-tocopherol, and ascorbic acid were used to compare the reducing power. The higher the absorbance, the better was the reducing power of the sample.

2.9. Antioxidant activity using thiocyanate assay

The antioxidant activity of the extract was determined using the thiocyanate method (Endrini, Rahmat, Ismail, & Yun Hin, 2002; Pyo, Lee, Logendra, & Rosen, 2004). The linoleic acid emulsion was prepared by homogenizing 0.28 g of linoleic acid, 0.28 g of Tween-40, and 50 ml of phosphate buffer (0.2 M, pH 7.0). Test samples were prepared in 80% methanol. Different test samples (0.5 ml) were mixed with 2.5 ml of linoleic acid emulsion, and 2.5 ml of phosphate buffer (0.2 M, pH 7.0). The samples were then incubated at 37 °C for 150 h. Mixtures prepared without the test sample served as control. Aliquots (0.1 ml) were drawn from the incubation mixture at 24 h intervals and mixed with 5.0 ml of 75% ethanol, 0.1 ml of 30% ammonium thiocyanate, and 0.1 ml of 20 mM ferrous chloride in 3.5% HCl and allowed to stand at room temperature for 3 min. The emerging colour was measured at 500 nm in a spectrophotometer. The degree of linoleic acid peroxidation was calculated at 96 h (when the absorbance of the control reached its maximum level) using the following relationships:

$$\text{Antioxidant activity(AA, \%)} = 100 - (\text{Increase in the absorbance of sample} / \text{Increase in the absorbance of control}) \times 100.$$

BHT and α-tocopherol were included as standard antioxidants for comparison.

2.10. Accelerated oxidation of sunflower oil

The plant extract and BHT were mixed with 20 g of oil into a 50 ml glass flask (samples: A: +500 ppm PE and 200 ppm BHT; B: +500 ppm PE; C: +250 ppm PE and 200 ppm BHT; D: +250 ppm PE; E: +200 ppm BHT; F: control), thoroughly homogenized, and placed in an oven at 60 ± 3 °C (Abdalla & Roozen, 1999). According to Moure et al. (2000), higher temperature assays (100 °C) are unreliable because hydroperoxides decompose at high temperatures, leading to volatile synthetic antioxidants, and thus present poor activity. The peroxide value (PV) that can be used as an indicator of the primary oxidation of oils was determined using the AOAC method (Helrich, 1990). Inhibition of oil oxidation (IO) was expressed as

$$\text{IO}(\%) = 100 - [(\text{peroxide value increase})\text{sample} / (\text{peroxide value increase})\text{control}] \times 100.$$

Synthetic antioxidant, BHT, was also used as the reference standard.

2.11. Statistical analysis

All tests and analyses were run in duplicate or in triplicate. The deviation from the mean at the 99% confidence level was employed for determining the differences in results. SPSS statistical software was employed for statistical analysis.

3. Results and discussion

3.1. Extract yield

The amount of extractable components (extract yield) obtained from extracts was 218 ± 1.06 mg/g of dry plant material.

3.2. Amount of total phenolics

Total phenolic content in the methanol extract of the samples amounted to 1.03 ± 0.01 mg TAE g^{-1} dry matter. Li et al. (2006) studied the polyphenol content of pomegranate peel and pulp extracts to find high polyphenol quantities in peel extracts (249 ± 17.2 g TAE g^{-1} dry matter), while they reported phenol concentrations of 24.4 ± 2.7 g TAE g^{-1} dry matter for pulp extracts. They demonstrated that the total phenolics content of peel extract was nearly 10 times higher than that of pulp extract. Pistachio hulls are another source of phenolic antioxidants and may contain up to 34 mg TAE g^{-1} dry weight (Balasundram, Sundram, & Samman, 2006). Chapuis-Lardy, Contour-Ansel, and Bernhard-Reversat (2002) showed the amount of total phenolics extracted with methanolic solutions from *Eucalyptus uropellita* to be 138 mg TAE g^{-1} dry matter, whereas distilled water extracts contained 126 mg TAE g^{-1} dry matter plant.

3.3. Total flavonoid content

Total flavonoid content in the plant sample was 0.595 ± 0.06 mg/g dry weight. The total flavonoid content in *Ocimum gratissimum* L. was reported by Vieira, Grayer, Patonb, and Simona (2001). Total concentrations of flavonoids among different accessions varied greatly from 0.01 mg/g dried leaf to 2.9, showing an increase by a factor of 290. The disparity in the results may be explained by differences of cultivars and growing conditions (Häkkinen et al., 1999).

3.4. DPPH radical-scavenging activities of plant polyphenols

DPPH assays reflect the ability of the compounds present to scavenge hydrophilic free radicals (Aehle et al., 2004). To evaluate the scavenging effect of the extract in our study, DPPH inhibition was investigated and the results were evaluated as relative activities against control. The DPPH radical-scavenging activity of extracted polyphenols is shown in Fig. 1, expressed as percent reduction of the initial DPPH absorption by the test compound. The results showed that ascorbic acid (100, 50 ppm) was an excellent DPPH radical-scavenger, with about 71.4% of DPPH scavenged under experimental conditions. This is while the plant extract and α-tocopherol showed moderate activities. The plant extract at a concentration of 100 ppm was as effective as α-tocopherol (100, 50 ppm) and they were both significantly ($\rho < 0.01$) better than BHT. Percent inhibition of DPPH did not significantly differ between the extract concentrations of 500 and 250 ppm (Fig. 2), indicating that a critical concentration of phenolics is sufficient to obtain the desired antioxidant activity, beyond which there is a saturation effect and the presence of additional phenolics does not contribute to increased antioxidant activity. The EC_{50} value of the methanol extract was found to be 250 ppm whereas the synthetic antioxidant BHT did not reach the EC_{50} value, even at a concentration of 500 ppm. Chun et al. (2005) reported that DPPH showed similar radical-scavenging activities in water and in 60% ethanol oregano extracts, even though total phenolic contents in the two extracts were different. The percent inhibition of DPPH varied from 80% to 82%. This suggests that the physicochemical nature of the individual phenolic in the extracts may be more important in contributing to the antioxidant activity than the total phenolics content measured by the Folin-Ciocalteu assay. Kulkarni, Aradhya, and Divakar (2004) showed that, among the different solvent extracts of pith and carpellary membrane of pomegranate fruit, the methanol extract showed excellent DPPH free radical-scavenging activity (93.2%), followed by the ethyl acetate extract (92.1%). Both extracts are very close

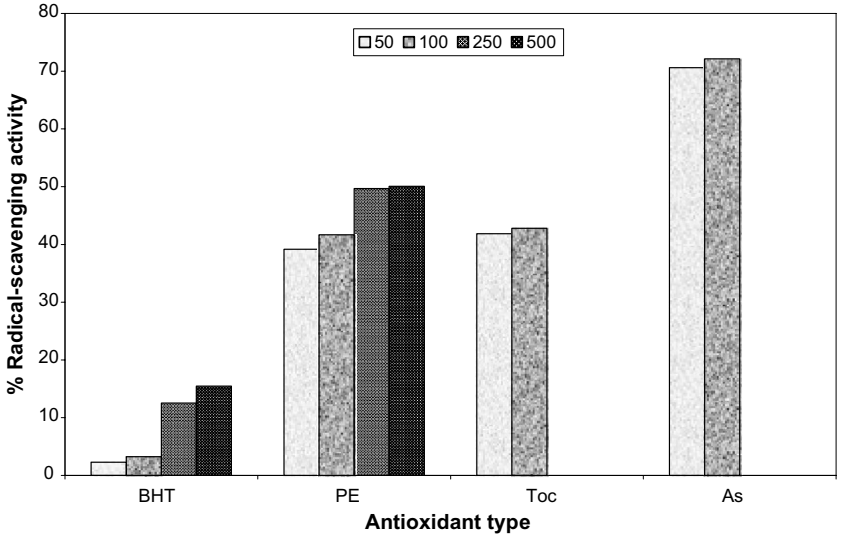

Fig. 1. Radical-scavenging activities of plant extract (PE), ascorbic acid (As), α-tocopherol (Toc) and BHT by DPPH method at different concentrations (ppm).

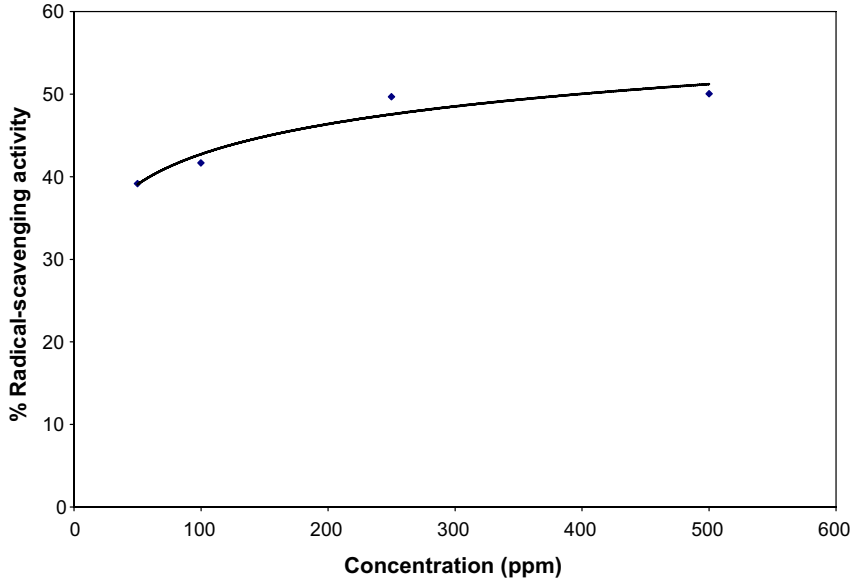

Fig. 2. Correlation between concentration of phenolic compounds in extract and its radical-scavenging activity.

to the radical-scavenging ability of the synthetic antioxidant BHA (94.5%).

3.5. β-Carotene bleaching (BCB) method

The BCB method is based on the loss of the yellow colour of β-carotene due to its reaction with radicals formed by linoleic acid oxidation in an emulsion. The rate of β-carotene bleaching can be slowed down in the presence of antioxidants (Kulisic et al., 2004). The antioxidant activity of the plant extract, measured by β-carotene bleaching, was compared with those of BHT, α-tocopherol and ascorbic acid. The antioxidant power decreased in the order: BHT $\geqslant$ plant extract > α-tocopherol > ascorbic acid. In comparison, the α-tocopherol showed a relatively significant antioxidant effect, while ascorbic acid showed no antioxidant activity. Except for the case of ascorbic acid, the concentration influenced the antioxidant power of each sample. Fig. 3 shows the decrease in absorbance of β-carotene in the presence of plant extract, as well as BHT, α-tocopherol and ascorbic acid. The control sample, without addition of antioxidant, oxidized most rapidly and a descending bleaching rate was observed in the presence of BHT > plant extract > α-tocopherol > ascorbic acid.

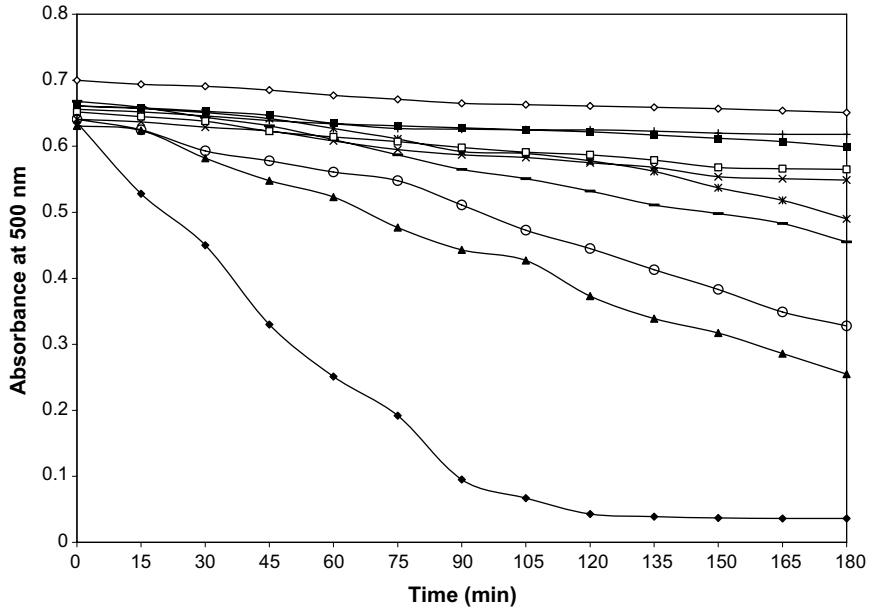

Fig. 3. Rate of β-carotene bleaching in control without antioxidant (♦), plant extract at 75 (×), 150 (■) and 300 (◇) ppm, BHT, α-tocopherol and ascorbic acid at 50 (□), (−), (○) and 100 (+), (✻), (▲) ppm, respectively.

In spite of the fact that a polar compound, such as ascorbic acid, is well known for its antioxidant effect, the β-carotene bleaching test failed to show its antioxidant properties. This interesting phenomenon, formalised as the "polar paradox", has been reported in earlier studies. The polar antioxidants remaining in the aqueous phase of the emulsion are more diluted in the lipid phase and are, thus, less effective in protecting the linoleic acid (Kulisic et al., 2004). The "polar paradox" has been repeatedly reported. It postulates that hydrophilic antioxidants are more effective than lipophilic antioxidants in bulk oil, whereas lipophilic antioxidants show greater activity in emulsions (Moure et al., 2001). Singh et al. (2002) reported that pomegranate peel and seed extracts, prepared by different solvents, exhibited various degrees of antioxidant activity. At a concentration of 50 ppm, EtOAc, MeOH and water extracts of peel were shown to exhibit antioxidant activity levels of 53%, 82%, and 64%, respectively. At a concentration of 100 ppm, EtOAc, MeOH and water extracts of seed exhibited 39%, 22% and 57% antioxidant activity, respectively. β-Carotene assay is used to measure the ability of the antioxidant to prevent the oxidative deterioration of lipids and fatty acids. Therefore, the higher antioxidant activity of the phenolic antioxidants from the *K. odoratissima* M. in this assay suggests a possible biological functionality in preventing the oxidative degradation of membrane lipids.

Table 1
Reducing activity of plant extract in comparison with ascorbic acid, BHT and α-tocopherol

Concentration (ppm)	Absorbance[a]			
	PE[b]	AS[c]	BHT	Toc[d]
50	–	0.74	0.24	0.04
100	0.08	1.69	0.64	0.21
300	0.50	–	–	–
500	0.91	–	–	–

[a] Absorbance at 700 nm.
[b] Plant extract.
[c] Ascorbic acid.
[d] α-Tocopherol.

3.6. Reducing activity

The reducing powers of various amounts of plant extract and standards are given in Table 1. The reducing power decreases in the order: As (100 ppm) > PE (500 ppm) > As (50 ppm) > BHT (100 ppm) > PE (300 ppm) > BHT (50 ppm) > α-Toc (100 ppm) > PE (100 ppm) > α-Toc (50 ppm). The methanol extract of the plant at 500 ppm exhibited a greater reducing power than did 50 ppm of ascorbic acid. It appears that antioxidant activity maintains a correlation with reducing effect and that it exponentially increases as a function of the enhancement of the reducing power (Duh & Yen, 1997). Yuan, Bone, and Carrington (2005) reported that one milligram of the dulse extract, having a polyphenol content equivalent to 10.3 μg gallic acid, exhibited a reducing activity equivalent to 9.68 μg of L-ascorbic acid.

3.7. Thiocyanate method

The highest activity of the plant extract was observed at 500 ppm (AA = 86.9%), which was less than that of BHT (AA = 93.3%, 100 ppm) but significantly ($\rho < 0.01$) greater than that of α-tocopherol at concentrations of 100 and 50 ppm (AA = 82.7%, 77.5%), respectively. The individual

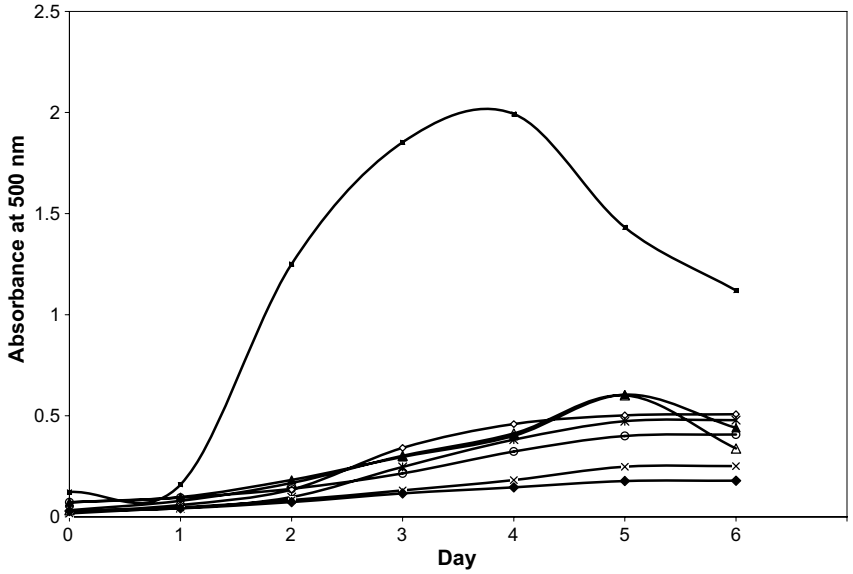

Fig. 4. Absorbance of control without antioxidant (■), plant extract at 100 (◇), 300 (✻) and 500 (○) ppm, BHT and α-tocopherol at 50 (×), (△) and 100 (♦), (▲) ppm, respectively, using thiocyanate method.

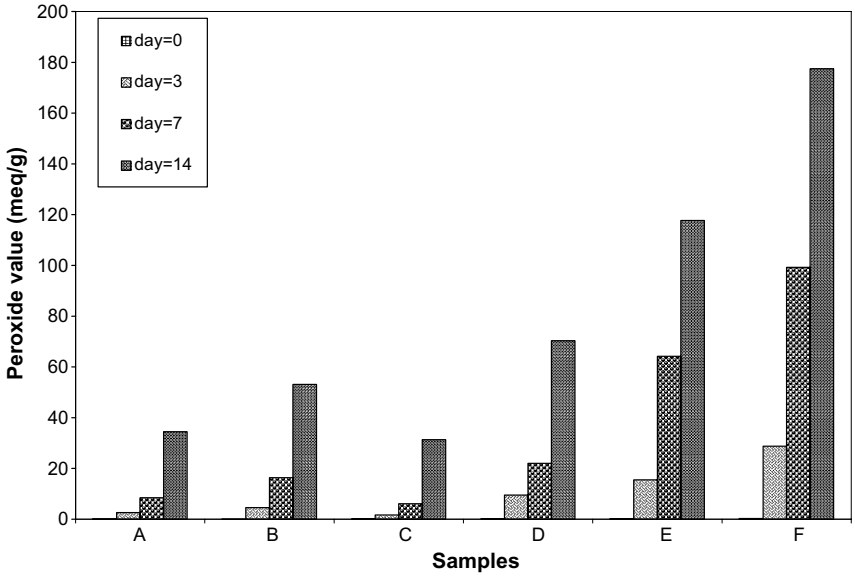

Fig. 5. Oxidation of sunflower oil treated with plant extract and BHT during storage at 60 ± 3 °C: (A) 500 ppm PE+200 ppm BHT, (B) 500 ppm PE, (C) 250 ppm PE + 200 ppm BHT, (D) 250 ppm PE, (E) 200 ppm BHT and (F) control.

activity of BHT, by the thiocyanate method, showed that BHT gave the least increase in absorbance values, followed by plant extract and α-tocopherol from day 1 to day 3 but, in the case of α-tocopherol, the levels increased on day 3, reaching a maximum on day 5 and finally dropping on day 6 due to the presence of malonaldehyde (Fig. 4). The low absorbance value indicated the high level of total antioxidant activity. These results suggest that the plant extract might react with free radicals, particularly with peroxy radicals which are the major propagators of the autooxidation of fat, thereby terminating the chain reaction.

Lee and Lim (2000) predicted that both water and ethanol extract of ginger might have antioxidative activity. The antioxidant activity of ginger was reported to be 40.3% and 56.0% for water (at a concentration of 2 mg/ml) and ethanol extract, respectively. Pyo et al. (2004) reported that the red leaf extracts of Swiss chard at a concentration of 1000 mg/ml exhibited an antioxidant activity level (AA = 72.5%) below that of BHT (AA = 88.2%) and nearly equal to or stronger than that of α-tocopherol (AA = 71.8%) at identical concentrations.

3.8. Inhibition of lipid oxidation

The course of sunflower oil oxidation for 14 d at 60 ± 3 °C is presented in Fig. 5. Oxidation was not noticeable until d 3 for any of the tested samples, after which it rose to 99.2 and 64.2 meq/kg for F (as defined in 2.10.) and E samples, respectively. Sample C retained its antioxidant activity over prolonged oxidation periods. A dramatic decrease in antioxidant efficiency due to high temperature was observed for BHT. Plant extract showed more thermal stability than did BHT. It may be concluded that BHT and extract have synergistic effects at low concentrations. Abdalla and Roozen (1999) reported that sage extract showed the highest antioxidant activity during primary and secondary oxidation of both traditional sunflower oil containing 570 ppm natural α-tocopherol and its oil-in-water emulsion in the dark at 60 °C. Oregano and thyme extracts were reported to have strong antioxidant effects in stabilizing lard.

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