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Evaluation of antioxidant activity of *Malus domestica* fruit extract from Kashan area

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Antioxidants are considered as the main factors in the inhibition of unwanted oxidation reactions. In this research the antioxidant potential of the fresh fruits of 4 cultivars of *Malus domestica* cultivated in the Kashan, Qamsar area was evaluated. The antioxidant activity of the samples were evaluated using two complementary antioxidant assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *β*-carotene/linoleic acid tests and the results were compared with the synthetic standard antioxidant butylated hydroxytoluene (BHT). Total phenolic contents of the samples are also estimated by Folin-Ciocalteu's phenol test. In both DPPH *β*-carotene/linoleic acid tests in the concentration of 2 mg/ml, only samples from Hossain cultivar showed moderate antioxidant activity with 63.92 ± 0.42 and 6.02 ± 0.03 inhibition percentages, respectively and other samples were only weekly active. The Folin-Ciocalteu's phenol test was also showed very little phenolic compounds for the fruits. In conclusion, week antioxidant activity was estimated for the studied apple cultivars.

Key words: Apple, *Malus domestica*, extract, antioxidant activity, total phenolic content.

INTRODUCTION

Free radicals are present in biological systems and may oxidize all the biological molecules present in our body, such as nucleic acids, proteins, lipids, initiating degenerative diseases (Cook and Samman., 1996; Harborne and Williams.,2000; Heim et al.,2002). Antioxidants are substances that neutralize free radicals and their negative effects. Antioxidants can inhibit or delay the oxidation of oxidizable substrates and this appears to be very important in the prevention of oxidative stress which is suggested as the leading cause of many oxidation related diseases (Bamoniri et al., 2010). Also antioxidants are substances that are able to prevent or retard the oxidation of lipids, proteins and DNA; and to protect the compounds or tissues from damage caused by oxygen or free radicals (Hasbay et al., 2007). Therefore, their health promoting effects reduce the risk of various diseases (Manach et al., 2004). Recently, antioxidant activity has been determined in

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many species of fruits, vegetables, herbs, cereals, sprouts and seeds (Kahkonen et al., 1999; Velioglu et al., 1998).

A special attention is paid to fruits, as rich sources of phenolic compounds (Kalt et al., 1999; Robards et al., 1999; Wang and Lin., 2000). Among others, the antioxidant properties of apple polyphenols have been extensively examined (Ju and Bramlage, 1999; Lu and Foo, 2000; Robards et al., 1999). Apples have the highest levels of antioxidant activity (Chinnici et al., 2004). Activity and concentration of antioxidants in fruit differ among cultivars, the part of the fruit, the growth stage and environmental conditions (Awad et al., 2001a,b,c; Sluis et al., 2001). Apple fruit contain several health and sensory related constituents including dietary fibre, sugars, vitamins and phenolic compounds (Hagen et al., 2007). The antioxidant capacity of apple is mostly attributed to phenolic compounds such as flavonoids and phenolic acids (Eberhardt et al., 2000; Lee et al., 2003).

Malus domestica Borkh. is one of the most commonly consumed fruit worldwide (Shoji et al., 2004) and we collected samples named Hossain, Sayyed Babaeei, Shekareh and Golab from Iran. These samples have been cultivated since foretime are medium in size with a circular shape. The yellow–pink skins are thin, rather wax-like, and the white fleshes are soft, juicy, aromatic and sweet. Because of staying on the tree, the skin color of these 4 apple cultivars changes gradually and becomes red. Thus, the present research reports the *in vitro* profile of the antioxidant activity of the fruit extracts using two complementary assays: DPPH radical and β-carotene linoleic acid tests; the total phenolic content of the fruit extracts, expressed as gallic acid equivalents.

MATERIALS AND METHODS

Fruit collection

Fresh fruit samples from Hossain, Sayyed Babaeei, Shekareh and Golab apple cultivars were collected in the Kashan, Qamsar area in the June 2008 when the fruit had just been harvested.

Extraction procedure

Apples characterized by plant taxonomist, immediately transported to the laboratory, washed, dryed, cut manually with a knife into small pieces, whole fruit except seeds extracts were obtained using a kitchen-type blender (Moulinex, France) and concentrated with a rotary evaporator.

Solvents and chemicals

2,2-Diphenyl-1-picrylhydrazyl (DPPH) radical, β -carotene, linoleic acid, 2,6-di-tert-butyl-4-methylphenol butylated hydroxytoluene (BHT) and gallic acid were procured from Sigma–Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol, and dimethylsulfoxide (DMSO), HPLC grade chloroform, standard Folin–Ciocalteu's phenol reagent, sodium carbonate, Tween 40, and all cultures media were obtained from Merck (Darmstadt, Germany). Ultra pure water was used for the experiment.

Antioxidant activity

DPPH radical scavenging

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical assay usually involves hydrogen atom transfer reaction but, based on kinetic data, an electron transfer mechanism has also been suggested for this assay (Huang et al., 2005; Foti et al., 2004). Radical scavenging activities of the plant essential oil and extract were determined using a published DPPH radical scavenging activity assay method (Sarker et al., 2006) with minor modifications.

Briefly, stock solutions (10 mg/ml each) of the extracts and the synthetic standard antioxidant BHT were prepared in methanol. Dilutions are made to obtain concentrations ranging from 1 to 5×10^{10} mg/ml. Diluted solutions (1 ml each) were mixed with 1 ml of a freshly prepared 80 µg/ml DPPH radical methanol solution and allowed to stand for 30 min in the dark at room temperature for any reaction to take place. Absorbance values of these solutions were recorded on an ultraviolet and visible (UV–Vis) spectrometer (Cintra 6, GBC, Dandenong, Australia) at 517 nm using a blank containing the same concentration of DPPH radicals. Inhibitions of DPPH radical in percent (I%) were calculated as follow:

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

In this research, dilution was not performed due to low concentration of extracts and low inhibitory percentage.

Where A_{blank} is the absorbance value of the control reaction (containing all reagents except the test compound) and A_{sample} is the absorbance values of the test compounds. The sample concentration providing 50% inhibition (half-maximal inhibitory concentration, IC_{50}) was calculated by plotting inhibition percentages against concentrations of the sample. It is interesting to note that in this research, the related graphs and some other necessary calculations like IC_{50} performed due to low concentration of extracts and inhibitory percentage.

β-Carotene/linoleic acid bleaching

The β-carotene/linoleic acid test evaluates the inhibitory effect of a compound or a mixture on the oxidation of β-carotene in the presence of molecular oxygen (O_2). Assay of the remained β-carotene gives an estimation of the antioxidant potential of the sample. The method described by Miraliakbari and Shahidi (2008), was used with slight modifications. A mixture of β-carotene and linoleic acid was prepared by adding together of 0.5 mg β-carotene in 1 ml chloroform (HPLC grade), 25 µl linoleic acid and 200 mg Tween 40. The chloroform was then completely evaporated under vacuum and 100 ml of oxygenated distilled water was subsequently added to the residue and mixed gently to form a clear yellowish emulsion.

The essential oil, extract and BHT (positive control) were individually dissolved in methanol (2 g/L) and 350 µl volumes of each of them were added to 2.5 ml of the above emulsion in test tubes and mixed thoroughly. The test tubes were incubated in a water bath at 50°C for 2 h together with a negative control (blank) contained the same volume of methanol instead of the extracts. The absorbance values were measured at 470 nm on an ultraviolet and visible (UV–Vis) spectrometer (Cintra 6, GBC, Dandenong, Australia). Antioxidant activities (inhibitions percentage, I%) of the samples were calculated using the following equation:

$$I\% = (A_{\beta\text{-carotene after 2-h assay}}/A_{\text{initial }\beta\text{-carotene}}) \times 100$$

Where $A_{\beta\text{-carotene after 2-h assay}}$ is the absorbance values of β-carotene after 2 h assay remaining in the samples and $A_{\text{initial }\beta\text{-carotene}}$ is the absorbance value of β-carotene at the beginning of the

Table 1. DPPH radical scavenging activity (percentage ± SD) of 4 apple cultivars in the concentration of 2 mg/ml.

Sample	Inhibition (%)
Hossain	63.92 ± 0.42
Sayyed Babaeei	39.60 ± 0.75
Shekareh	19.99 ± 0.24
Golab	43.16 ± 1.92
BHT[a]	96.65 ± 0.15

[a] In concentration of 0.5 mg/ml.

Table 2. Antioxidant activity of β-carotene/linoleic acid bleaching assay method (percentage ± SD) of 4 apple cultivars in the concentration of 2 mg/ml.

Sample	β-carotene bleaching (%)
Hossain	6.02 **±** 0.03
Sayyed Babaeei	4.24 **±** 0.56
Shekareh	1.00 **±** 0.05
Golab	3.16 **±** 0.08
BHT	96.40 **±** 0.07

Table 3. The contents of total phenol of 4 apple cultivars.

Sample	Total phenol contents (µg/mg)
Hossain	0
Sayyed Babaeei	0
Shekareh	0
Golab	1.5

experiments. All tests were carried out in triplicate and inhibition percentages were reported.

Total phenolics

Total phenolics content was determined using Folin-Ciocalteu reagent as reported in the literature (Slinkard and Singleton, 1977). A solution of the extract (0.1 ml) containing 1000 µg of the extract was pipetted into a 50 ml volumetric flask, 46 ml distilled water and 1 ml Folin–Ciocalteu's phenol reagent were added, and the flask was thoroughly shaken. After 3 min, 3 ml of 2% Na_2CO_3 solution was added and the mixture was allowed to stand for 2 h with intermittent shaking. Absorbance values were measured at 760 nm. The same procedure was repeated for all the standard gallic acid solutions (0–1000 lg/0.1 ml) and a standard curve obtained with the following equation:

Absorbance = 0.0012 × gallic acid (µg) + 0.0033

Total phenols of the extract, as gallic acid equivalent, was determined by using the absorbance value of the extract measured at 760 nm as input to the standard curve and the equation. Test was carried out in triplicate and gallic acid equivalent value was reported.

RESULTS AND DISCUSSION

DPPH

DPPH radical scavenging activity potentials of fruit extract were evaluated for the assessment of their antioxidant capacities and compared with BHT (the standard commercial synthetic antioxidant). Among the extracts, the best radical scavenging effect against DPPH was observed in Hossain cultivar (63.92 ± 0.42%) in the concentration of 2 mg/ml. The results of 4 apple cultivars and BHT are presented in Table 1.

β-Carotene/linoleic acid

The potential of the plant to inhibit lipid peroxidation was evaluated using the β-carotene/linoleic acid bleaching test. In *β*-carotene/linoleic acid tests in the concentration of 2 mg/ml, only samples from Hossain cultivar showed 6/015 ± 0/003 inhibition percentages. The results of 4 apple cultivars and standard (BHT) are presented in Table 2.

Total phenolic constituents

Total phenolic content of the plant extracts were determined using a colorimetric assay method based on Folin–Ciocalteu reagent reduction. The Folin-Ciocalteu's phenol test was also showed very little phenolic compounds for the fruits. The amounts of total phenols found in the fruit extracts are shown in Table 3.

DPPH assay and β-Carotene/linoleic acid

The measurement of the antioxidant capacity of food extracts and pure compounds is commonly performed using several methods. Each method relates to the generation or use of a different radical that is directly involved in the oxidative process, acting through a variety of mechanisms. Among the various assays, we selected the DPPH and β-Carotene/linoleic acid assays to determine the antioxidant activity of fruit extracts.

During 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical test, the capacity of the samples to donate hydrogen atom and/or electron to this blue/purple stable radical and converting it to yellow diphenylpicrylhydrazine molecule was measured (Tepe et al., 2005). This reaction is used for measuring the ability of the extracts or pure molecules (such as BHT) to scavenge free radicals. Our results estimate a mild antioxidant potential for the Hossain cultivar while other samples were weakly active.

Results of antioxidant test of 4 apple cultivars showed that none of 4 samples have high antioxidant properties at 2 mg/ml concentration but only Hossain cultivar showed 64% inhibitory power. It is to be noted that

extracts were prepared with high concentration, therefore samples were not diluted. These findings are in agreement with measured total phenolic contents of the samples (Drogoudi et al., 2008; Lata, 2007; D´Abrosca et al., 2007; Tsao et al., 2005; Vieira et al., 2009). β-Carotene/linoleic acid test of 4 apple cultivars showed the same results as antioxidant test with the exception 0f Hossain cultivar which showed greater inhibitory power 6% compared to DPPH procedure. This finding is in contradiction with the findings of Garcia et al. (2009); Lata et al. (2009); Lee et al. (2003); Bandoniene and Murkovic (2002) and Kondo et al. (2002); which might be due to different cultivars they have selected under different climatic condition.

Total phenolic contents

The basic structure of the phenols and other structural factors play a fundamental role in the mechanism by which these compounds are able to scavenge free radicals (Sadeghipour et al., 2005). As underlined also by Lata et al. (2009) and Lata (2008), it is difficult to compare the content of apple phenolic among different studies, as many variations can be principally caused by different growth period, geographic location, storage type, genetic diversity and many other factors. The results, expressed as gallic acid equivalents, were 0 and 1.5 ± 0.6 µg/mg for the extracts of apples, respectively.

These values are comparable to the values reported in literature for other apple cultivars, such as Golden Delicious, Stark Delicious, Mora, Nesta, Panaia-red and Ruggine (Iacopini et al., 2009) and others. Phenolic compounds normally play main role in the antioxidant activity of the plant extracts, thus, low DPPH antioxidant activity of our samples may be related to their negligible total phenolic compounds contents. Folin–Ciocalteu test showed that there is low percentage of phenolic compounds in all samples which is in accordance with antioxidant tests. Overall conclusions was that all samples did not show high antioxidant power however Hossain cultivar showed higher antioxidant power, which might be due to presence of phenolic compounds.

Conclusions

Fruits have long been regarded as having considerable health benefits, particularly due to their antioxidant properties, which can protect the human body against cellular oxidation reactions. In our study, we have focused on antioxidant activity and total phenolic compounds of apples. They are the most common compounds in fruits and vegetables and have a strong antioxidant capacity. Fresh fruits of 4 cultivars of *M. domestica* cultivated in the Kashan, Qamsar area was selected. The antioxidant activity of the samples were evaluated using two complementary antioxidant assays: 2,2-diphenyl-1-picrylhydrazyl (DPPH) and *β*-carotene/linoleic acid tests and the results were compared with the synthetic standard antioxidant BHT. Total phenolic contents of the samples are also estimated by Folin-Ciocalteu's phenol test. In both DPPH *β*-carotene/linoleic acid tests in the concentration of 2 mg/ml, only samples from Hossain cultivar showed moderate antioxidant activity with 63.92 ± 0.42 and 6.02 ± 0.03 inhibition percentages, respectively and other samples were only weekly active. The Folin-Ciocalteu's phenol test also showed very little phenolic compounds for the fruits.

We therefore conclude that the phenolic content, the radical-scavenging and antioxidant properties of old local apple varieties demonstrate that these neglected cultivars could be a good source of phytochemicals, bioactive compounds with important protective properties. This local apple cultivars could be also considered as an important source of genes for apple breeding program and for the production of value added apple cultivar. So that being, further studies on local and ancient varieties have to be encouraged so that those varieties with the most technological interest can be selected.

Conflict of Interest

The authors have not declared any conflict of interest.

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