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Free Radical Scavengering and Phenolic Compounds of Peel and Pulp of Quince

Marzieh Babashpour-Asl[1*] and Marzieh Piryaei[2]

1. Department of Horticultural Sciences, Islamic Azad University, Maragheh Branch, Maragheh, Iran
2. Department of Chemistry, Faculty of Science, University of Maragheh, Maragheh, Iran

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ABSTRACT
This study aimed to determine the amount of flavonoids, anthocyanins, total phenolic compounds, ascorbic acid and antioxidant activity of the peel and pulp of the quince (*Cydonia oblonga* Mill.) in the polar and nonpolar sub-fractions of methanol extracts with two extraction methods, Soxhlet and microwave. The antioxidant properties were assessed by the ability to quench the stable free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric reducing antioxidant power (FRAP) and β-carotene bleaching assays. The results showed that the Soxhlet method extracted higher amount of the phytochemicals. In the DPPH system, the highest radical scavenging activity was seen by the polar sub-fraction of the methanol extract in the peel of quince fruit (IC_{50}=52±7.3 µg/mL). Among all the extracts analyzed, the polar sub-fraction of the peel extract exhibited a significantly higher total flavonoid (62.33±3.1 mg CTE/100 g), anthocyanin (1.54±0.24 µg C-3-GE/100 mg fresh weight), phenolic content (108.14 µg GAE/mg) and antioxidant activity than other samples with Soxhlet extraction method. Fruit has a number of bioactive ingredients and many of them were concentrated in the peel of the fruit rather than the flesh. Therefore, enjoy the benefits that unpeeled fruit offers! The present study revealed that the peel of quince fruit has higher phytochemicals and antioxidant properties when compared to the pulp.

Keywords: Total phenols, flavonoids, anthocyanin, ascorbic acid, antioxidant, quince.

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Introduction

Most phytochemicals in natural agricultural sources have been generally recognized as bioactive or health-promoting compounds. Antioxidant phytochemicals have recently become an attractive subject for scientists in many different research areas (Gu, 2012). Renewed attention has been given to the importance and role of antioxidants due to the increasing experimental, clinical, and epidemiological data which show the beneficial effects of these antioxidants against oxidative stress-induced degenerative and age-related diseases, aging and cancer (Charles, 2013).

Quince (*Cydonia oblonga* Mill.) belongs to the Maloideae subfamily of the Rosaceae family, which includes commercially important fruits such as apples and pears (Wojdylo et al. 2013). It is native to Anatolia, Persia, Northern Caucasia and Caspian Sea (Acikgoz, 2010). Its fruits are often used in the food industry due to their aroma, which is similar to that of orange and pineapple. Furthermore, quince is a valuable source of biologically active substances.

* Corresponding Author, Email; babashpour@gmail.com

These compounds provide antioxidant, antibacterial, antiulcer, cardioprotective, anti-inflammatory, antidiabetic and antiallergic properties (Muzykiewicz et al., 2018).

In the past decade, several studies on *C. oblonga* fruit and its derivatives have been performed by a research group from Portugal. It has been revealed that fruit contains famous antioxidants such as caffeoylquinic acids (79.6 mg/kg) and rutin (5.5 mg/kg), while its peel is also rich source of caffeoylquinic acid (291.6 mg/kg) along with other important flavonoids such as kaempferol 3-glucoside (92.9 mg/g), quercetin 3-galactoside (100.8 mg/g), and kaempferol-3-rutinoside (61.1 mg/g) (Silva et al., 2002). The presence of ascorbic, citric, malic, D-(-)-quinic, fumaric, and L-shikimic acids was also confirmed in both peel and pulp (Ashraf et al., 2016). Qualitative and quantitative analyses of quince fruit (pulp and peel) collected from different regions of Portugal and evaluated their antioxidant potential using DPPH assay was shown that phenolic fractions possessed strong free radical scavenging activity than that of organic acid fractions and whole methanolic fractions (Silva et al., 2004).

Phenolic profiles of methanolic extracts of seed, peel and pulp were reported by Magalhaes et al. (2009) in which the total phenol contents were 0.4, 6.3 and 2.5 g/kg, respectively. Alesiani et al. (2010) isolated 59 phytochemicals from quince peels (including five newly characterized phytochemicals. Total phenolic content of methanolic extract of leaf was the highest (27.96 g/kg dried) followed by peel (7.41 g/kg), pulp (1.17 g/kg) and seed (0.52 g/kg) as reported by Carvalho et al. (2010). These bioactive components, which are important for health and nutrition, are obtained from plants by various extraction methods. Recent studies showed that the quantity and quality of the plant extracts and their bioactivity depend on the conditions under which the extraction process is carried out (Benmeziane et al., 2014; Szydlowska-Czerniak and Tulodziecka, 2015; Wu et al., 2017).

The interest in edible plants as source of natural bioactive compounds prompted us to investigate antioxidant constituents of *C. oblonga* fruit, such as flavonoids, anthocyanin, ascorbic acid and phenolic compounds, and to evaluate its antioxidant potential. For this purpose, two extraction methods were compared.

Materials and Methods

Chemicals and plant material

Linoleic acid, 2, 6 -di- tert-butyl- 4-methylphenol (butylated hydroxytoluene, BHT), 2, 2-diphenyl-1- picrylhydrazyl (DPPH, 95%), gallic acid, oxalic acid, ascorbic acid (AA), catechin, PVPP (polyvinyl polypyrrolidone), cyanidin -3-glucoside and β-carotene, were procured from Sigma-Aldrich Chemie (Steinheim, Germany). Analytical grade methanol, ethanol, and HPLC grade chloroform, standard Folin–Ciocalteu's phenol reagent, anhydrous sodium sulfate, ferric chloride, sodium carbonate, potassium ferricyanide, phosphate buffer solution (PBS), and Tween 40 were obtained from Merck (Darmstadt, Germany). The ripen fruits of *C. Oblonga* cv. Esfahan were gathered in the summer of 2018 from Maragheh region, East Azerbaijan, Iran.

Soxhlet extraction

200 g of the plant material (peel and pulp of *C. oblonga*) were extracted with methanol by using Soxhlet apparatus at 60 °C for 21 h. The extract was filtered and concentrated under vacuum at 40 °C by using a rotary evaporator (Heidolph, Laborota 4000, Schwabach, Germany).

Microwave extraction

200 g of plant material put in the central position of microwave oven in order to minimize the effect of field pattern variation in the oven. Samples were heated for several minutes (10 to 20 min) and were added to 500 mL of solvent for 2 h.

Then, all extracts were filtered and concentrated same as the Soxhlet method.

Determination of antioxidant properties

1. Diphenyl-2-picrylhydrazyl radical (DPPH) scavenging activity assay

The free radical-scavenging activities of extract were measured by using 2, 2-diphenyl-1-picryl-hydrazyl (DPPH) as described by Sharififar et al. (2007) with some modification. 3 mL of various concentrations of the extract (500, 1000, 1500, 2000 and 2500 µg/mL) was added to 1 mL of a 0.5 mM methanol solution of DPPH. The mixture was strongly shaken and left to stand at room temperature for 60 min in the dark. Then, the absorbance was measured at 517 nm against the blank. Inhibition of free radical, DPPH, in percent (I%) was calculated according to formula: I% = ((Ab- As)/Ab) × 100. Where Ab is the absorbance of the control reaction (containing all reagents except the test compound), and As is the absorbance of the test compound. The sample concentration providing 50% inhibition (IC_{50}) was calculated from the graph of inhibition percentage against sample concentrations. Decreasing absorbance values indicated higher free radical scavengering activity. Ascorbic acid (AA) was used as the positive control. Tests were carried out in triplicate.

β –Carotene/linoleic acid assay (Relative Antioxidant Activity, RAA)

The antioxidant activity was evaluated according to the method described by Miller (1971) with some modifications. Briefly, 1.5 mL of β-carotene solution (1 mg/mL in chloroform), 3 mL of linoleic acid solution (10 mg/mL in chloroform), and 1.0 mL of Tween 40 solution (300 mg/mL in chloroform) were pipetted into a 250 mL flask. The chloroform was removed by rotary vacuum evaporator, and 150 mL deionized water was added to the residue and the mixture was shaken to form an emulsion. 350 µL of test sample in methanol (2 mg/mL) was mixed with 2.5 mL of this reagent, and the emulsion system was incubated for up to 24 h at room temperature. The same procedure was repeated with the synthetic antioxidant, BHT as positive control, and a blank containing only 350 µL of methanol. After this incubation period, absorbance of the mixtures was measured at 490 nm. Antioxidative capacities of the extract were compared with those of BHT and blank.

Ferric reducing antioxidant power (FRAP) assay

The reducing power of extract was determined according to the method of Jahanban-Sfahlan et al. (1965) with some modifications. Different concentrations of methanolic extract (polar and nonpolar) of fruits in methanol (1.0 mL) were mixed with 2.5 mL of phosphate buffer (200 mM, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50 $^{\circ}$C for 20 min. A portion (2.5 mL) of trichloroacetic acid (10%) was added to the mixture to stop the reaction, which was then centrifuged at 3000 g for 10 min. The upper layer of solution (2.5 mL) was mixed with distilled water (2.5 mL) and $FeCl_3$ (0.5 mL, 0.1%), and the absorbance was measured at 700 nm. Higher absorbance indicates higher reducing power. Ascorbic acid was used as positive control.

Determination of total phenolic contents

Total phenolic contents of the extracts were determined using the Folin–Ciocalteu reagent according to the method of Singleton and Rossi (1965) using gallic acid as standard, with some modifications. The extract solution (0.1 mL) containing 1000 µg of the extract was mixed with 46 mL of distilled water in a volumetric flask and 1 mL Folin–Ciocalteu reagent was added, and the flask was thoroughly shaken. The mixture was allowed to react for 3 min and 3 mL aqueous solution of 2% Na_2CO_3 was added. At the end of incubation of 2 h at room temperature,

absorbance of each mixture was measured at 760 nm. The same procedure was also applied to the standard solutions of gallic acid, and a standard curve was obtained. Total phenol contents were expressed as µg gallic acid equivalents per mg of the extract. All tests were carried out in triplicate, and gallic acid equivalent (GAE) values were reported as X ± SD of triplicates.

Determination of total flavonoids

A modified protocol of that described by Kim et al. (2003) was employed. A 0.1 mL aliquot of methanolic extract, appropriately diluted, was mixed with 0.4 mL distilled water in a 1.5 mL micro-centrifuge tube 0.03 ml of 5% $NaNO_2$ was added and the mixture allowed to react for 5 min. Following this, 0.03 mL of 10% $AlCl_3$ was added and the mixture kept for a further 5 min. Finally, the reaction mixture was treated with 0.2 mL of 1 M Na_2CO_3 and 0.24 mL distilled water, and the absorbance at 510 nm was obtained against a blank prepared similarly, by replacing extract with distilled water. Total flavonoid content was calculated from a calibration curve using catechin as standard, and expressed as mg catechin equivalents (CTE) per 100 g plant (Dourtoglou et al., 2006).

Determination of ascorbic acid content

Ascorbic acid of the extracts was determined using ascorbic acid as standard, with some modifications. The samples (1 g) and 4 ml oxalic acid (1%) were mixed, homogenized for 1 min, and filtered. PVPP (polyvinyl polypyrrolidone) (100 g) was added to 2.5 mL of the filtered sample, to remove phenols, and 2–3 drops of H_2SO_4 (25%) were added, to reduce the pH to below 1. Absorbance of the mixture was determined at 254 nm. Results were expressed as µg ascorbic acid (AA)/100 mg fresh weight (FW) (Pantelidis et al., 2007).

Determination of anthocyanin

Total anthocyanin content was measured with the pH differential absorbance method, as described by Cheng and Breen (1991). Briefly, absorbance of the extracts was measured at 510 and 700 nm in buffers at pH 1.0 (hydrochloric acid–potassium chloride, 0.2 M) and 4.5 (acetate acid–sodium acetate, 1 M). Anthocyanin content was calculated using a molar extinction coefficient of 29,600 (cyanidin-3-glucoside) and absorbance of $A = [(A_{510} - A_{700})\ pH_{1.0} - (A_{510} - A_{700})\ pH_{4.5}]$. Results were expressed as µg cyanidin-3-glucoside equivalents (C-3-GE) per 100 mg FW (Pantelidis et al., 2007).

Statistical analysis

The experiment was conducted in completely randomized design (CRD) with three replications. Statistical analyses were made using the SAS 9.1 program. Data were subjected to analysis of variance and means were separated using LSD test at $P< 0.01$ significance level (Software Version 9.1 SAS).

Results

Antioxidant properties

Free radical, DPPH, scavenging activity assay

During extraction, it was seen that maximum extraction yield (27% W/W) was achieved with Soxhlet extraction method. The polar sub-fraction in the peel of quince fruit extract exhibited the highest radical scavenging activity with the lowest IC_{50} value of 52.34±7.3 µg/ml for Soxhlet method (Fig. 1). In addition, DPPH scavenging abilities of the methanolic extracts were lower than that of synthetic antioxidant BHT. Ascorbic acid and BHT were used as standards. In the Figures 1 and 2 were shown the antioxidant activity in the polar and non-polar sub-fraction of peel and pulp extract of *C. oblonga* fruit with Soxhlet and microwave extraction methods.

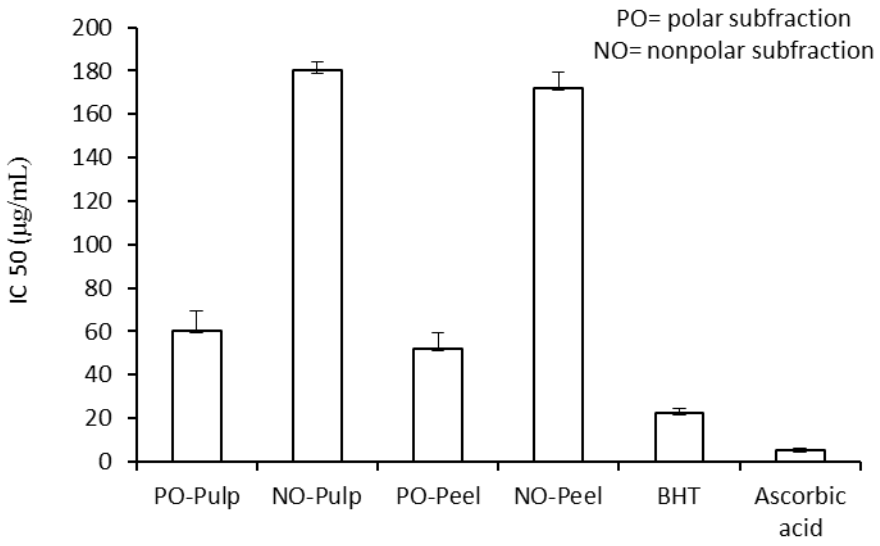

Fig. 1. Antioxidant activity of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits measured by DPPH in Soxhlet extraction method

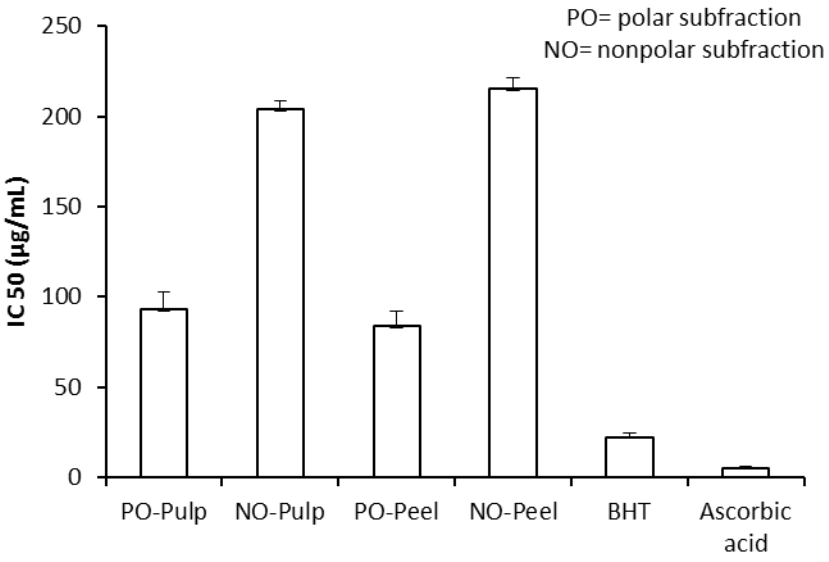

Fig. 2. Antioxidant activity of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits measured by DPPH in microwaves extraction method

β-carotene/ linoleic acid assay (RAA)

The relative antioxidative activities (RAAs) of the extracts were calculated from the equation, RAA=A sample/A BHT, where A BHT is the absorbance of the control (BHT) and A sample is the absorbance of the extract. The calculated RAAs of the extracts with Soxhlet and microwave methods are given in Figures 3 and 4. In both extraction methods, the polar sub-fraction of methanolic extract in the pulp of *C. oblonga* showed better antioxidative capacity than the other sub-fractions (RAAs for Soxhlet: 61.37% and for microwave: 48.36%).

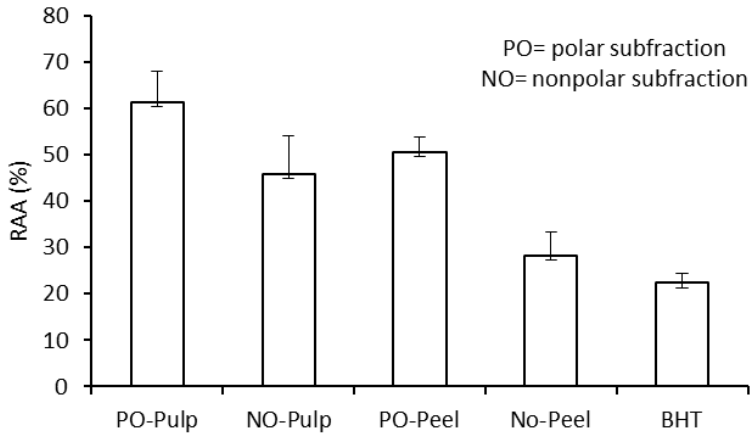

Fig. 3. Relative antioxidative activities (RAAs) of the polar and non-polar sub-fractions of peel and pulp extract of *Cydonia oblonga* Mill. fruit with Soxhlet extraction method

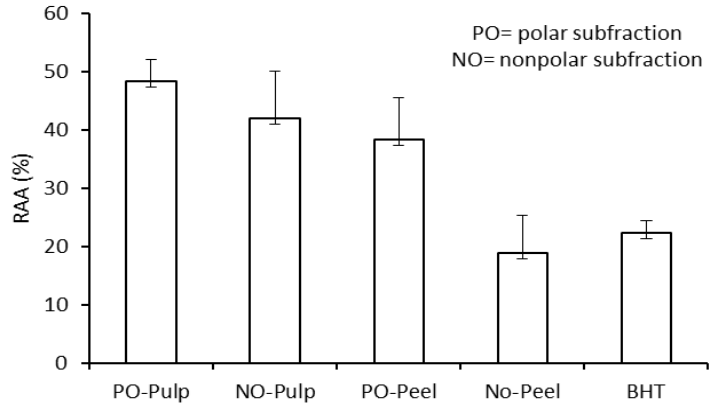

Fig. 4. Relative antioxidative activities (RAAs) of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits with microwave extraction method

FRAP assay

Figures 5 and 6 shows the reducing power of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* with two extraction methods. It was found that the reducing power of extract elevated with the increase of their concentrations.

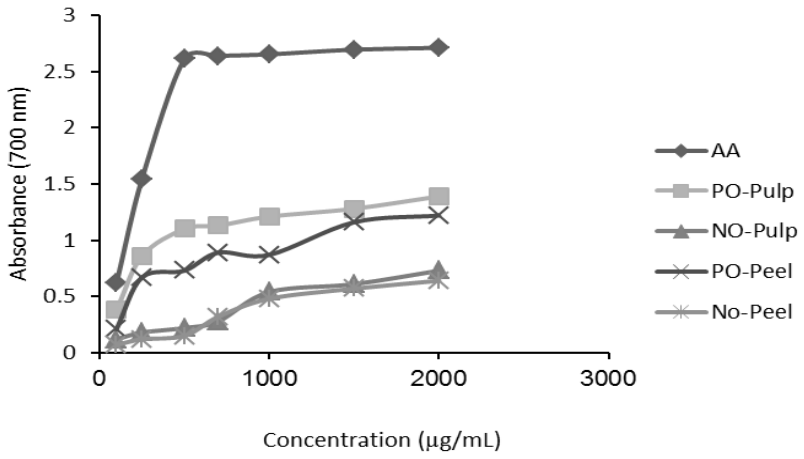

Fig. 5. Reducing power of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits with Soxhlet extraction method

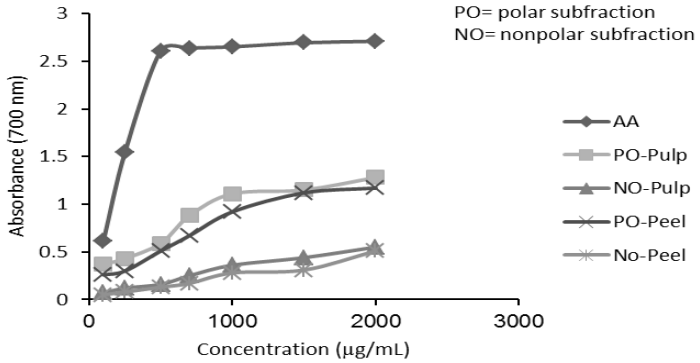

Fig. 6. Reducing power of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits with microwave extraction method

Total phenolic compounds

The amounts of total phenols found in the plant methanolic extract are shown in Figure 7 (Soxhlet extraction) and Figure 8 (microwave extraction). The results indicated that the polar sub-fraction of methanolic extract in peel of *C. oblonga* with Soxhlet method has higher total phenolic compounds (108.14 µg /mg) than the other sub-fractions (Fig. 7). In addition, according to these results, there is a relationship between total phenols content and antioxidant activity. Phenolic compounds, biologically active components, are the main agents that can donate hydrogen to free radicals and thus break the chain reaction of lipid oxidation at the first initiation step.

Total flavonoid content

The standard curve equation for determination flavonoids with catechin is y (absorbance) = 0.004 × catechin (µg) + 0.0495. The amounts of total flavonoids for peel and pulp of *C. oblonga* with two extraction methods were shown in Table 1.

Total anthocyanin content

Significant differences in anthocyanin content were recorded, since these pigments are responsible for the red and blue color. The polar sub-fraction in the peel of *C. oblonga* fruit contained the highest anthocyanin in Soxhlet (1.54±0.24 µg cyanidin-3-glucoside equivalents /100 mg fresh weight) and microwave (1.39±1.15 µg cyanidin-3-glucoside equivalents/100 mg fresh weight) extraction methods. The results were shown in Table 2.

Ascorbic acid content

Significant differences in ascorbic acid content among the different sub-fraction were recorded Table 2. The polar sub-fraction in the pulp of fruits had the highest content of ascorbic acid (4.65 ±1.28 mg/100 g fresh weight for Soxhlet extraction and 4.39±1.55 mg/100 g fresh weight for microwave extraction).

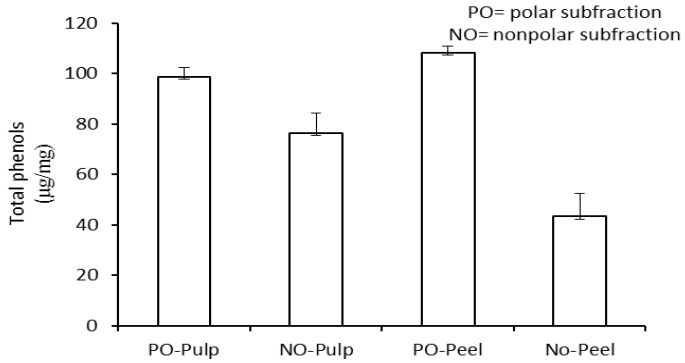

Fig. 7. Total phenols content of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits with Soxhlet extraction method

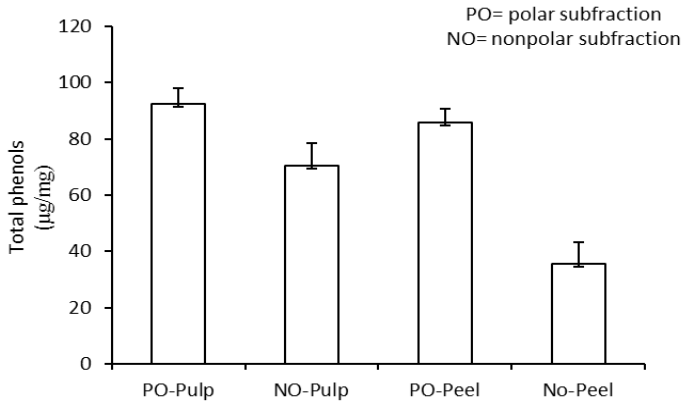

Fig. 8. Total phenols content of the polar and non-polar sub-fractions of peel and pulp extracts of *Cydonia oblonga* fruits with microwave extraction method

Table 1. Flavonoids content in methanolic extract of pulp and peel of *Cydonia oblonga* fruits by two extraction methods

Fruit part	Extraction method	Flavonoid content (mg CAE/100 g plant)
Pulp	Soxhlet	50.51± 5.8
	Microwave	49.68±9.4
Peel	Soxhlet	62.33±3.1
	Microwave	57.15±7.6

Table 2. Anthocyanin and ascorbic acid contents in methanolic extract of pulp and peel of *Cydonia oblonga* fruits by two extraction methods

Extraction method	Fruit part	Fraction	Ascorbic acid[a]	Anthocyanin[b]
Soxhlet	Pulp	Polar sub-fraction	4.65±1.28	1.13±0.41
		Nonpolar sub-fraction	1.15±0.22	0.45±0.08
	Peel	Polar sub-fraction	5.37±1.03	1.54±0.24
		Nonpolar sub-fraction	2.26±0.58	0.62±0.10
Microwave	Pulp	Polar sub-fraction	4.39±1.55	1.06±0.13
		Nonpolar sub-fraction	1.21±0.34	0.30±0.11
	Peel	Polar sub-fraction	4.01±0.63	1.39±0.15
		Nonpolar sub-fraction	1.15±0.41	0.51±0.18

[a] Results are expressed as µg cyanidin-3-glucoside equivalents /100 mg fresh weight (fw).
[b] Results are expressed as µg ascorbic acid (AA)/100 mg fresh weight (fw)

Discussion

The effect of antioxidant on DPPH radical scavenging was thought to be due to their hydrogen donating ability or radical-scavenging activity (Sharififar et al, 2007). When a solution of DPPH is mixed with that of a substance that can donate a hydrogen atom, then this gives rise to the reduced form 1, 1-diphenyl-2-picryl hydrazine (non-radical) with the loss of this violet color (Molyneux, 2004). DPPH scavenging activity is usually presented by IC_{50} value, defined as the concentration of the antioxidant needed to scavenge 50% of DPPH present in the test solution. Lower IC_{50} value reflects better DPPH radical-scavenging activity. In our research, the polar sub-fraction in the peel of quince fruit extract exhibited the highest radical scavenging activity with the lowest IC_{50} value (52.34±7.3 µg/ml). The antioxidant activities of the plant extract were also evaluated by the spectrophotometric β-carotene bleaching test. In a β-carotene/linoleic acid model system, β-carotene undergoes rapid discoloration in the absence of an antioxidant. β-carotene bleaching method is based on the loss of the

yellow color 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 values obtained clearly reflect that the peel showed greater scavenging capacity, this might be due to presence of more phenolic compounds (Silva et al., 2004) and higher concentration of sugar acids (ascorbic acid).

Different studies have indicated that the electron donation capacity reflects the reducing power of bioactive compounds in associated with antioxidant activity. Antioxidants can be explained as reducers, and inactivation of oxidants by reducers can be described as redox reaction in which one reaction species is reduced at the expense of the oxidation of the other. Fe^{3+} reduction is often used as an indicator of electron donating activity, which is an important mechanism of phenolic antioxidant action (Yildirim et al., 2001). In the reducing power assay, the presence of antioxidants in the sample would result in the reduction of Fe^{3+}–Fe^{2+} by donating an electron. Amount of Fe^{2+} complex can be then monitored by measuring the formation of Perl's Prussian blue (Fe_4 [- Fe (CN) $_6]_3$) at 700 nm. The methanolic extracts of quince fraction displayed concentration dependent antioxidant potential in the reducing power assay. These results showed methanolic extracts may act as an electron donor and therefore react with free radicals, convert them to more stable products and terminate radical chain reaction. The change in absorbance is therefore, directly related to the combined or "total" reducing power of the electron donating antioxidants present in the reaction mixture (Amin et al., 2013). Total phenolics content in the extract of the peel and pulp of *C. oblonga* were determined spectrometrically according to the Folin-Ciocalteu procedure and calculated as gallic acid equivalents. Gallic acid is a water-soluble polyhydroxy phenolic compound that can be found in various natural plants. The standard curve equation was, y (absorbance) = 0.0003 × gallic acid (µg) + 0.00534. The absorbance value was inserted in the above equation and the total amount of phenolic compound was calculated. The result showed that the total phenolic values in the peels were higher than in the fresh pulps. This high potential of phenolic compounds to scavenge radicals may be explained by their phenolic hydroxyl groups (Sun et al., 2002). Phenolic compounds might tend to accumulate in the dermal tissues of the plant body due to their potential role in protecting against ultraviolet radiations, acting as attractants in fruit dispersal, and as defense chemicals against pathogens and predators (Toor and Savage, 2005). This study showed the highest total flavonoid, anthocyanin and ascorbic acid contents in the quince peel. The results indicate that peel which otherwise is wasted, is effective in scavenging free radicals and can serve as potential antioxidants.

Conclusions

The results obtained from this research clearly showed the antioxidant activity of different parts of quince fruit by various antioxidant methods. The polar sub-fraction of quince peel methanolic extract was richer in total phenolic, flavonoid, anthocyanin and ascorbic acid contents than fruit pulp. Fruit has a number of bioactive ingredients and many of them are concentrated in the peel of the fruit rather than the flesh inside. Therefore, enjoy the benefits that unpeeled fruit offers! The antioxidant activity of plant extracts should be evaluated in a variety of model systems using several different indices to ensure the effectiveness of such antioxidant materials.

Acknowledgments

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Conflict of Interests

The authors indicate no conflict of interest for this work.

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