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Full Length Research Paper

Antioxidant characteristics of different solvent extracts from almond (*Prunus dulcis* L.) shell

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The extracts from almond (*var.* katha badam and kaghzi badam) shell, produced by different extraction solvents (80% methanol, 100% methanol, 80% ethanol, and 100% ethanol), were investigated for their antioxidant activity and total phenolic (TP) contents. Antioxidant activity (AA) of the extracts was determined by measuring the reducing power, inhibition of linoleic acid peroxidation and 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH) scavenging capacity. The shell extracts exhibited considerable amount, ranging from 1.36 to 7.21 mg, gallic acid equivalents (GAE) /100g of dry matter, of total phenolics. The reducing power (absorbance data at 12.5 mg/mL extract concentration) ranged from 0.31 to 1.83 while inhibition of linoleic acid peroxidation and DPPH radical scavenging capacity varied over 38.22 to 82.80% and 12.15 to 57.90%, respectively. The present results showed significant ($p < 0.05$) variations in relation to the extracting solvents and almond varieties tested. Efficacy of the employed solvents towards extraction of potent antioxidant components from almond shell followed the order: 80% methanol > 80% ethanol >100% methanol > 100% ethanol. These results support the potential uses of almond shells (an agrowaste) for the isolation of antioxidants which could be explored for food preservation and pharmaceutical uses.

Key words: Almond shell, solvent extraction, antioxidant components, total phenolics, linoleic acid peroxidation, 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical.

INTRODUCTION

Presently, the use of synthetic antioxidants, such as butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary butyhydroquinone (TBHQ) is becoming limited in the food industry due to their perceived carcinogenic potential (Jeong et al., 2004). On the other hand, plant-derived natural anti-oxidants, because of the anticarcinogenic attributes and other associated medicinal benefits, are gaining much appreciation (Iqbal et al., 2007; Sultana et al., 2009).

Plants are recognized as one of the most potential sources of natural antioxidants (Shahidi, 1997). Various studies and books reveal the antioxidant potential of plants due to the occurrence of different valuable bioactives, especially the phenolic compounds (Shahidi, 1997; Buricova and Reblova., 2008; Sultana et al., 2009). The role of dietary antioxidants, including Vitamin C, tocopherols and polyphenols in improving the health is now well accepted supporting the fact that diets rich in fruits and vegetables are associated with the reduced risk of chronic diseases (Lana and Tijkskens, 2006). Regular usage of nuts in the diet can be associated to reduce the risk of certain diseases including cancer and diabetes (Pinelo et al., 2004). Such health promoting properties of nuts might be linked to the presence of bioactive compounds such as flavonoids, isoflavones, and other phenolics (Subashinee et al., 2002).

Almond (*Prunus dulcis* L.) is one of the species of *Prunus* belonging to the subfamily *Prunoideae* of the

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family Rosaceae. Nutritionally and medicinally almond is a valuable food commodity. In addition to LDL-cholesterol lowering effect, the consumption of almond is also associated with the reduced risk of heart diseases (Wijeratne et al., 2006). Such health functions related to almond consumption can be attributed to the antioxidant activity of vitamin E and monounsaturated fats as well as to the presence of phenolics such as catechin, protocatechuic acid, prenylated benzoic acid, 2-prenylated benzoic acid, 2-prenyl-4-O-β-D-glucopyranosyl-oxy-4-hydroxybenzoic acid in this valuable nut (Subashinee et al., 2002).

Currently, much interest has been focused on exploring the antioxidant potential of agrowastes (Bandoniene et al., 2000). For example, Anwar et al. (2006) examined the antioxidant efficacy of various agrowastes using different antioxidant assays. Sultana et al. (2007) examined the antioxidant activity of corn cob extracts with the aid of different antioxidant models. The antioxidant activity of methanolic extracts of peanut hulls from various cultivars has also been examined by Yen and Duh (2007). In another study, Pinelo et al. (2004) evaluated the antioxidant phenolics from almond hulls and pine sawdust.

Different solvent systems have been used for the extraction of antioxidant components from various plant materials. The yield and antioxidant activity of the extracted plant materials are strongly affected by the nature of extraction solvents (Sultana et al., 2009; Anwar et al., 2010). As almond is a potential source of bioactives, it would be interesting to evaluate the efficacy of different extraction solvents towards recovery of potent antioxidants from almond shell (often discarded as an agrowaste). The present study, therefore, evaluates the effects of different extraction solvents on the recovery of extractable components, phenolics and antioxidant activity of shell from two locally available varieties of almond.

MATERIALS AND METHODS

Collection of samples

Almond samples (*var.* katha and kaghazi) known as thick and thin shell, respectively were purchased from local dry fruit market of Faisalabad, Pakistan. Manually removed almond shells were dried at ambient conditions and used for extraction purpose.

Chemicals and reagents

All the reagents used in the present experiments were analytical grade from Merck or Sigma unless specified otherwise. 2,2-diphenyl-1-picrylhydrazyl free radical (DPPH), linoleic acid, food grade synthetic antioxidant BHT, Folin-Ciocalteu reagent were purchased from Sigma Chemicals Co. (St. Louis, MO, USA). All other chemicals (analytical grade) that are anhydrous sodium carbonate, sodium hydroxide, sodium nitrite, ferrous chloride, ammonium thiocyanate, potassium dihydrogen phosphate and dipotassium hydrogen phosphate used in this study were purchased from Merck (Darmstadt, Germany).

Extracting solvent system

The dried samples of almond shell were ground into a fine powder using a commercial blender **(**TSK-949, Westpoint, France). The material passing through 80-mesh sieve was used for extraction purposes. Four solvent systems: 80% methanol (methanol: water, 80:20 v/v), 100% methanol, 80% ethanol (ethanol: water, 80:20 v/v) and 100% ethanol were employed for extraction purpose.

Extraction of almond shell antioxidant components

The ground shell material (20 g) was extracted with 200 mL of each of the extracting solvents (80% methanol), (100% methanol), (80% ethanol) and (100% ethanol) at room temperature for 6 h in an orbital shaker (Gallenkamp, UK). The residues were separated from the extracts by filtering through filter paper (Whatman No. 1); the residues were further extracted with fresh solvent. The extracts recovered from both the extractions were pooled and then freed of solvent under vacuum at 45°C, using a rotary evaporator (EYELA, SB-651, Rikakikai Co. Ltd. Tokyo, Japan). The semi solid crude concentrated extracts (CCE) recovered were weighed to calculate the yield and stored in a refrigerator (-4°C), until used for further analyses (Sultana et al., 2009).

Evaluation of antioxidant activity of almond shell extract

Determination of total phenolics content (TPC)

The amount of TP was estimated colorimetrically with the aid of Folin-Ciocalteu reagent following the method as described by Sultana et al. (2009). In this test, 50 mg of crude extract was mixed with 0.5 mL of Folin-Ciocalteu reagent and 7.5 mL deionized water. The mixture was kept at room temperature for 10 min, and then 1.5 mL of 20% sodium carbonate (w/v) was added. The mixture was heated in a water bath at 40°C for 20 min and then cooled in an ice bath; the absorbance was recorded at 755 nm using a spectrophotometer (U-2001, Hitachi Instruments Inc., Tokyo, Japan). Amount of TP was calculated from gallic acid standard calibration curve (R^2 = 0.9984). The results were expressed as gallic acid equivalents gallic acid equivalents (GAE) g/100g of dry matter.

DPPH radical scavenging assay

2,2–diphenyl–1-picrylhydrazyl (DPPH) free radical scavenging activity of the extracts was assessed following the procedure used earlier (Iqbal et al., 2005). Briefly, into extract (1.0 mL) containing 25 µg/mL of extract dry mass in methanol, 5.0 mL of freshly prepared DPPH free radical solution (0.025 g/L) was added. Absorbance at different time periods (0.5, 1.0, 2.0, 5.0 and 10.0 min) of the reaction mixture was recorded at 515 nm using a spectrophotometer. Absorbance taken at 5th min was used for comparison of radical scavenging activity of the extracts. Percentage DPPH radical scavenging activity was calculated with the help of the following equation:

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

In this equation A_{blank} denotes the absorbance of control while A_{sample} is the absorbance of the test reaction mixture.

Antioxidant activity determination in linoleic acid system

The antioxidant activity of the tested almond shell extracts was also determined by the magnitude of inhibition of linoleic acid peroxidetion (Iqbal et al., 2005). For this purpose, 5 mg of the extract was added into a solution mixture of linoleic acid (0.13 mL), 99.8% ethanol (10 mL) and 10 mL of 0.2 M sodium phosphate buffer (pH 7). The mixture was made up to 25 mL by the addition of distilled water and then incubated at 40°C up to 360 h. Extent of oxidation was measured by peroxide value according to thiocyanate method as described by Yen et al. (2000). In a volumetric flask, 10 mL of ethanol (75% v/v), 0.2 mL of aqueous solution of ammonium thiocyanate (30% w/v), 0.2 mL of sample solution and 0.2 mL of ferrous chloride ($FeCl_2$) solution (20 mM in 3.5% HCl; v/v) were added successively. After stirring for 5 min, the absorbance of the reaction mixture was noted at 500 nm using a spectrophotometer. A control containing all the reagents except the test extract was also processed under similar conditions. A synthetic antioxidant compound namely BHT was employed as a positive control. Percentage inhibition (I) of linoleic acid peroxidation was calculated with the help of following equation:

(% I) = 100 – [(Abs. increase of sample at 360 h / Abs. increase of control at 360 h) / 100]

Determination of reducing power

The reducing power of almond shell extracts was assessed according to the procedure described by Yen et al. (2000) with slight changes. The extract (in the concentration range between 2.5 and10.0 mg/mL) was mixed with sodium phosphate buffer (5.0 mL, 0.2 M, pH 6.6) and potassium ferricyanide solution (5.0 mL, 1.0%); the mixture was incubated at 50°C for 20 min. Then 5 mL of 10% trichloroacetic acid was added and the mixture centrifuged at 1000 × *g* for 10 min at 5°C in a refrigerated centrifuge machine (CHM-17; Kokusan Denki, Tokyo, Japan). The upper /top layer (5.0 mL) of was decanted and diluted further with 5.0 mL distilled water and ferric chloride (1.0 mL, 0.1%). The absorbance of the final mixture, recorded at 700 nm, using a spectrophotometer was used to express the extracts reducing power in terms of absorbance data.

Statistical analysis

Three samples for each almond *var.* were assayed and analyzed individually in triplicate and the data reported as mean ± SD (n = 3 × 3 × 1). Data were analyzed using Minitab 2000 Version 13.2 statistical software (Minitab Inc. Pennsylvania, U.S.A) at 5% significant level.

RESULTS AND DISCUSSION

Antioxidant yield

The percentage yield and total phenolic contents of shell extracts from the tested varieties (thick shell and thin shell) of almonds are depicted in Table 1. The amount of antioxidant components extracted from almond shells using aqueous methanol (80% methanol), 100% methanol, aqueous ethanol (80% ethanol) and 100% ethanol varied from 2.08 to 8.92% showing significant difference among extraction solvents used ($P > 0.05$). Aqueous ethanol (80%) ethanol extract from both the shell varieties (thick and thin shell) exhibited highest extract yield 6.65 and 8.92%, respectively. It is assumed that almond shell contained antioxidant compounds which are more soluble in aqueous ethanol and aqueous methanol as against the pure counterpart solvents.

The percentage yield of almond shell antioxidant components as obtained in our present analysis was higher than those reported earlier (0.25 to 4.46 %) by Pinelo et al. (2004) for almond hull using ethanol, methanol and water.

Differences in the yield of extracts from almond shell might be ascribed to the differing polarities of the solvents used as well as to the availability of extractable components of varying nature defined by their chemical composition (Sfahlan et al., 2009). Based on the present results, the ability of different solvents to extract antioxidant components from almond shells followed the order: 80% ethanol > 80% methanol > 100% ethanol > 100% methanol.

Total phenolic contents (TPC)

The interest in the plant phenolics has increased greatly due to their prominent free radical scavenging activity (Sultana and Anwar, 2008). The determination of TPC in almond shell extracts was carried out by Folin-Ciocalteau method and the results expressed as gallic acid equivalent per dry matter. The given colorimetric method was chosen due to its efficacy, simplicity and quickness to quantify the total phenolics.

TPC of pure and aqueous methanolic and ethanolic extracts from thick and thin shell almond are given in Table 1. The amount of total phenolics recovered in different almond shell extracts varied from 1.36 to 7.21 gallic acid equivalents (GAE) mg/g of dry matter showing considerable variation among the solvents and between the varieties tested. Phenolic contents as determined in the present analysis were found to be in close agreement with those reported by Pinelo et al. (2004) in ethanol extract (2.31 to 7.21 mg gallic acid equivalents (GAE) /g) and in methanol extract (1.06 to 4.12 mg gallic acid equivalents (GAE) /g) produced from almond hulls. Conversely, Safhalan et al. (2009) investigated much higher (18.4 to 62.7 mg gallic acid equivalents (GAE) /g extract) TPC in methanol extracts from different varieties of almond shell.

When compared the data between the two varieties of almond, thin shell variety has shown relatively higher values for TPC as compared to thick shell. In case of both the varieties, aqueous methanol and aqueous ethanol extracted greater amount of total phenols which could be supported by the literature. According to several reports aqueous methanol and ethanol have been proven as effective solvents to extract the phenolic compounds from different plant matrices (Siddhuraju and Becker, 2003; Sultana et al., 2009; Sultana and Anwar, 2008; Anwar et al., 2010).

Table 1. Yield (g/100g of dry weight) and total phenolic contents (GAE) mg/g of dry matter) of solvents extracts from almond shell.

Extract	Percentage yield (g/100g of dry matter)		Total phenolic contents (GAE) mg/g of dry matter)	
	Thick shell	Thin shell	Thick shell	Thin shell
100% methanol extract	2.50 ± 0.30	2.08 ± 0.05	2.07 ± 0.30	3.78 ± 0.05
80% methanol extract	5.40 ± 0.21	3.36 ± 0.13	2.26 ± 0 .21	7.21 ± 0.20
100% ethanol extract	3.30 ± 0.32	3.87 ± 0 .16	1.36 ± 0.01	2.31 ± 0.50
80% ethanol extract	6.65 ± 0.43	8.92 ± 0.40	1.47 ± 0.03	2.87 ± 0.20

Values are mean ± SD of three samples analysed individually in triplicate (p <0.05).

Table 2. DPPH radical scavenging capacity and inhibition of linoleic acid peroxidation of different solvent extracts from almond shell.

Extract (%)	% DPPH radical scavenging activity		% Inhibition of linoleic acid peroxidation	
	Thick shell	Thin shell	Thick shell	Thin shell
100 methanol extract	17.64 ± 0.28	37.01 ± 0.70	68.15 ± 1.30	64.14 ± 2.90
80 methanol extract	18.22 ± 0.21	57.90 ± 0.58	82.80 ± 0.90	76.25 ± 2.00
100 ethanol extract	14.92 ± 0.45	15.32 ± 0.73	38.22 ± 1.90	61.80 ± 1.80
80 ethanol extract	12.15 ± 0.34	36.22 ± 0.96	73.25 ± 2.10	58.20 ± 1.40

Values are mean ± SD of three samples analyzed individually in triplicate (p < 0.05).

DPPH radical scavenging assay

DPPH, a violet color stable organic free radical, shows absorption maximum around 515 to 528 nm. Upon receiving proton from hydrogen donor substances such as phenolics, it loses its chromophore and changes into yellow color. With the increase in concentration of phenolic compounds or the degree of hydroxylation of the phenolic compounds, DPPH free radical scavenging capacity and thus antioxidant activity increases (Sultana and Anwar, 2008). Percentage DPPH free radical scavenging activity of different almond shell extracts as affected by the extracting solvent is depicted in Table 2. Absorbance values in this test were recorded during 0.5 to 10 min time intervals from the initiation of the reaction. The observed scavenging activity was comparable at the initiation of the reaction and altered with the increase in the reaction time until it became constant at 10th min. Major differences (p< 0.05) of $DPPH^{\cdot}$ scavenging capacity among different solvent extracts were recorded at 5th min of the reaction.

A higher percentage $DPPH^{\cdot}$ scavenging capacity is correlated to a higher antioxidant activity of extracts (Sultana et al., 2009). $DPPH^{\cdot}$ scavenging activity for almond extracts varied widely ranging from 12.15 to 18.22% in case of thick shell and 15.32 to 57.9% in case of thin shell extracts. Maximum $DPPH^{\cdot}$ scavenging activity was observed for 80% methanol extracts from both varieties showing greater efficacy of this solvent towards extraction of potent radical scavengers. This free radical scavenging activity of shell extracts might be related to the presence of phenolic compounds as determined in the related assays. The results of the present study were comparable with those reported by Pinelo et al. (2004) which indicated $DPPH^{\cdot}$ scavenging activity as much as 2.15 to 36.21% in pure ethanol extract and 14.92 to 58.05% in pure methanol extract of almond hull. The present scavenging activity was significantly (P<0.05) affected by extraction solvents used.

Antioxidant activity in linoleic acid system

The antioxidant activity of almond shell extracts in the present investigation was also assessed by measuring the percentage inhibition of linoleic acid oxidation. Linoleic acid(C18:2), a poly unsaturated fatty acid, upon oxidation produces peroxides which oxidize ferrous (Fe^{2+}) to ferric (Fe^{3+}), the later yields complex with thiocyanate (SCN), the concentration of which is estimated colorimetrically by measuring the absorbance at 500 nm. A higher magnitude of peroxides formed during the reaction gives higher absorbance thus relating to lower antioxidant activity. The results for percentage inhibition of linoleic acid peroxidation, after incubation time of 360 h, are presented in Table 2.

Synthetic antioxidant, BHT was used as a positive control to compare the antioxidant activity of almond shell extracts. The shell extracts from both the varieties of almond exhibited appreciable inhibition of peroxidation ranging from 38.22 to 82.80% in case of thick and 58.20 to 76.25% for thin shell. As expected aqueous (80%) methanol and aqueous (80%) ethanol extracts were found to be more effective towards inhibition of peroxidation. The antioxidant activity in terms of the ascribed test varied significantly in relation to varieties and solvents tested (P<0.05).

Table 3. Reducing power of different extracts from almond shell.

Conc. mg/mL	Extract								BHT
	100% Methanol		80% Methanol		100% Ethanol		80% Ethanol		
	Thick shell	Thin shell	Thick shell	Thin shell	Thick shell	Thin shell	Thick shell	Thin shell	
2.5	0.31 ± 0.01	0.40 ± 0.01	0.55 ± 0.01	0.55 ± 0.01	0.08 ± 0.01	0.08 ± 0.01	0.21 ± 0.01	0.20 ± 0.01	0.74 ± 0.04
5.0	0.46 ± 0.01	0.46 ± 0.01	0.62 ± 0.02	0.61 ± 0.02	0.12 ± 0.02	0.12 ± 0.02	0.37 ± 0.08	0.37 ± 0.08	0.94 ± 0.05
7.5	0.59 ± 0.04	0.50 ± 0.04	0.73 ± 0.03	0.72 ± 0.03	0.27 ± 0.02	0.27 ± 0.02	0.38 ± 0.10	0.38 ± 0.10	1.13 ± 0.06
10.0	0.92 ± 0.05	0.90 ± 0.05	0.85 ± 0.03	0.84 ± 0.03	0.29 ± 0.04	0.28 ± 0.04	0.41 ± 0.11	0.41 ± 0.11	1.53 ± 0.08
12.5	1.54 ± 0.06	1.44 ± 0.06	1.83 ± 0.04	1.82 ± 0.04	0.32 ± 0.04	0.31 ± 0.04	0.43 ± 0.13	0.53 ± 0.13	1.70 ± 0.09

Values are mean ± SD of three samples analysed individually in triplicate (P <0.05).

Reducing power of extracts

Measurement of reducing potential is a useful indicator to studying some important aspects of antioxidant activity of the plant extracts. Mechanistically, in this method, ferric ions undergo reduction to form ferrous ions with consequent change in color from yellow to bluish green. The intensity of color and thus absorption directly depend on the reducing potential of the compounds present in the reaction medium. Greater intensity of the color gives higher absorption relating to greater antioxidant activity (Sultana and Anwar, 2008). The data in Table 3 depict the reducing potential of two varieties of almond shell extracts produced by aqueous and pure alcohols. The reducing potential of the extracts (over the concentration range of 2.5 to 12.5 mg/mL) increased in a concentration dependent manner. At 12.5 mg/mL extract concentration, the reducing potential ranged from 0.32 to 1.83 (absorbance values). Reducing potential was higher for 80% methanolic extracts of thick and thin shells, 1.83 and 1.82, respectively. The statistical analysis showed significant variation of reducing potential as function of extraction solvent and varieties tested. Sfahlan et al. (2009) reported the reducing power of almond shell extracts using methanol as extracting solvent to be 0.151 to 0.228 lower than the values found in the present analysis. Reducing potential of plant extracts may vary within the varieties due to difference in genetic makeup of almond varieties as well as due to other factors such as maturity at harvest and processing conditions (Sultana and Anwar, 2008; Sultana et al., 2009; Anwar et al., 2010).

It could be concluded from the results of the present study that the antioxidant potential of almond shell extracts varied considerably in relation to the varieties tested and the extraction solvents employed. Aqueous (80%) methanol has been found to be the best solvent for extraction of potent antioxidant components from almond shell. This investigation suggests the exploration of almond shell as a cheap raw material for extraction of valuable antioxidants.

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