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Antioxidant and antiradical activities of phenolic extracts from Iranian almond (*Prunus amygdalus* L.) hulls and shells

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Abstract: Wild almonds of Iran show genetic diversity and have very a large distribution. Different species of these almonds have more variation in genes resistant to dryness, saltiness, diseases, pests, and other factors. In order to compare the antioxidant and antiradical activity of wild almond hull and shell phenolic extracts, 4 wild almond species (*Amygdalus lycioides* Spach, *A. kotschyi* Boiss. & Hohen, *A. pabotti* Browicz, *A. trichamygdalus* (Hand.-Mzt) Woronow) were selected from Shahindezh and Qasemloo Valley, West Azerbaijan province, Iran, in 2007. The fruits of these almonds were collected, their hulls and shells were dried and then ground, and then methanolic extracts were prepared from these hulls and shells. Total phenolic content was determined using the Folin–Ciocalteu (F–C) method. The extracts' reducing power and scavenging capacity for radical nitrite, hydrogen peroxide, and superoxide were evaluated. Hull and shell extracts, respectively, had a range of 122.2 ± 3.11-75.9 ± 1.13, 46.6 ± 0.94-18.1 ± 0.15 mg/g gallic acid equivalents/g extract in total phenolic content, 0.667-0.343, 0.267-0.114 AU at 700 nm in reducing power, 94.9 ± 0.97%-63.7 ± 1.14%, 65.7 ± 0.64%-24.2 ± 1.31% in hydrogen peroxide, 90.6 ± 1.11%-60.7 ± 2.13%, 56.7 ± 1.33%-28.5 ± 1.65% in superoxide, and 85.2 ± 1.21%-53.4 ± 2.69%, 53.5 ± 0.86%-24.9 ± 1.63% in nitrite radical scavenging percentage. The results show that the antioxidant and antiradical activities of the almond hull are higher than those of its shell phenolic extract among different species of almond. In addition, the reducing power of almond hull and shell phenolic extract was positively correlated with the phenolic content and radical scavenging capacities of wild almond hull and shell extracts in different species were positively correlated with phenolic content and reducing power.

Key words: Wild almonds, hull, shell, phenolic content, antioxidant, antiradical

İran bademinin gövde ve kabuklarındaki fenolik özütlerinin antioksidant ve antiradikal aktiviteleri

Özet: İran yabani bademleri, genetik çeşitlilik gösterirler ve çok geniş yayılışa sahiptirler. Bu bademlerin farklı türleri kuraklık, tuzluluk, hastalık, pestisitler ve diğer faktörlere dirençli genlerde daha fazla varyasyona sahiptir. Yabani badem gövde ve kabuklarının fenolik özütlerinin antioksidan ve antiradikal aktivitelerini karşılaştırmak için, dört yabani badem türü (*Amygdalus lycioides* Spach, *A. kotschyi* Boiss. & Hohen, *A. pabotti* Browicz, *A. trichamygdalus* (Hand.-Mzt) Woronow), Shahindezh ve Qasemloo vadisi, batı Azerbaycan, 2007 yılında İran'dan seçilmiştir. Bademlerin meyveleri toplandı, gövde ve kabukları kurutuldu, dövüldü ve daha sonra metanolik özütleri gövde ve kabuklardan hazırlandı. Toplam fenolik miktarı Folin-Ciocalteu (F-C) metodu kullanılarak belirlenmiştir. Radikal nitrit, hidrojen peroksit ve süperoksit için, özütlerin indirgeme gücü ve süpürme kapasitesi değerlendirilmiştir. Gövde ve kabuk özütleri için bu

değerler sırasıyla, toplam fenolik içerikte, 122,2 ± 3,11-75,9 ± 1,13, 46,6 ± 0,94-18,1 ± 0,15 mg/g gallik asit eşdeğer/g özütü, 700 nm de indirgeme gücü 0,667-0,343, 0,267-0,114 AU, hidrojen peroksit % 94,9 ± 0,97-% 63,7 ± 1,14, % 65,7 ± 0,64-% 24,2 ± 1,31, süperoksit % 90,6 ± 1,11-% 60,7 ± 2,13, % 56,7 ± 1,33-% 28,5 ± 1,65 ve nitrit radikal süpürme yüzdesi % 85,2 ± 1.21-% 53,4 ± 2,69, % 53,5 ± 0,86-% 24,9 ± 1,63 olarak bulunmuştur. Sonuçlar, bademin farklı türleri arasında, badem gövdesinin antioksidan ve antiradikal aktivitelerinin, badem kabuklarının fenolik özütlerininkinden daha büyük olduğunu gösterdi. Ayrıca badem gövde ve kabuk fenolik özütünün indirgeme gücü fenolik içeriği ile ilişkiliydi ve farklı yabani badem türlerinin gövde ve kabuk özütlerinin radikal süpürme kapasiteleri, fenolik içerik ve indirgeme gücü ile de pozitif ilişkiliydi.

Anahtar sözcükler: Yabani badem, gövde, kabuk, fenolik içerik, antioksidan, antiradikal

Introduction

Synthetic antioxidants such as butylated hydroxyanisole, butylated hydroxytoluene, and tertiarybutylhydroquinone are widely used in foods. The use of synthetic antioxidants in foods, however, is discouraged because of their toxicity and carcinogenicity (1). Therefore, special interest has been focused on the use of natural antioxidants that can remove free radicals, which cause various diseases, carcinogenesis, and aging (2). Natural antioxidant compounds such as flavonoids, tannins, coumarins, curcuminoids, xanthons, and terpenoids are found in fruits, leaves, seeds, and oils of various plant products (3), and some of these are as effective as synthetic antioxidants in different models (4).

Phenolic compounds range in size from monomers to long-chain polymers such as tannins, and usually exist bound to carbohydrates or as part of repeating subunits of high molecular weight polymers. Various phenolic compounds have been detected in almond by-products. 4 different flavonol glycosides—isorhamnetin rutinoside, isorhamnetin glucoside, kaempferol rutinoside, and kaempferol glucoside—have been reported in almond seedcoats. Other investigators have likewise identified phenolic compounds in almond skins and shells, including quercetin glycosylated to glucose, galactose and rhamnose, kaempferol, naringenin, catechin, protocatechuic acid, vanillic acid, and a benzoic acid derivative. Phenolic compounds act as antioxidants by scavenging free radicals and chelating metals in foods. These natural antioxidants convey wholesomeness to consumers (5).

The aim of this study was to determine and compare phenolic content in different species of wild almond hull and shell, evaluating their potential antioxidant and antiradical activity.

Methods

Preparation and extraction of samples

Fruit samples of *Amygdalus lycioides* Spach, *Amygdalus kotschyi* Boiss. & Hohen, *Amygdalus pabotti* Browicz, and *Amygdalus trichamygdalus* (Hand.-Mzt) Woronow were supplied by the Agricultural and Natural Research Center of West Azerbaijan province from Shahindezh and Qasemloo Valley, West Azerbaijan province, Iran. The hulls and shells of wild almonds were separated, dried at room temperature, and then reduced to coarse powder. This powder (6 g) was extracted with methanol (100 mL) in a Soxhlet apparatus for 30 min at 80 °C. The methanol extract of almond hull and shell was evaporated to dryness using a rotary evaporator and stored in the dark at 4 °C until use (6).

Determination of total phenolics

The content of total phenolics was determined colorimetrically using Folin-Ciocalteus's phenol reagent, as described by Singleton and Rossi (7). Briefly, 2.5 mL of 10-fold diluted Folin-Ciocalteu reagent, 2 mL of 7.5% sodium carbonate, and 0.5 mL phenolic extract were mixed well. The absorbance was measured at 765 nm after 15 min heating at 45 °C. A mixture of water and reagents was used as a blank. The phenolic content was expressed as mg gallic acid equivalents per g of extract.

Reducing power

The reducing power of almond hull and shell phenolic extracts was determined according to the method of Oyaizu (8). Almond hull and shell phenolic extract (1 mg/mL), phosphate buffer (1 mL, 0.2 M, pH 6.6), and potassium ferricyanide (1.0 mL, 10 mg/mL) were mixed together and incubated at 50 °C for 20 min. Trichloroacetic acid (1.0 mL, 100 mg/mL) was added to the mixture and centrifuged at 13,400

×g for 5 min. The supernatant (1.0 mL) was mixed with distilled water (1.0 mL) and ferric chloride (0.1 mL, 1.0 mg/mL), and then the absorbance was measured at 700 nm.

Hydrogen peroxide radical scavenging assay

A modified version of the method described by Ruch et al. (9) was used to determine the hydrogen peroxide-scavenging ability of almonds hull and shell extracts. Extracts (1 mg/mL) were dissolved in 3.4 mL of a 0.1 M phosphate buffer (pH 7.4) solution and mixed with 600 µL of a 43 mM solution of hydrogen peroxide (prepared in the same buffer). The concentration of hydrogen peroxide was measured by reading absorbance values at 230 nm of the reaction mixtures. For extracts, a blank sample devoid of hydrogen peroxide was used for background subtraction. Reduction of absorbance in a hydrogen peroxide solution alone due to its degradation was recorded and values were corrected accordingly. The concentration of hydrogen peroxide in the assay medium was determined using a standard curve, and the hydrogen peroxide- scavenging effects of the extracts were calculated using the following equation:

$$H_2O_2\text{ - scavenging effect (\%)} = 100 - \left[\frac{H_2O_2\text{ concentration of medium containing the additive}}{H_2O_2\text{ concentration of the control medium}}\right] \times 100$$

Superoxide radical scavenging assay

For superoxide anion radical assay, the superoxide anion radicals were generated by a pyrogallol autoxidation system (10). A volume of 9 mL of Tris-HCl buffer solution (50 mmol/l, pH 8.2) was added into a test tube, and the test tube was incubated in a water bath at 25 °C for 20 min. A volume of 40 µL of pyrogallol solution (45 mmol/L of pyrogallol in 10 mmol/L of HCl), which was also pre-incubated at 25 °C, was injected to the above test tube with a microliter syringe and mixed. The mixture was incubated at 25 °C for 3 min and then a drop of ascorbic acid was dripped into the mixture promptly to terminate the reaction. The absorbance at 420 nm marked as A_0 was measured 5 min later, and this A_0 denotes the speed of pyrogallol autoxidation. The A_1 autoxidation speed was obtained applying the above method and with the addition of a certain concentration (1 mg/mL) of extract into the Tris-HCl buffer solution. Simultaneously, a blank control of reagent was obtained as A_2. The scavenging percentage was calculated according to the following formula:

$$\text{Superoxide radical scavenging effect (\%)} = A_0 - (A_1 - A_2) \times 100/A_0$$

Nitric oxide radical scavenging assay

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (11). In this investigation, Griess Illosvoy reagent was modified by using naphthyl ethylene diamine dihydrochloride (0.1% w/v) instead of 1-napthylamine (5%). The reaction mixture (3 mL) containing sodium nitroprusside (10 mM, 2 mL), phosphate buffer saline (0.5 mL), and almond hull and shell phenolic extract (1 mg/mL) was incubated at 25 °C for 150 min. After incubation, 0.5 mL of the reaction mixture was mixed with 1 mL of sulfanilic acid reagent (0.33% in 20% glacial acetic acid) and allowed to stand for 5 min for completing diazotization. Then 1 mL of naphthyl ethylene diamine dihydrochloride was added, mixed, and allowed to stand for 30 min at 25 °C. A pink coloured chromophore is formed in diffused light. The absorbance of these solutions was measured at 540 nm against the corresponding blank solutions. The nitric oxide-scavenging capacities of the extracts were calculated using the following equation:

$$\text{Nitric oxide radical scavenging effect (\%)} = A_{\text{Blank}} - A_{\text{Sample}} \times 100/A_{\text{Sample}}$$

Statistical analysis

All experiments were performed with at least 3 replicates. One-way ANOVA was applied to determine the significance of results between different treatments. All the statistical analyses were done using SPSS v.11.5 for Windows.

Results and discussion

Phenolic compounds are the most active natural antioxidants in plants (12). They are known to act as antioxidants not only because of their ability to donate hydrogen or electrons but also because they are stable radical intermediates (13). Generally, the outer layers of plants such as the peel, shell, and hull contain large amount of polyphenolic compounds to protect the inner materials. A number of phenolic acids are linked to various cell wall components such as arabinoxylans and proteins (14).

Free radicals possess an unpaired electron, which makes them highly reactive. Antioxidants neutralize free radicals by donating a hydrogen atom to them. Attempts have been made to evaluate the effectiveness of antioxidants in scavenging free radicals such as ABTS radical anion ($ABTS^{\bullet-}$) and $DPPH^{\bullet}$, hydroxyl $HO^{\bullet}$, and superoxide $O2^{\bullet-}$ radicals (15). In all these methods, antioxidant efficacies are measured at room temperature, thus eliminating any risk of thermal degradation of the molecules being tested.

Different solvent extractions provide different types of compounds because of their variable chemical nature and sensitivity toward extraction or hydrolysis methods. As seen with almonds, diethyl ether (16), methanol (17), ethyl acetate, and *n*-butanol (18), which have been used to extract phenolic compounds in almond skins, shells and hulls, have resulted in different components in their extraction. Pinelo et al. (19), in order to optimize the yield of phenolic compounds in almond hull and pine sawdust under different experimental conditions, showed that among the 3 solvents (ethanol, methanol, and water) ethanol was the most favourable for total extractables, although methanol was more selective for extracting poly phenolics. Therefore, in this study we used methanol as a solvent for the extraction of phenolic compounds from almond hulls and shells.

In this study, the average of total phenolic content in 4 wild almond hull phenolic extracts was 97.1 ± 1.83 mg gallic acid equivalents/g extract. The maximum total phenolics content in hull extract was 122.2 ± 3.11 mg/g for *A. kotschyi* and minimum total phenolics content was 75.9 ± 1.13 mg/g for *A. pabotti*. The content of total phenolics in almond hull extract reported by Siriwardhana and Shahidi (20), Wejerante et al. (21), and Subhashinee et al. (21) were 71.1 ± 1.74 mg catechin equivalents/g extract and 71 ± 2 mg quercetin equivalents/g extract, respectively. The average of total phenolics content in 4 wild almond shell phenolics extract was 31.8 ± 0.92 mg gallic acid equivalents/g extract. The highest total phenolics content of shell extract, 46.6 ± 0.94 mg/g, was obtained for *A. lycioides*, and the lowest phenolics content of shell extract 18.1 ± 0.15 mg/g for *A. trichamygdalus.* Total phenolics content for almond shell extract reported by Moure et al. (22) was 2.2 g gallic acid equivalents/100 g shell. This study shows that the average of total phenolics content in 4 almond hulls extract (97.1 ± 1.83 mg/g) was 3-fold higher than that of their shell extract (31.8 ± 0.92 mg/g). This indicates that the total phenolic of almond hull extract was higher than that of its shell in different almond species.

The antioxidant properties of phenolic compounds are associated with their reducing power (23), which is associated with the presence of reductones (24). The reducing power of almond hull and shell in different species increases significantly with phenol content. In addition, the phenolic content and reducing power of wild almond hull and shell vary among species (Figures 1 and 2). The average reducing power values in 4 wild almond hulls phenolics extract was 0.504 AU at 700 nm. Maximum reducing power was 0.667 for *A. kotschyi* and minimum reducing power was 0.343 for *A. pabotti*. The average reducing power values in 4 wild almond shell phenolics extract of 0.191 was achieved. Maximum reducing power was 0.267 for *A. lycioides* and minimum reducing power among almonds shell extract was 0.114 for *A. trichamygdalus*. The average of reducing power values in almond hull extract (0.504) was higher than that of their shell extract (0.191). Accordingly, the reducing power of almond hull extract was higher than that of its shell extract in different species of almond.

The hydrogen peroxide scavenging activity of almond hull and shell methanolic extracts were phenol content dependent; species with high phenolic content scavenged most of the hydrogen peroxide (Figure 3). In this investigation the average of hydrogen peroxide radical scavenging percentage in 4 wild almond hull phenolics extract was 78.7 ± 1.03%. Maximum hydrogen peroxide radical scavenging percentage was 94.9 ± 0.97% for *A. kotschyi* and the minimum hydrogen peroxide radical scavenging percentage was 63.7 ± 1.14% for *A. pabotti*. Hydrogen peroxide radical scavenging percentage for almond hull extract reported

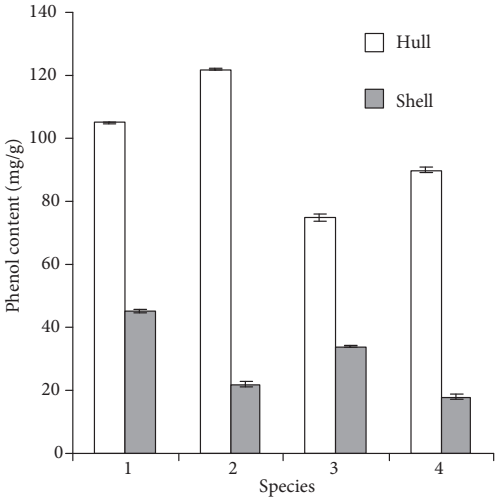
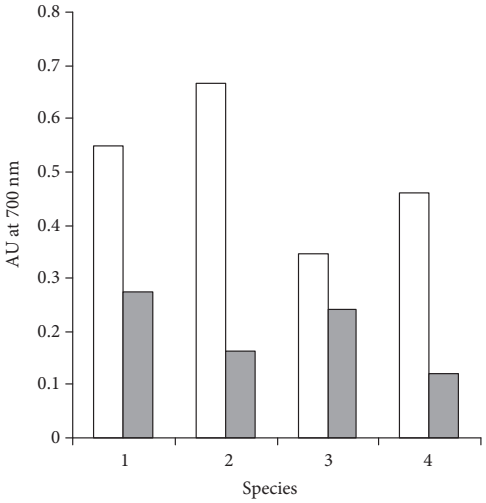


Figure 1. Phenolic content of 4 wild almond hull and shell extracts (mg Gallic acid equivalents/g methanolic extract): 1-*A. lycioides,* 2- *A. kotschyi,* 3-*A. pabotti,* 4-*A. trichamygdalus.* Means of 3 replicates with standard errors, P < 0.05.



Figures 2. Reducing power of 4 wild almond hull and shell phenolic extracts: 1-*A. lycioides,* 2- *A. kotschyi,* 3-*A. pabotti,* 4-*A. trichamygdalus.* (mean ± S.D., n = 3), P < 0.05.

by Siriwardhana and Shahidi (20) was 66 ± 1% at 100 ppm and 91 ± 1% at 200 ppm. The average of hydrogen peroxide radical scavenging percentage in 4 wild almond shell extract was 45.3 ± 0.99%. The maximum hydrogen peroxide radical scavenging percentage was 65.7 ± 0.64% for *A. lycioides* and minimum hydrogen peroxide radical scavenging percentage among almond shell extract was 24.2 ± 1.31% for *A. trichamygdalus*. The average of hydrogen peroxide radical scavenging percentage of 4 wild almond hull phenolics extract (78.7 ± 1.03%) was higher than that of their shell extract (45.3 ± 0.99%). This indicates that hydrogen peroxide radical scavenging percentage of almond hull phenolics extract was higher than that of its shell in different wild almond species. The rates of hydrogen peroxide scavenging of hull and shell varied among species too. Thus, hydrogen peroxide scavenging activity of almonds hull and shell extracts in some species (for example of hull *A. kotschyi* and for shell *A. lycioides*) would contribute to their inhibition of lipid peroxidation and thereby protect cells from oxidative damage.

We evaluated the scavenging capacity of almond hull and shell extracts towards superoxide anion radicals by using a pyrogallol autoxidation system. Pyrogallol can autoxidate fast in alkali conditions and release superoxide anions and the superoxide anions can accelerate the autoxidation. However, the superoxide anions can be scavenged by adding some scavenger or antioxidant, the autoxidation will thus be depressed. As shown in Figure 4, the inhibition effects of wild almonds hull and shell extracts on the autoxidation of pyrogallol were relatively feeble in species with lower phenolic content, but the wild almond hull and shell extract with high phenolic content exhibited strong inhibition activities. This indicates that wild almond hull and shell, in particular hull extract has a strong inhibition effect on the autoxidation of pyrogallol. In other words, it can scavenge the superoxide anion radicals generated by the pyrogallol autoxidation system effectively. The average of superoxide radical scavenging percentage in 4 wild almond hull phenolic extract 74.3 ± 1.37% was obtained. Maximum superoxide radical scavenging percentage was 90.6 ± 1.11% for *A. kotschyi* and minimum superoxide radical scavenging percentage was 60.7 ± 2.13% for *A. pabotti*. Superoxide radical scavenging percentage for almond hull extract reported by Siriwardhana and Shahidi (20) was 97 ± 1% at 100 ppm and 99 ± 1% at 200 ppm. The average of superoxide radical scavenging percentage in 4 wild almond shell extract was 41.7 ± 1.18%. The maximum superoxide radical scavenging percentage was 56.7 ± 1.33% for *A. lycioides*, and the minimum hydrogen peroxide radical scavenging percentage among almond shell extract was 28.5 ± 1.65% for *A. trichamygdalus*. This study shows that

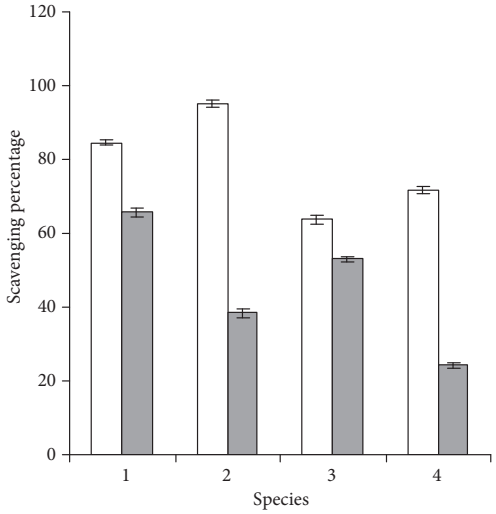


Figure 3. Scavenging percentage for radical peroxide in 4 wild almond hull and shell extracts: 1-*A. lycioides,* 2- *A. kotschyi,* 3-*A. pabotti,* 4-*A. trichamygdalus.* (mean ± S.E., n = 3), P < 0.05.

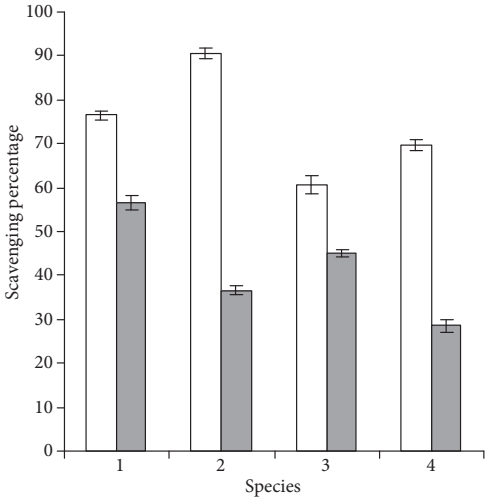


Figure 4. Scavenging percentage for radical superoxide in 4 wild almond hull and shell extracts: 1-*A. lycioides,* 2- *A. kotschyi,* 3-*A. pabotti,* 4-*A. trichamygdalus.* (mean ± S.E., n = 3), P < 0.05.

the superoxide radical scavenging percentage of almond hull phenolic extract was higher than that of its shell in different wild almond species.

Nitrite has long been applied in the processing of meat products as a preservation agent and color agent. The nitrate occurring in many vegetables can also be transformed to nitrite by reduction reactions with the action of bacteria in human bodies. These nitrites may be transformed into nitrosamines combining with secondary and tertiary amines in human bodies. These nitrosamines are procarcinogenic substance (25). Thus, if some substance can scavenge nitrosamine or its precursor substance such as nitrite, it probably has protective functions. In this investigation the average nitric oxide radical scavenging percentage in 4 wild almond hull phenolic extract was 68.9 ± 1.42%. The maximum nitric oxide radical scavenging percentage was 85.2 ± 1.21% for *A. kotschyi* and the minimum nitric oxide radical scavenging percentage was 53.4 ± 2.69% for *A. pabotti*. The average nitric oxide radical scavenging percentage in 4 wild almond shell extract was 39.2 ± 1.19%. The maximum nitric oxide radical scavenging percentage was 53.5 ± 0.86% for *A. lycioides*, and the minimum nitric oxide radical scavenging percentage among almond shell extracts was 24.9 ± 1.63% for *A. trichamygdalus*. This investigation shows that the nitric oxide radical scavenging percentage of almond hull phenolic extract was higher than that of its shell in different wild almond species. In addition, the nitric oxide radical scavenging activity of almond hull extract has for the first time shown that almond hull phenolic extract is a potent scavenger of nitric oxide. As shown in Figure 5, the scavenging capacities of wild almond hull and shell phenolic extracts towards nitrite varies relatively among species, and the scavenging activities are

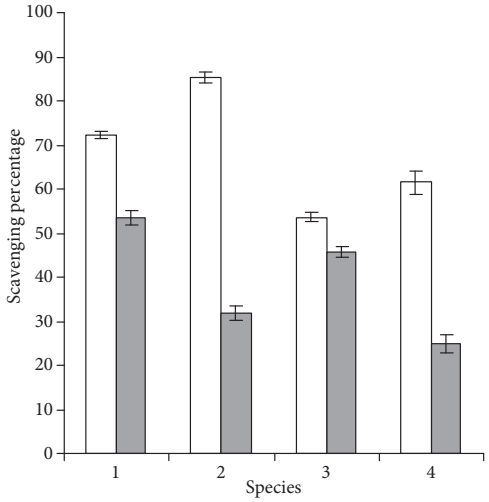


Figure 5. Scavenging percentage for radical nitrate in 4 wild almond hull and shell extracts: 1-*A. lycioides,* 2- *A. kotschyi,* 3-*A. pabotti,* 4-*A. trichamygdalus.* (mean ± S.E., n = 3), P < 0.05.

obviously stronger in species with high phenolic content. This indicates that wild almond hull extract and shell phenolic extract, in particular hull extract, are also excellent nitrite scavengers.

The results of correlation analyses between the total phenolic content, reducing power antioxidant, and antiradical activities are depicted in Figures 6 and 7. A statistically significant ($P < 0.05$) correlation was found with total phenolics versus antiradical activity and reducing power. Using a 4-point correlation between total phenolics and antioxidant activity, the data were significant at $P < 0.05$. In the case of leguminous seed extracts, a statistically significant ($P \leq 0.01$) correlation was determined for total phenolics versus total antioxidant activity (TAA) (26). The strong correlation between the content of total phenolics and reducing power has been found in the extracts of selected plant species from the Canadian prairies, as reported by

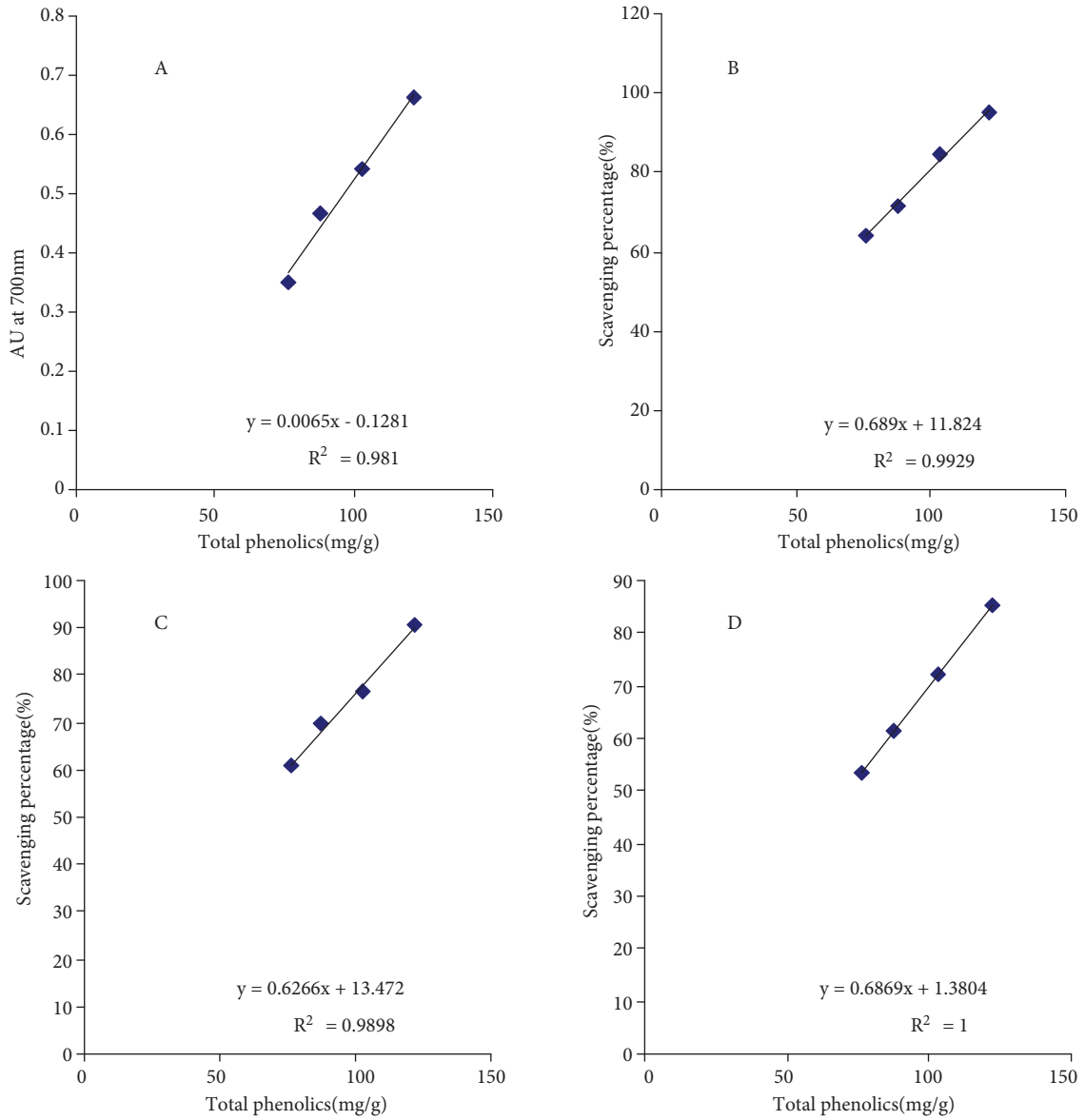


Figure 6. Correlations between the total phenolic content of almonds hull and observed antioxidant activity from reducing power (A), scavenging percentage for hydrogen peroxide (B), superoxide (C), and nitrite (D) radicals, $P < 0.05$.

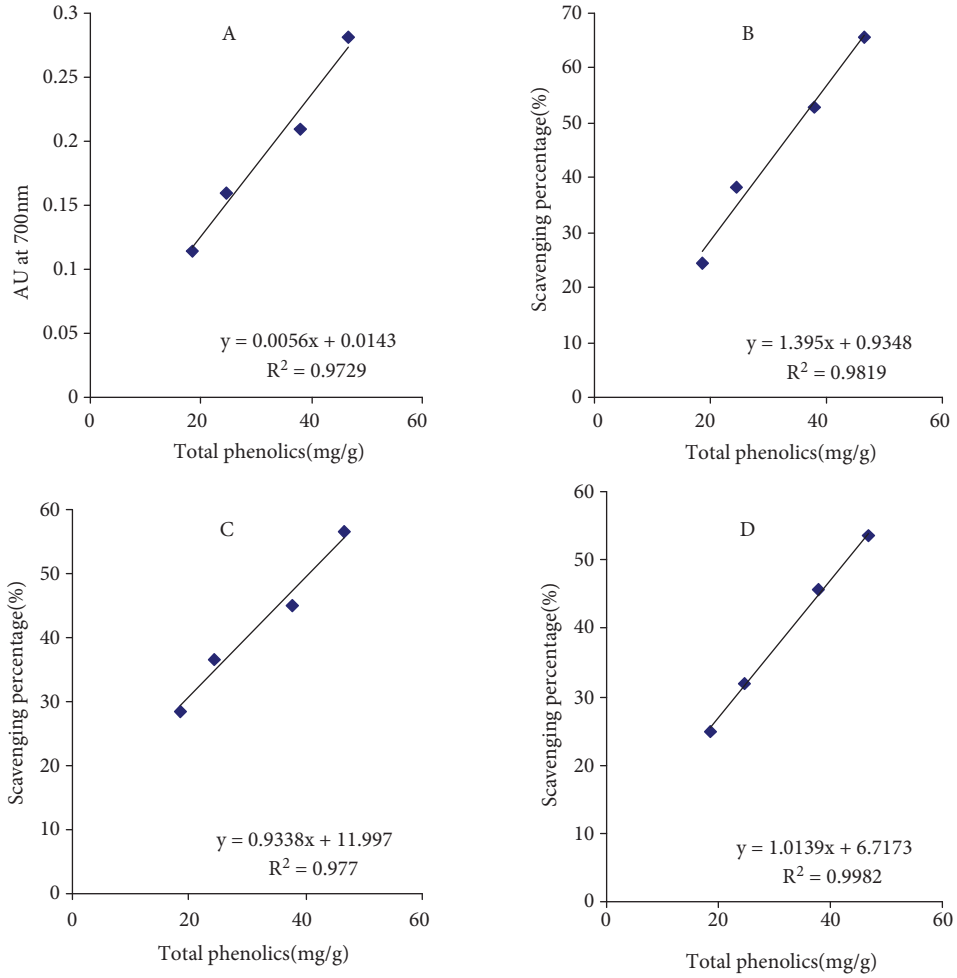


Figure 7 Correlations between the total phenolic content of almonds shell and observed antioxidant activity from reducing power (A), scavenging percentage for hydrogen peroxide (B), superoxide (C), and nitrite (D) radicals, $P < 0.05$.

Amarowicz et al. (27). Velioglu et al. (28) examined 28 plant products and found a significant relationship between the total antioxidant activity and total phenolics in flaxseed and cereal products.

Conclusion

In this study, it was demonstrated that methanolic extracts from wild almond hulls and shells possess antioxidant and antiradical activity, which could vary in different species, and this phenolic extract may be helpful in preventing or slowing the progress of various oxidative stress-related diseases. However, to use the extracts of these phenolic compounds as an antioxidant in foods, methanol should be substituted with some harmless solvent, although water is not as effective as organic solvents to extract useful compounds from plants by-products.

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