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Antioxidants and antiradicals in almond hull and shell (*Amygdalus communis* L.) as a function of genotype

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ABSTRACT

To compare the antioxidant and antiradical activity of *Amygdalus communis* L. hulls and shells phenolic extracts in different genotypes, 18 *A. communis* L. genotypes were selected from those in Qooshchi, Qalgachi, Qovarchin Qale, Najaf Abad, Jamal Abad, Kahriz, Sfahlan of West and East Azerbayjan provinces of Iran in 2007. The fruits of these almonds were collected, their hulls and shells dried, ground and then methanolic extracts 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. Significant differences were found in phenolic content of hulls and shells among various genotypes, radical scavenging capacity percentage varied significantly among genotypes and their hulls and shells. S_3-7 genotype with the highest phenolic content and antioxidant activity in its hulls represents a valuable genotype for procuring antioxidant phenolic compounds.

1. Introduction

Free radical reactions are involved in many biological processes that cause damage to lipids, proteins, membranes and nucleic acids, thus giving rise to a variety of diseases. Reactive oxygen species (ROS) have been recognised as playing an important role in the initiation and/or progression of various diseases such as atherosclerosis, inflammatory injury, cancer and cardiovascular disease. Thus, recent studies have investigated the potential of plant products to serve as antioxidants against various diseases induced by free radicals. Additionally, it has been determined that the antioxidant effect of plant products is mainly due to phenolic compounds, such as flavonoids, phenolic acids, tannins and phenolic diterpenes. There have been numerous studies on the biological activities of phenolics, which are potent antioxidants and free radical scavengers (Chung, Wong, Huang, & Lin, 1998; Datta, Sinha, & Chattopadhyay, 2000; Heinonen, 1999; Hou et al., 2003; Kahkonen et al., 1999; Yu-Tang, Jyh-Horng, Yueh-Hsiung, & Shang-Tzen, 2007).

Almond, scientifically known as *Prunus dulcis*, belongs to the family Rosaceae and is also related to stone fruits such as peaches, plums and cherries. It is the number one tree nut produced on a global basis, and the United States, specifically California, is the major producer (Sathe et al., 2002; Wijerante, Abou-Zaid, & Shahidi, 2006).

Almond, with or without the brown skin, is consumed as the whole nut or used in various confectioneries and chocolates; its discarded components are used as livestock feed (Takeoka et al., 2000). Extracts of whole almond seed, brown skin, and green shell cover possess potent free radical scavenging capacities (Siriwardhana & Shahidi, 2002). These activities may be related to the presence of flavonoids and other phenolic compounds in nuts. Almond hulls have been shown to serve as a rich source of triterpenoids, betulinic, urosolic and oleanolic acids (Takeoka et al., 2000), as well as flavonol glycosides and phenolic acids (Sang, Lapsley, Rosen, & Ho, 2002). The aim of this study was to determine and compare phenolic content in different genotypes of *A. communis* L. hulls and shells and evaluate their potential antioxidant and antiradical activity.

2. Methods

2.1. Extraction

Samples were supplied by the Agricultural and Natural Research Center of West and East Azerbaijan provinces from different location (Qooshchi, Qalgachi, Qovarchin Qale, Najaf Abad, Jamal Abad, Kahriz, Sfahlan). The green shells cover (Hulls) and inner shells of *A. communis* L. genotypes were separated, dried at room temperature and then reduced to coarse powder. This powder

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(3 g) was extracted with methanol (50 ml) in soxhlet apparatus for 30 min at 80 °C (Wijerante et al., 2006).

2.2. Determination of total phenolics

The total phenolics were assayed colorimetrically by means of the Folin–Ciocalteu method, as modified by Singleton and Rossi (1965). Ten-fold diluted Folin–Ciocalteu reagent (2.5 ml), 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 content of phenolics was expressed as mg gallic acid equivalents (GAE) in per gram extract.

2.3. Reducing power

The reducing power of almond hull and shell methanolic extract was determined according to the method of Oyaizu (1986). Almond hull and shell methanolic extract (1 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.

2.4. Hydrogen peroxide radical inhibition assay

A modified version of the method described by Ruch, Cheng, and Klauring (1989) was used to determine the hydrogen peroxide scavenging ability of almonds hulls and shells extracts. Extracts 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). Catechin was used as the reference compound. The concentration of hydrogen peroxide was measured by reading the 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 hydrogen peroxide-scavenging capacities of the extracts were calculated using the following equation:

$$\text{Scavenging percentage} = 100 - \left[\frac{\text{H}_2\text{O}_2\text{concentration of medium containing the additive}}{\text{H}_2\text{O}_2\text{concentration of the control medium}}\right] \times 100$$

2.5. Superoxide radical inhibition assay

For superoxide anion radical assay, the superoxide anion radicals were generated by a pyrogallol autoxidation system (Jing & Zhao, 1995). 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 microlitre syringe and mixed up. 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 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:

$$\text{Scavenging percentage} = A_0 - (A_1 - A_2) \cdot 100/A_0$$

2.6. Nitric oxide radical inhibition assay

Nitric oxide radical inhibition can be estimated by the use of Griess Illosvoy reaction (Garrat, 1964). 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 extract (0.5 ml) was incubated at 25 °C for 150 min. After incubation, 0.5 ml of the reaction mixture 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.

$$\text{Scavenging percentage} = A_{\text{Blank}} - A_{\text{Sample}} \times 100/A_{\text{Sample}}$$

2.7. Statistical analysis

All the assays were carried out in triplicate. The results are expressed as mean values and standard error (SE) of the mean or standard deviation (SD) of the mean. The differences between the almond genotypes were analyzed using one-way analysis of variance (ANOVA). This treatment was carried out using SPSS v. 11.5 program.

3. Results and discussion

3.1. Determination of total phenolics

Free radicals have attracted interest of scientists in the past decade. Their broad range of effects in biological systems has drawn the attention of many experimental works. It has been proved that these mechanisms may be important in the pathogenesis of certain diseases and ageing. There are many reports that support the use of antioxidant supplementation in reducing the level of oxidative stress and in slowing or preventing the development of complications associated with diseases (Rose, Creighton, Stewart, Sanwal, & Trevithick, 1982). Many synthetic antioxidant components have shown toxic and/or mutagenic effects, which have shifted the attention towards the naturally occurring antioxidants. Numerous plant constituents have proven to show free radical scavenging or antioxidants activity (Aruoma & Cuppett, 1997). Flavonoids and other phenolic compounds (hydroxyl cinnamic derivatives, catechines etc.) of plant origin have been reported as scavengers and inhibitors of lipid peroxidation (Formica & Regelson, 1995).

Almonds their hulls and shells possess powerful free radical scavenging capacities (Frison-Norrie & Sporns, 2002), and these activities could be due to the triterpenoids, flavonoids and phenolic acids that are present in almond by-products. The production of almond hulls which are mainly used in livestock feed, is estimated to exceed 6 million tons annually (Takeoka et al., 2000), thus being a potentially good source from which to extract antioxidants that are present, if any, in high quantities (Subhashinee, Amarovicz, & Shahidi, 2006; Wijerante et al., 2006).

Plant phenolics, in general, are highly effective free radical scavengers and antioxidants. The content of total phenolics in each

genotype of almond hull and shell was determined spectrometrically according to the Folin–Ciocalteu method and calculated as gallic acid equivalents (GAE). Accordingly, the total phenolic content of the hulls were significantly higher than that of the shells in each genotype as shown in Fig. 1 and Table 1. These results indicated that the methanolic extracts of hulls could be effectively in the free radical scavenging activity. Thus, the S_3-7 genotype and other genotypes with high phenolic content in hulls were further investigated for their phytochemical characteristics and in vitro antioxidant activity.

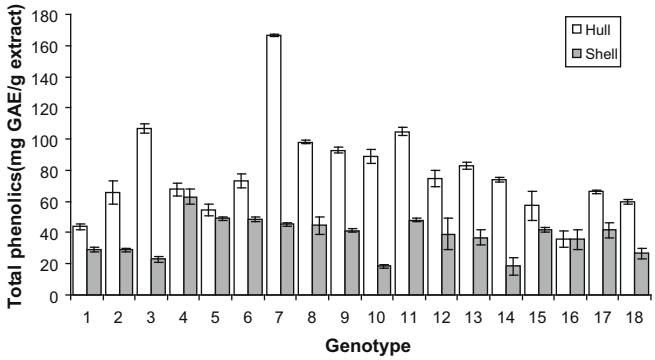

Fig. 1. Phenolic content of 18 genotypes of *A. communis* L. hulls and shells: 1: S_1-1, 2:S_1-2, 3:S_1-3,4: S_1-4, 5:S_2-5, 6:S_2-6, 7:S_3-7, 8:S_3-8, 9:S_4-1, 10:S_4-2, 11:S_4-3, 12:S_4-4, 13:S_4-5, 14:S_4-6, 15:J_1-1, 16:J_1-2, 17:N_1-1, 18:K_1-1. Means of three replicates with S.E, $p < 0.05$.

3.2. Reducing power

For the measurements of the reductive ability, we investigated the Fe^{3+} to Fe^{2+} transformation in the presence of methanolic extract using the method of Oyaizu (1986). The reducing power increased with increasing the phenolic content of extract and reducing power was positively correlated with phenolic content. The reducing capacity of compound may serve as a significant indicator of its potential antioxidant activity (Meir, Kanner, Akiri, & Hadas, 1995). The absorbance values of the shells extract at different genotypes were found to be less than that of hulls phenolic extract (Fig. 2 and Table 1). This result indicates that polyphenol present in almond hull and shell could be partly responsible for the beneficial effects. Compelling evidence indicates that increased consumption of dietary antioxidants or fruits and vegetables with antioxidant properties may contribute to the improvement in quality of life by delaying onset and reducing the risk of degenerative diseases associated with ageing.

Table 1
The comparison of phenol content (mg gallic acid equivalents (GAE) in per gram extract), reducing power and scavenging percentage for radical's hydrogen peroxide, superoxide and nitrite in each genotype of *A. communis* L. hull and shell.

Genotype	Phenol content (mg GAE/g extract)	Reducing power (700 nm)	Peroxide scavenging (%)	Superoxide scavenging (%)	Nitrite scavenging (%)
Hull					
1	43.9 ± 1.79	0.251	29.7 ± 0.14	56.5 ± 0.63	40.2 ± 0.07
2	65.8 ± 7.52	0.508	57.4 ± 0.34	63.4 ± 1.57	57.0 ± 2.11
3	106.7 ± 2.74	0.637	98.7 ± 0.68	93.4 ± 0.67	84.8 ± 0.43
4	67.7 ± 4.33	0.530	57.7 ± 0.18	66.2 ± 1.42	62.9 ± 1.22
5	48.9 ± 3.48	0.337	38.2 ± 0.68	60.3 ± 0.55	52.2 ± 0.54
6	73.2 ± 4.35	0.510	77.3 ± 1.22	70.0 ± 0.75	65.3 ± 0.61
7	166.7 ± 1.05	0.831	93.9 ± 5.93	97.3 ± 0.55	87.0 ± 0.37
8	98.2 ± 1.24	0.752	76.7 ± 3.41	86.5 ± 0.43	84.5 ± 0.27
9	92.8 ± 1.91	0.636	67.6 ± 2.72	84.6 ± 1.76	81.4 ± 0.85
10	89.1 ± 4.61	0.504	73.9 ± 9.12	79.8 ± 1.11	80.9 ± 0.73
11	104.8 ± 2.53	0.661	87.2 ± 8.87	89.4 ± 1.32	84.4 ± 0.51
12	74.1 ± 5.25	0.556	65.4 ± 0.53	69.6 ± 1.33	65.2 ± 0.65
13	82.9 ± 2.51	0.547	63.7 ± 1.11	73.4 ± 1.21	79.3 ± 0.64
14	74.1 ± 1.47	0.545	62.2 ± 4.81	68.1 ± 1.34	71.5 ± 1.09
15	57.4 ± 9.31	0.416	40.0 ± 9.14	58.0 ± 1.25	56.5 ± 1.48
16	35.9 ± 4.93	0.163	35.0 ± 2.32	52.2 ± 1.51	31.0 ± 0.72
17	66.2 ± 1.11	0.490	55.9 ± 1.22	63.4 ± 1.44	70.4 ± 0.85
18	59.6 ± 1.33	0.470	52.1 ± 1.21	60.0 ± 1.28	60.5 ± 1.13
Mean	78.2 ± 3.41	0.519	62.9 ± 3.08	71.8 ± 1.11	67.5 ± 0.79
Shell					
1	28.8 ± 1.40	0.151	32.8 ± 0.34	50.8 ± 1.44	35.2 ± 1.82
2	28.8 ± 1.19	0.160	31.9 ± 1.62	50.7 ± 1.11	28.8 ± 1.51
3	22.9 ± 1.91	0.148	30.0 ± 1.24	47.0 ± 1.23	31.7 ± 1.50
4	62.7 ± 4.91	0.400	63.3 ± 1.71	74.8 ± 1.11	59.2 ± 1.91
5	54.5 ± 1.43	0.350	63.5 ± 2.11	74.5 ± 0.92	59.9 ± 1.93
6	48.4 ± 1.51	0.277	58.6 ± 0.16	63.2 ± 3.55	54.2 ± 1.29
7	45.5 ± 1.13	0.265	52.7 ± 1.22	59.9 ± 1.12	44.5 ± 1.52
8	44.5 ± 5.72	0.271	50.8 ± 1.22	59.4 ± 1.21	44.7 ± 1.19
9	41.4 ± 1.05	0.277	45.9 ± 0.96	58.4 ± 1.81	37.5 ± 1.77
10	18.4 ± 1.30	0.140	29.3 ± 1.41	32.2 ± 0.92	27.5 ± 1.11
11	48.1 ± 1.42	0.266	55.8 ± 1.23	65.1 ± 1.63	54.1 ± 0.98
12	39.0 ± 9.91	0.248	39.3 ± 1.31	58.1 ± 1.82	33.4 ± 1.38
13	36.8 ± 4.72	0.228	38.8 ± 1.24	58.4 ± 1.21	33.4 ± 1.62
14	18.5 ± 5.71	0.137	30.3 ± 1.19	34.1 ± 2.64	28.9 ± 1.81
15	41.8 ± 1.69	0.280	50.3 ± 1.21	58.1 ± 1.91	40.3 ± 1.50
16	35.6 ± 6.38	0.223	46.1 ± 0.82	59.5 ± 0.92	31.6 ± 1.62
17	41.7 ± 4.80	0.144	55.3 ± 1.11	58.0 ± 1.33	45.1 ± 1.43
18	26.5 ± 3.39	0.143	33.3 ± 1.01	34.9 ± 1.62	38.4 ± 1.33
Mean	38.0 ± 3.30	0.228	44.6 ± 1.17	55.4 ± 1.52	40.5 ± 1.51

The values are means of three replicates with standard errors (Mean ± S.E, $n = 3$), $p < 0.05$.

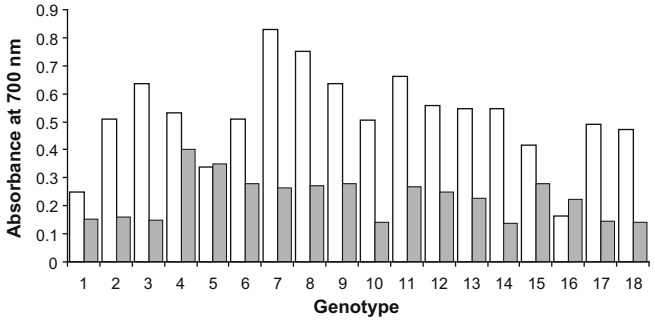

Fig. 2. Reducing power in 18 genotypes of *A. communis* L. hulls and shells: 1: S_1-1, 2:S_1-2, 3:S_1-3, 4: S_1-4, 5:S_2-5, 6:S_2-6, 7:S_3-7, 8:S_3-8, 9:S_4-1, 10:S_4-2, 11:S_4-3, 12:S_4-4, 13:S_4-5, 14:S_4-6, 15:J_1-1, 16:J_1-2, 17:N_1-1, 18:K_1-1. (Mean ± S.D, $n = 3$), $p < 0.05$.

3.3. Hydrogen peroxide radical scavenging percentage

Hydrogen peroxide exhibits weak activity in initiating lipid peroxidation; however, its potential to produce highly ROS, such as hydroxyl radical through Fenton reaction, is very high. Hydrogen peroxide is poorly reactive in aqueous solutions at physiological concentrations, is toxic to cells at 10–100 μM levels, and can cross biological membranes rapidly to form cytotoxic hydroxyl radicals (Siriwardhana & Shahidi, 2002). The hydrogen peroxide-scavenging activity of almond hulls and shells methanolic extracts were phenol content dependent and in genotypes with high phenolic content, spatially hulls extracts most of the hydrogen peroxide was scavenged, as shown in Fig. 3 and Table 1. The rates of hydrogen peroxide scavenging of hulls and shells vary among genotypes. Hydrogen peroxide-scavenging activity of the hull was higher than of the shell in each genotype. Thus, hydrogen peroxide-scavenging activity of almond hulls and shells extracts, spatially hulls extracts would contribute to their inhibition of lipid peroxidation and thereby protect cells from oxidative damage.

3.4. Superoxide radical scavenging percentage

The superoxide radical is a powerful oxidising agent that can react with biological membranes and induce tissue damage. It also decomposes to singlet oxygen, hydroxyl radical or hydrogen peroxide (Siriwardhana & Shahidi, 2002). Superoxide anion is an initial free radical and plays an important role in the formation of other reactive oxygen species such as hydrogen peroxide, hydroxyl radical, or singlet oxygen in living systems (Stief, 2003). It can also react with nitric oxide and form peroxynitrite, which can generate

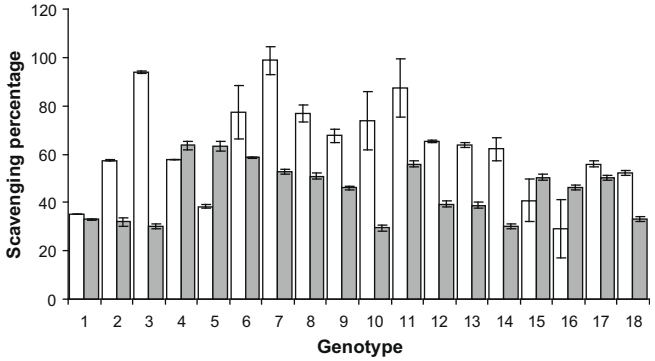

Fig. 3. Scavenging percentage for radical peroxide in 18 genotypes of *A. communis* L. hulls and shells: 1: S_1-1, 2:S_1-2, 3:S_1-3, 4: S_1-4, 5:S_2-5, 6:S_2-6, 7:S_3-7, 8:S_3-8, 9:S_4-1, 10:S_4-2, 11:S_4-3, 12:S_4-4, 13:S_4-5, 14:S_4-6, 15:J_1-1, 16:J_1-2, 17:N_1-1, 18:K_1-1. (Mean ± S.E, $n = 3$), $p < 0.05$.

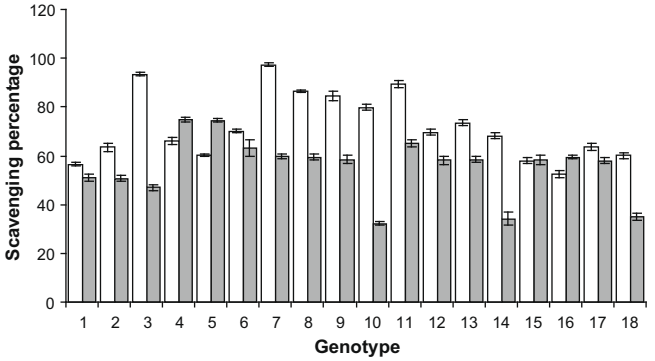

Fig. 4. Scavenging percentage for radical superoxide in 18 genotypes of *A. communis* L. hulls and shells: 1: S_1-1, 2:S_1-2, 3:S_1-3, 4: S_1-4, 5:S_2-5, 6:S_2-6, 7:S_3-7, 8:S_3-8, 9:S_4-1, 10:S_4-2, 11:S_4-3, 12:S_4-4, 13:S_4-5, 14:S_4-6, 15:J_1-1, 16:J_1-2, 17:N_1-1, 18:K_1-1. (Mean ± S.E, $n = 3$), $p < 0.05$.

toxic compounds such as hydroxyl radical and nitric dioxide (Halliwell, 1997). We evaluated the scavenging capacity of almond hulls and shells extract towards superoxide anion radicals by using a pyrogallol autoxidation system. Pyrogallol can autoxidate fast in alkali conditions and release superoxide anions, and, in return, 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 Fig. 4, the inhibition effects of almond hulls and shells extract on the autoxidation of pyrogallol were relatively feeble at genotypes with low phenolic content, but the almond hulls and shells extract, spatially hulls extract, with high phenolic content exhibited strong inhibition activities. The maximum inhibition percentage was 97.3 ± 0.55% for S_3-7 (Table 1). This indicates that this almond 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.

3.5. Nitrite radical scavenging percentage

Nitric oxide (NO) is a highly reactive molecule that participates in signal transduction in the cardiovascular and immune systems. It is often characterised by contrasting actions as it can exhibit antioxidant and pro-oxidant functions as well as anti-apoptotic and proapoptotic effects. Also, the role of NO in carcinogenesis has not been entirely clarified and observations have been reported demonstrating its ability to both stimulate and inhibit tumour growth (Sokolowska, Rokita, & Wlodek, 2003). Nitric oxide radical inhibition study proved that almond outer green shell extract is a

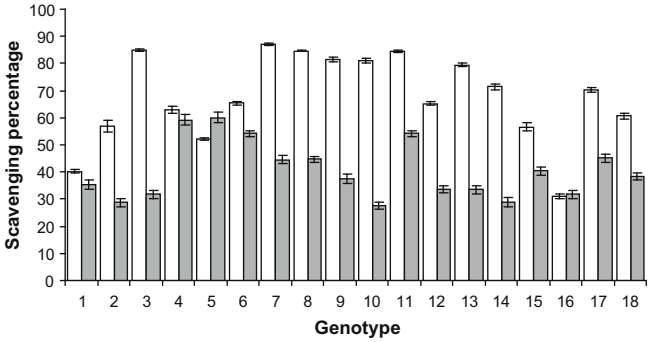

Fig. 5. Scavenging percentage for radical nitrite in 18 genotypes of *A. communis* L. hulls and shells: 1: S_1-1, 2:S_1-2, 3:S_1-3, 4: S_1-4, 5:S_2-5, 6:S_2-6, 7:S_3-7, 8:S_3-8, 9:S_4-1, 10:S_4-2, 11:S_4-3, 12:S_4-4, 13:S_4-5, 14:S_4-6, 15:J_1-1, 16:J_1-2, 17:N_1-1, 18:K_1-1. (Mean ± S.E, $n = 3$), $p < 0.05$.

potent scavenger of nitric oxide too (Fig. 5). The maximum nitrite radical scavenging percentage was 87.0 ± 0.37% for S_3-7 genotype (Table 1). This nitric oxide generated from sodium nitroprusside reacts with oxygen to form nitrite. The extract inhibits nitrite formation by competing with oxygen to react with nitric oxide directly and also to inhibit its synthesis. Scavengers of nitric oxide compete with oxygen leading to reduced production of nitric oxide (Marcocci, Packer, Droy-Lefai, Sekaki, & Gardes-Albert, 1994). The scavenging of nitric oxide by almond hull and shell extract was in a phenolic content-dependent manner.

4. Conclusions

Almond (*Amygdalus communis* L.) fruit hull and shell phenolic extract possess antioxidant activity, which might be vary in different genotypes and this phenolic extract helpful in preventing or slowing the progress of various oxidative stress-related diseases. Methanolic extract from almonds hull and shell showed a strong antioxidant activity. However, to use the extracts of these phenolic compounds as 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. Further investigation on the isolation and identification of antioxidant component(s) in the different almonds genotypes may lead to chemical entities with potential for clinical use.

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