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Assessment of Chemical Composition and in vitro Antioxidant Activity on Different Varieties of Almond (Prunus amygdaloides schltr.)

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ABSTRACT

Six different varieties of Almond (Prunus amygdaloides) such as Patasa, Surkh talwar, Safaid talwar, Aroo, Kaati and Pewand of Loralai Balochistan were analyzed for determination of heavy metals (Zn, Fe, Cr, Co, Pb, Cd and Cu), by atomic absorption spectrometry and total phenolic content (TPC), total flavonoid content (TFC), antioxidant activity by DPPH (2,2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing ability of plasma) assay. By following two step extraction method free and bound fractions were obtained from almonds (with and without brown skin individually) with two solvents i.e. acetone and water. A minute difference was found between free and bound phenolics whereas contents of bound flavonoids were much more high than free flavonoids. Comparison between acetone and water extracts of with and without skin samples showed that acetone extracts of almond samples (with skin) represents comparatively high TPC, TFC and antioxidant activity. 'Patasa' was ranked first for displaying high antioxidant capacity as well as high concentration of TPC (920.95 ± 9.3 mgGAE/100g DW) and TFC (117.99 ± 1.9 mgQE/100g DW) among all the varieties. Strong positive correlations between phytochemicals and antioxidant activity proved that phenolics and flavonoids either in free or in bound form are good scavengers of free radicals and help in the inhibition of diseases that are caused by oxidative stress. The results for heavy metals indicated that the amount of Cu was high in all varieties while the Surkh talwar accumulated higher concentration of Zn, Cd and Cu.

Introduction

Phytochemicals

In this modern era synthetic drugs are being made which are easily available but people still rely on using medicinal herbs and plants due to its less or no harmful effects on human health (Iqbal et al., 2015). Studies have shown that these medicinal plants have anticancer, antitumor, antibacterial, antiviral and so many other activities due to the presence of great variety of compounds, especially those of secondary metabolites (Iqbal et al., 2015). The largest category of phytochemicals is polyphenols. It is the collective term used for many sub-groups of phenolic compounds (Tsao, 2010). Different epidemiological studies strongly suggests that consumption of foods rich in plant polyphenols can reduce the risk of development of many diseases such as cardiovascular, cancer, osteoporosis and neurodegenerative diseases. The main classes of polyphenols are phenolic acids, flavonoids, lignans and stilbenes (Pandey and Rizvi, 2009). Phenolic acids are the naturally occurring secondary metabolites extensively spread throughout the plant kingdom (Saxena et al., 2013). Phenolic compounds were long considered to have a negative effect on health but now from various studies it is cleared that presence of phenolic compounds in food is important for protection from oxidative stress and microbes (Marinova et al., 2005). Phenolics exist in two different forms i.e. free phenolics and bound phenolics. The free phenolics can be easily extracted by any solvent whereas bound phenolics are bonded covalently with plant matrix to the polysaccharides, pectin and cellulose through ester bond and cannot be extracted easily with water or any other solvent, therefore other methods such as acidic, alkaline hydrolysis methods are used to extract bound phenolic compounds (Su et al., 2014). The activities of phenolic compounds that are in bound form in different fruits, could be underestimated to a great extent if they are not considered in some way (Su et al., 2014). The most abundant type of polyphenols in human diet are flavonoids. More than 4000 types of flavonoids have been identified so far (Bahadoran et al., 2013). The best known property of flavonoids is their potential to act as a powerful antioxidant by protecting the human body from free radicals (Saxena et al., 2013). Most of the recent researches have focused on the health benefits of flavonoids for humans and are found to have coronary heart disease prevention, anticancer activities, anti-inflammatory, hepatoprotective, while some exhibit potential antiviral activities (Kumar and Panday, 2013).

Antioxidant Activity

Oxygen is an important element needed for life. Cells take oxygen to generate energy by the breakdown of sugar molecules (Pham-Huy et al., 2008). The free radicals can be defined as reactive species, having an unpaired electron in its outermost shell, being capable to react with other compounds (Rahman, 2007). They are formed generally in the form of RNS i.e. reactive nitrogen species and ROS i.e. reactive oxygen species being highly reactive resulting from the cellular redox reaction which plays a dual role as both beneficial and harmful compounds. The low levels of ROS and RNS have beneficial effects on immune function and cellular responses while at high concentration they create oxidative stress which plays a major role in the development of chronic diseases like cancer, aging, cardiovascular and neurodegenerative diseases, autoimmune disorders and arthritis (Pham-Huy et al., 2008). The body has several functions which overcome the oxidative stress by the production of antioxidants. An antioxidant is a stable molecule that inhibits cellular damage through their free radical scavenging property (Lobo et al., 2010). The major enzymatic antioxidants (endogenous) that are involved in the neutralization of ROS and RNS are glutathione peroxidase, superoxide dismutase and catalase (Rahman, 2007). The exogenous that are the nonenzymatic antioxidants are vitamin E and C, natural flavonoids,

melatonin, carotenoids, thiol antioxidants (glutathione, lipoic acid, thioredoxin) and other compounds (McCall and Frei, 1999).

Trace metals

Heavy metals are found as a result of both human activities or natural everywhere in the environment (Wilson and Pyatt, 2007). Soil contamination results in the increased level of metal uptake by food crops. The excessive level of metal ions affects the plants such as growth inhibition, chlorosis, root damage and larger stomata (Posada et al., 2012). The use of medicinal plants has been on its rise throughout the world because of its therapeutic effectiveness and low cost (Parveen et al., 2017). The role of medicinal plants in treating different diseases ranging from common cold to cancer is particularly dependent on concentration of trace metals which are responsible for their healing as well as toxic properties (Tokalioglu, 2012). Much attention has been paid on organic constituents of medicinal plants and little work has been done on the trace metals contents (Ansari et al., 2004).

Almonds

Prunus amygdaloides Schltr. also named almond in English and badam in Urdu, belongs to the family Rosaceae. It is one of the most well-known dry fruit. The fruit is about 3.5-6cm long, fleshy (drupe) containing one or occasionally two nuts inside (Johnson, 2007). California having an ideal climate for almond growth with cool, rainy winters and hot, dry summers, is the world's largest producer of almonds exporting to over 80 countries (Johnson, 2007). The climate of Pakistan is such that nearly all types of fruits can be cultivated such as temperate, tropical and subtropical (Khan and Shaukat, 2006). Pakistan is also one of the exporter of almonds. The mountainous areas in Khyber Pakhtunkhwa and Balochistan are ideal for all types of temperate fruits (Laghari, 1998). Almonds grow with an elevation of about 2150m respectively (Khan and Shaukat, 2006).

Material and method

Sampling and Identification

The six cultivated varieties of almond having their local names, Patasa, Aroo, Kaati, Pewand, Safaid talwar and Surkh talwar were brought from Loralai. The botanical name was confirmed by the help of flora of Pakistan (http://www.tropicos.org/) and the Plant list (http://www.theplantlist.org/).

Peeling off and Grinding

Almonds were used with and without skin for phytochemical screening. The almonds used without skin were peeled off by knife. Both peeled and unpeeled almonds of each variety were ground to fine powder in mortar and pestle.

Extraction of Free and Bound Phenolic Compounds

Following the two-step extraction method by Ji et al., 2011 for free phenolics, approximately 5 gram of powdered sample was extracted with 25ml of water and acetone individually in triplicates. Keeping on a shaker overnight, the samples were centrifuged for 15 minutes at 8000 rpm and supernatant was collected in a 100 ml bottle. Same procedure was repeated three times and supernatants were collected.

The bound phenolic compounds were extracted following the method of Martinez et al., 2009 with some modifications. Briefly, the residues left from the above method were digested with 4M

NaOH solution for 1 hour on shaker and then neutralized with 4ml HCl (conc.). Hexane (5ml) was mixed to remove lipids and left for 2 hours and then discarded. The final solutions were extracted three times with ethyl acetate and supernatants were collected.

Determination of Total Phenolic Content

The presence of total phenolic content in the plant samples was determined by using spectrophotometric method (Saeed et al., 2012). 1ml of seed extract mixed with 1ml of 10% Folin ciocalteu's phenol reagent (10:90). After incubation for 5 minutes at room temperature, 2ml of Na2CO3 (6%) is added and thoroughly mixed. The solutions are then mixed and incubated for 90 minutes in the dark. Then the absorbance of the solutions read at 760nm on a UV-VIS spectrophotometer. The concentration of the samples can be determined by using seven different concentrations (5, 10, 20, 40, 60, 80 and 100 µg/ml) of gallic acid and expressed as µg of gallic acid equivalent (GAE) of sample.

Determination of Total Flavonoid Content

The total flavonoids were determined by using the method of Lin et al. (2011) with modifications. 1ml of seed extract was mixed with 0.5ml solution of NaNO2 (5%) followed by mixing 0.5ml of AlCl3.6H2O (10%). After incubation for 5 minutes at room temperature, 2ml solution of NaOH (1M) was added followed by mixing. Absorbance was read at 510nm on spectrophotometer. Using a quercetin standard solution and making concentrations (5, 10, 20, 40, 60, 80 and 100 µg/ml), a calibration curve was made to determine total flavonoid content. Results were expressed as µg of quercetin equivalents (QE) g-1 of samples. Experiments were performed in triplicates.

Antioxidant activity by DPPH assay

Assay will be used following the method of Zhu et al., 2006. Briefly the DPPH (2,2-diphenyl-1-picrylhydrazyl) solution (1ml) will be blended with 2ml of samples. After shaking, the reaction mixtures were incubated for 30 minutes at room temperature and the absorbance of decolorized samples read at 517nm against the blank. Scavenging percentage of antioxidants was calculated by:

Antioxidant activity by FRAP assay

FRAP (ferric reducing ability of plasma) assay applied following the method of Hazra et al. (2008) with modifications. 1ml extract was blended with 1ml of phosphate buffer (0.2M, pH 6.6) followed by the mixing of 1ml potassium ferricyanide (1%). The mixtures were incubated for 20 minutes in a water bath at 35oC and then mixed with 1ml trichloroacetic acid (10%). Pipette out 1ml of upper layer of mixture and mixed with 1ml of distilled water as well as with 1ml of ferric chloride (0.01%) and incubated for 20 minutes at room temperature. Absorbance was read at 700nm against the blank and results were expressed as concentration of antioxidants in 100 grams of samples dry weight. (mg/100g GAE, DW).

Determination of metal contents

The solutions were prepared in such a way that 1g of powdered sample was digested using 20ml of HNO3 and HClO4 mixture (5:1). This mixture was then heated on a hot plate till a clear transparent color obtained (Amde et al., 2013) The measurement of selected metals (Zn, Fe, Cr, Co, Pb, Cd and Cu) was performed on flame atomic absorption spectrophotometer (Model: AA-670 Shimadzu, japan) against the blank which was also digested in the same way with each batch

of samples. Stock solutions (1000 mg/L) of metals were used as standards for making calibration curve (Abbasi et al., 2015)

Statistical Analysis

The data were processed for statistical analysis to find out the individual variations and their mutual relationships. Statistical analyses were performed using SPSS software 13.0 (SPSS Inc., Chicago, IL, USA).

Results and Discussion

Metal contents

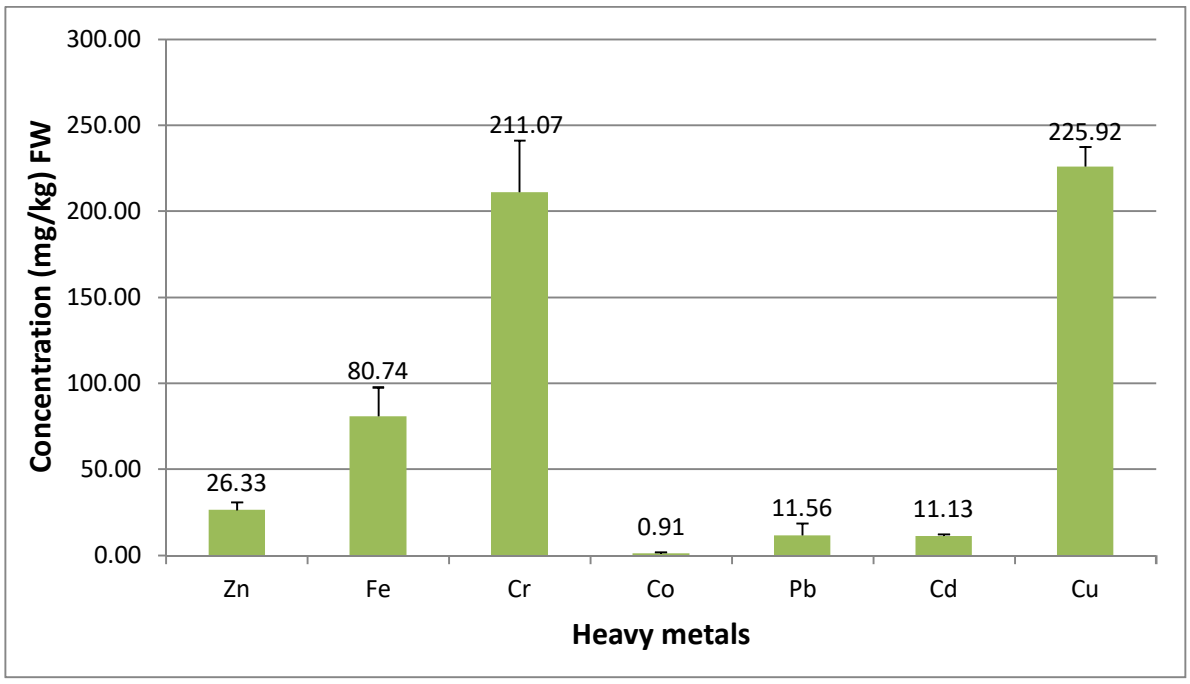

Figure 1. Average concentration of selected metals in almond varieties

The highest concentration was recorded for copper (225.92 ± 10.5 mg/kg FW) in all varieties, followed by chromium (211.07 ± 27.4 mg/kg) and considerable amount of iron, zinc, lead and cadmium was present (Fig. 1). Copper is regarded as an anti-infectious, anti-inflammatory and antiviral (Cosmulescu et al., 2009) and is the most abundantly found heavy metal in almond varieties and ranges from 238.08 ± 3.4 to 212.58 ± 3.8 mg/kg FW (Table 1). The concentration of chromium ranged between 243.32 ± 4.1 to 171.62 ± 1.5 mg/kg (FW) and was reported to be the second highest metal present. Higher concentration of iron was present in Kaati (104.69 ± 3.2 mg/kg FW) whereas the lowest concentration in Surkh talwar (58.72 ± 1.3 mg/kg). Iron is an important nutritional component for body especially in those parts of the world where anaemia is common (Abbasi et al., 2015). On the average basis zinc ranged from 33.98 ± 2.0 to 20.78 ± 0.8 mg/kg FW. It aids in the formation of proteins, DNA and RNA, healing the wounds and cell division (Cosmulescu et al., 2009). A narrow range of cobalt (from 2.03 ± 0.6 to 0.10 ± 0.1 mg/kg FW) was recorded. Cobalt is essential in minute amount to the body but higher intake can cause serious problems as it is a major cause of contact dermatitis (Basketter et al., 2003). Lead ranged from 23.25 ± 1.3 to 5.18 ± 2.8 mg/kg FW showing a benefit for health but it is toxic to brain and kidneys, whereas cadmium was present in low level in between 12.52 ± 0.3 to 9.52 ± 1.9 mg/kg FW in all varieties (Table 1).

Table 1. Heavy metals concentration (mg/kg FW) in almond varieties

Metals	P	Srt	Sft	Ar	K	Pew	Average
Zn	27.43 ± 2.6	33.98 ± 2.0	25.98 ± 1.0	22.67 ± 0.9	27.15 ± 0.5	20.78 ± 0.8	26.33
Fe	65.53 ± 0.3	58.72 ± 1.3	83.30 ± 1.3	92.15 ± 0.8	104.69 ± 3.2	80.02 ± 1.6	80.74
Cr	179.0 ± 10.4	171.62 ± 1.5	230.75 ± 2.6	243.32 ± 4.1	233.17 ± 7.7	208.55 ± 0.9	211.07
Co	1.60 ± 0.5	0.63 ± 0.1	0.43 ± 0.3	2.03 ± 0.6	0.68 ± 0.1	0.10 ± 0.1	0.91
Pb	23.25 ± 1.3	8.55 ± 1.9	8.65 ± 3.1	16.45 ± 1.1	7.25 ± 3.2	5.18 ± 2.8	11.56
Cd	10.93 ± 1.8	12.52 ± 0.3	12.33 ± 0.3	9.52 ± 1.9	10.07 ± 1.3	11.40 ± 0.3	11.13
Cu	212.58 ± 3.8	238.08 ± 3.4	234.13 ± 1.6	235.02 ± 2.3	222.95 ± 2.2	212.78 ± 4.0	225.92

Quantification of free phenolic content in water and acetone extracts

Almonds are a great source of phenolics, flavonoids, tannins and antioxidants for scavenging of free radicals (Milbury et al., 2006). Overall comparison between acetone and water extracts (Fig. 2) showed that acetone extracts depicted more phenolic content than the water extracts. Comparatively highest amount (920.95 ± 9.3mgGAE/100g (DW)) of free phenolics were found in the acetone extract of Patasa (WS) followed by water extract of kaati 897.74 ± 22.2 mgGAE/100g (DW) (WoS), acetone extracts of Safaid talwar and Aroo without skin respectively. Lower amount (249.83 ± 2.6 mgGAE/100g (DW)) of free phenolics were found in water extract of Aroo (WS) followed by water extract of Safaid talwar (WoS), water extract of Safaid talwar (WS) and acetone extract of Surkh talwar (WoS). Phenolic acids were proved to be the most bioaccessible class in almond as well as skin which can be included in diet through home made biscuits and crisp breads (Mandalari et al., 2016).

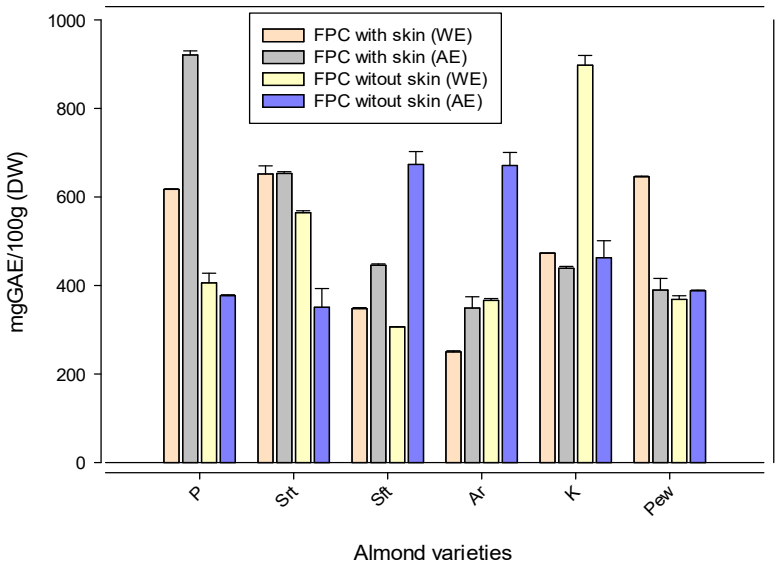

Figure 2. Comparison of free phenolic content in water and acetone extract

Quantification of Bound phenolic content (BPC) in water and acetone extracts

In case of bound phenolics, water extracts depicted high level of phenolics as compared to acetone extracts. Highest concentration (1127.72 ± 7.8 mgGAE/100g DW)) of bound phenolics were present in water extract of Patasa (WS) followed by water extracts of Surkh talwar, Pewand, Kaati, Aroo of with skin samples (929.51 ± 16.7, 710.85 ± 6.1, 663.64 ± 1.3, 640.43 ± 10.1 mgGAE/100g

(DW) respectively). Lowest bound phenolics were found in without skin samples of Pewand and Kaati (238.87 ± 18.6 and 284.00 ± 3.0 mgGAE/100g DW respectively) (Fig. 3).

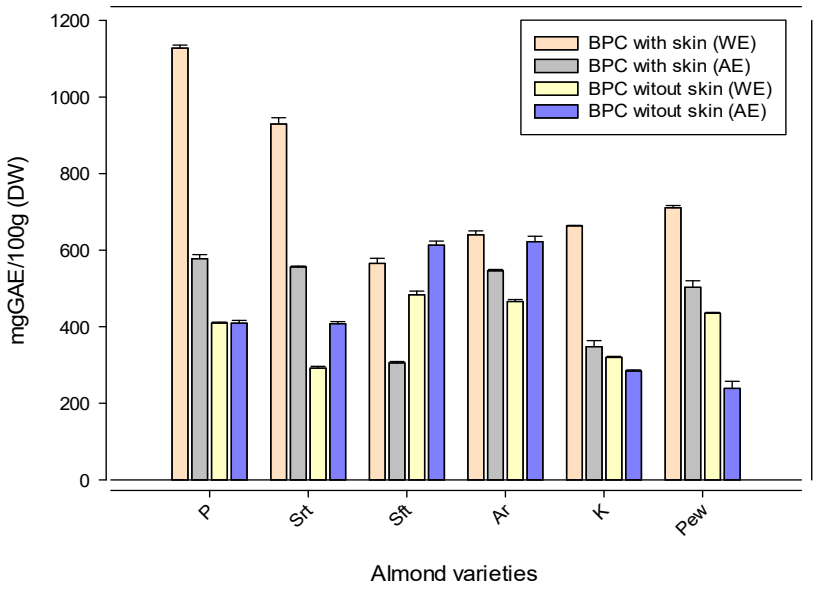

Figure 3. Comparison of bound phenolics content in water and acetone extract

Quantification of Free flavonoid content (FFC) in water and acetone extracts

It is important to add flavonoid rich food in our diet. Fruits and vegetables are the main sources of flavonoids for humans. Flavonoids were reported to be associated with the antioxidant activity, help in prevention from coronary heart disease and have anticancer activity (Yao et al., 2004).

Comparing the free flavonoids extracted by water and acetone, it is clear that the free flavonoids were extracted more by water. Highest concentration (117.99 ± 1.9 mgQE/100g DW) was found in acetone extract of Patasa (WS), water extract of Pewand (WS) (114.96 ± 15.9 mgQE/100g DW), water extract of Pewand (WoS) and Patasa (WoS) with a slight difference between them i.e. 108.82 ± 7.7 and 107.84 ± 7.6 mgQE/100g (DW) respectively . Lowest concentration (9.91 ± 0.5 mgQE/100g DW) of free flavonoids were found in acetone extract of Pewand (WoS) followed by acetone extracts of Safaid talwar (WS),and Kaati (WS) whereas all other samples were found with average amount of free flavonoids (Fig. 4).

Figure 4. Comparison of free flavonoids content in water and acetone extract

Quantification of Bound flavonoid content (BFC) in water and acetone extracts

Comparing the samples (water extracted) on the basis of with and without skin, the samples having skin showed a remarkable amount of bound flavonoids in all the six varieties with the highest values of 561.36 ± 9.9, 555.56 ± 14.5 mgQE/100g DW in Aroo and Pewand respectively. The minimum concentration (62.90 ± 4.7) of bound flavonoids were found in Kaati. Variations were found for bound flavonoids in acetone extracts among varieties. High concentration of flavonoids were present in Aroo and Pewand (WS) having the value 400.60 ± 9.4 and 363.61 ± 38.7 mgQE/100g DW, respectively. The lowest concentration (56.67 ± 9.8 and 75.13 ± 5.3) was found in without skin samples of Pewand and Kaati respectively. The water and acetone extracts of all

six varieties (with and without skin) are shown in Figure 5. Clearly it can be seen that the samples (WS) whether water or acetone extracted were found having a large amount of bound flavonoids. Highest concentration was found in water extracts of Aroo and Pewand (with skin) having minor difference. Lowest flavonoid content was found to be in acetone extract of Pewand (WoS) followed by samples in water extracts of Kaati and Surkh talwar (WoS) (Fig. 5).

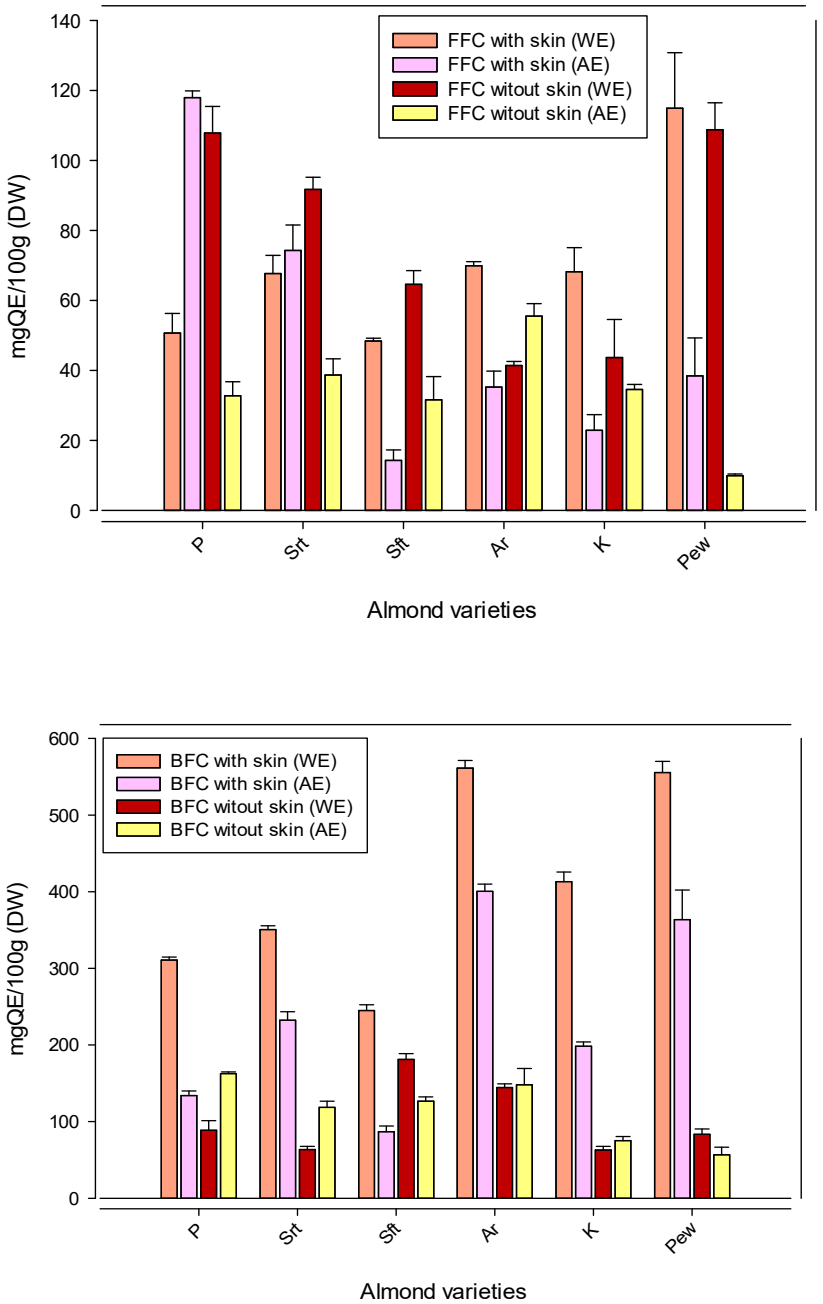

Figure 5. Comparison of bound flavonoids content in water and acetone extract

Antioxidant activity by DPPH assay

Wijeratne et al. (2006) evaluated the antioxidant power of whole almond seed, its brown skin and green shell cover extracts by checking the inhibition of human LDL oxidation, DNA scission and

activities of metal ion chelation. The total phenolic content of green cover and brown skin extracts were found to be 10 times greater than that of whole seed.

Percentage scavenging of DPPH in free fractions (with and without skin)

In comparison to water extracts, highest percentage of scavenging was recorded in acetone extracts of Safaid talwar (WS) and Patasa (WS) (91.1% ± 1.3 and 88.2% ± 1.1) respectively, with a marginal difference between them. (Fig. 6), but the lowest activity was found in water extracts (WoS) of Surkh talwar and Pewand (27.4 ± 1.1 and 40.0 ± 5.0) repectively (Fig. 7).

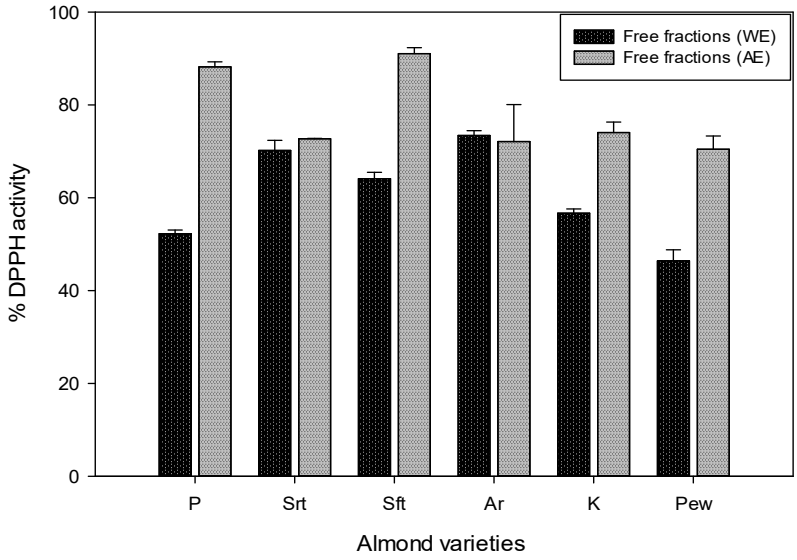

Figure 6. Percentage scavenging of DPPH in free fractions with skin

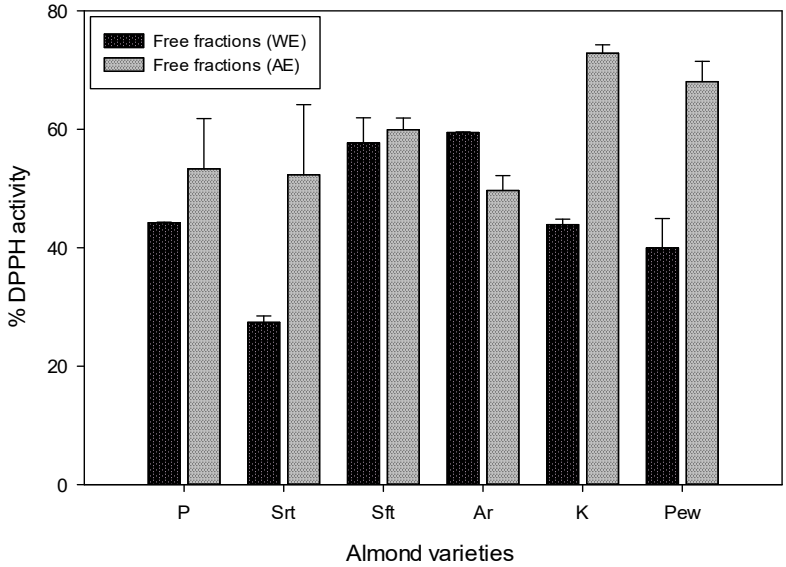

Figure 7. Percentage scavenging of DPPH in free fractions without skin

Percentage scavenging of DPPH in bound fractions (with and without skin)

Antioxidant activity of bound fractions showed an outstanding result as all the varieties (WS) showed scavenging activity above 60%. Highest activity was recorded in water extract (WS) of Aroo (81.7% ± 0.2) (Fig. 8), while the lowest activity was recorded in acetone extract (WoS) of Surkh talwar (49.8% ± 0.2) (Fig. 9). Mandalari et al. (2010) analysed natural and blanched almond skins for microstructural studies and the phenolic contents were found to be high in natural skin than that of blanched skin while both showed high antioxidant properties.

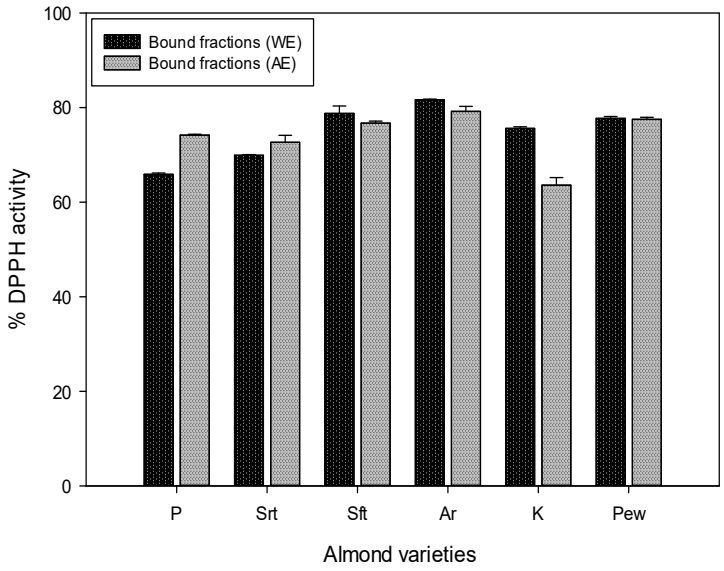

Figure 8. Percentage scavenging of DPPH in bound fractions with skin

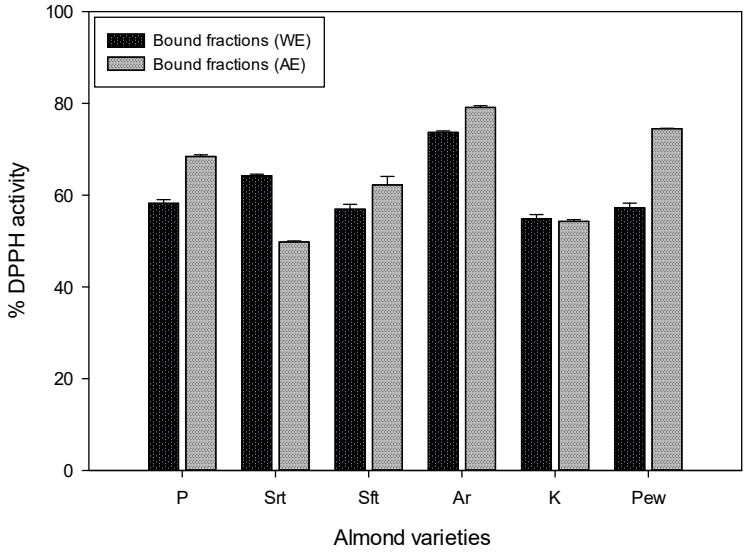

Figure 9. Percentage scavenging of DPPH in bound fractions without skin

Antioxidant activity by FRAP assay

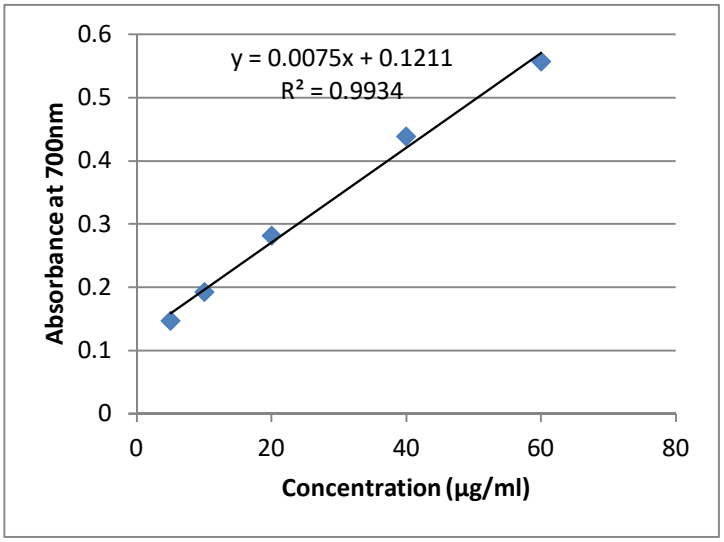

Figure 10. Total ferrous ion content in standard gallic acid.

Standard curve of gallic acid, regression equation and R2 value was constructed using standard chemical to measure the quantity of ferrous ion as antioxidants in almond varieties. The gallic acid solution of concentration (5-100 ppm) conformed to Beer's Law at 700 nm with a regression co-efficient (R2) = 0.993. The plot has a slope (m) = 0.007 and intercept = 0.121. The equation of standard curve is y = 0.007x + 0.121 (Fig. 10).

Ferric ion reducing activity in free fractions (with and without skin)

Acetone extracts of all the samples showed highest activity compared to the water extracts. Highest antioxidant power was observed in Patasa (WS) (753.16 ± 6.3 mgGAE/100g DW) followed by Surkh talwar (WS) (425.24 ± 6.0 mgGAE/100g DW) while the water extract (WS) of Aroo (28.47 ±1.0), is recorded with the lowest antioxidants (Fig. 11).

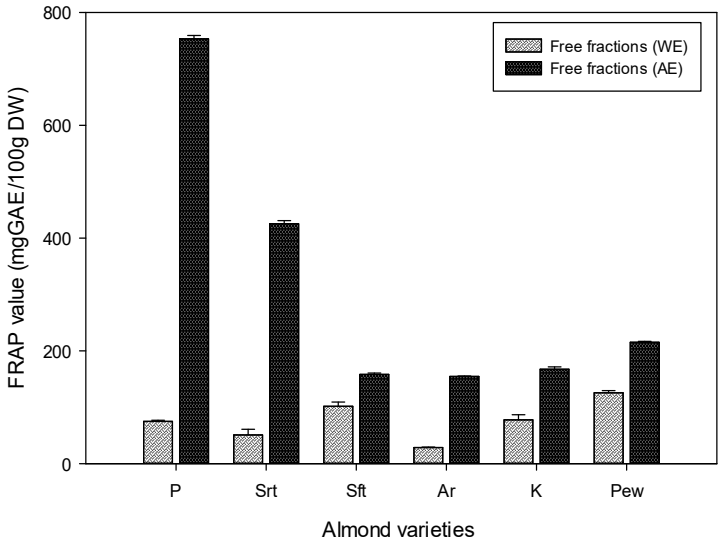

Figure 11. Ferric ion reducing activity in free fractions with skin

The acetone extracts (WoS) of Aroo (246.20 ± 3.7 mgGAE/100g DW) followed by Safaid talwar (231.89 ± 2.1) and Surkh talwar (223.66 ± 5.5) were reported to be highly antioxidative among all while the lowest of all were the water extracts of Aroo (34.14 ± 3.8 mgGAE/100g DW) followed by Safaid talwar and Surkh talwar (Fig. 12).

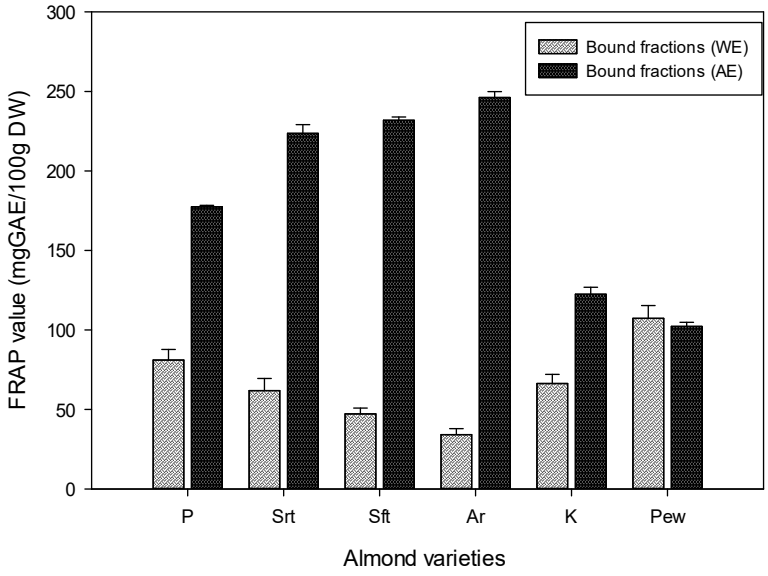

Figure 12. Ferric ion reducing activity in free fractions without skin

Ferric ion reducing activity in bound fractions (with and without skin)

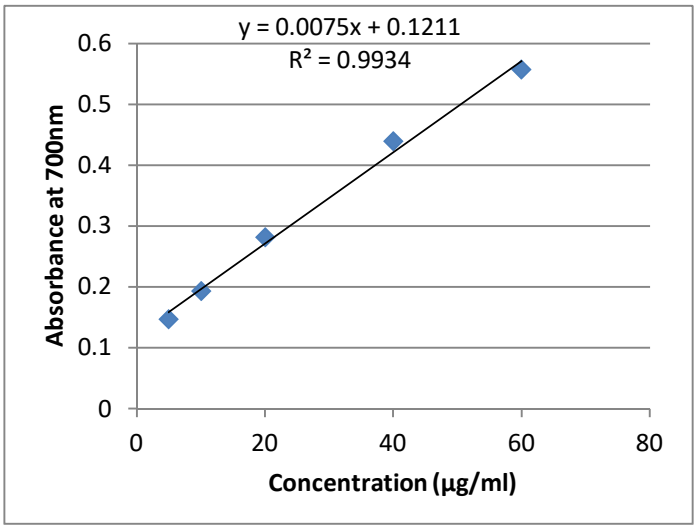

Figure 13. Total ferrous ion content in standard gallic acid.

Standard curve of gallic acid, regression equation and R^2 value was constructed using standard chemical to measure the quantity of ferrous ion as antioxidants in almond varieties. The gallic acid solution of concentration (5-100 ppm) conformed to Beer's Law at 700 nm with a regression co-efficient (R^2) = 0.993. The plot has a slope (m) = 0.007 and intercept = 0.121. The equation of standard curve is y = 0.007x + 0.121 (Fig. 13).

Variations among the varieties are shown in figure 14 and 15. The water extract sample (WS) of Patasa (643.39 ± 6.1 mgGAE/100g DW) and acetone extract of Pewand (WS) (480.20 ± 4.9 mgGAE/100g DW) showed high amount of antioxidants, (Fig. 14) whereas the water extract (WoS) of Patasa (36.84 ± 0.7) exhibited less antioxidants among all the samples (Fig. 15).

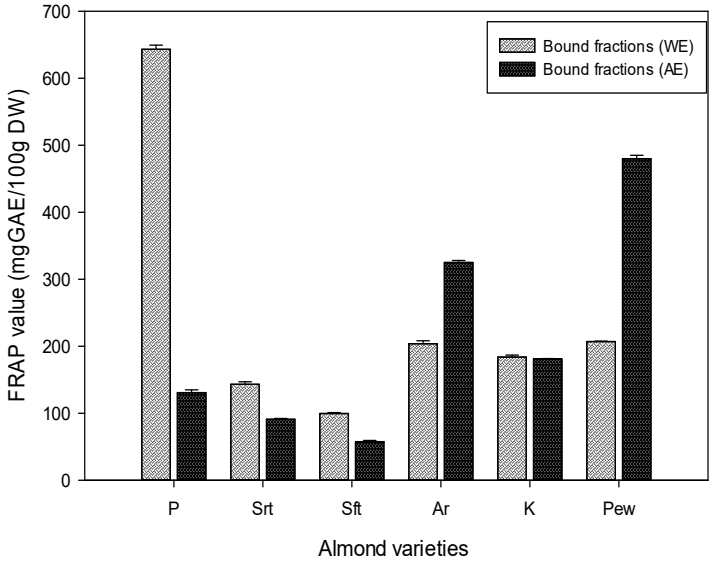

Figure 14. Ferric ion reducing activity in bound fractions with skin

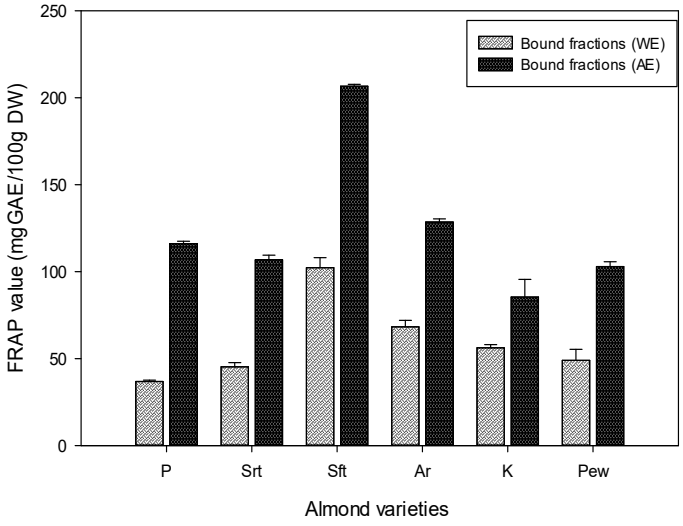

Figure 15. Ferric ion reducing activity in bound fractions without skin

Bolling et al. (2010) determined polyphenols and antioxidant activity (FRAP) in roasted and pasteurized almonds and found that pasteurization did not affect the total phenolics and antioxidants while in roasted almonds had 26% less total phenolics and 34% FRAP than raw.

Correlation analysis

The correlation coefficients among total phenolics, flavonoids and antioxidant activity in free and bound fractions of almond varieties (with and without skin) were calculated to scrutinize their positive and negative associations. Studies have explored the bioavailability of flavonoids from

almond skins and their effect on antioxidant activity (Chen et al., 2005). Isfahlan et al. (2008) determined antioxidant and antiradical activities of phenolic extracts from hulls and shells of Iranian almonds and it was found that radical scavenging capacities of wild almond hull and shell extracts in different species were positively correlated with phenolic content and reducing power.

Results show correlation analysis in water and acetone extracts of samples (without skin) are presented in table 2. Highly significant positive correlations were observed for following pairs: Water extract of free fraction DPPH and acetone extract of free phenolic (r=0.91), acetone extract of bound fraction FRAP and water extract of bound flavonoid (r=0.91), water extract of bound flavonoid and acetone extract of free phenolic (r=0.90). Similarly strong negative correlation were found between: water extract of free fraction FRAP and acetone extract of free flavonoid (r= -0.94), water extract of bound fraction FRAP and water extract of bound flavonoid (r = -0.87) (Table 2).

Results showing correlation analysis in water and acetone extracts of samples (with skin) are presented in table 3. Highly significant positive correlations were observed for following pairs: water extract of bound phenolic and acetone extract of free flavonoid (r = 0.99), acetone extract of free phenolics and acetone extract of free fraction FRAP (r=0.98), water extract of bound phenolic and acetone extract of free fraction FRAP (r=0.98), acetone extract of free flavonoids and acetone extract of free fraction FRAP (r=0.98). Likewise strong negative correlations were observed in: water extract of bound phenolic and water extract of bound fraction DPPH (r = -0.94), water extract of bound fraction DPPH and acetone extract of free fraction (r = -0.93), acetone extract of free flavonoid and water extract of bound fraction DPPH (r = -0.89). Hence it is proved throughout the research that consumption of all six varieties of almond, tested through different assays, are an excellent source of nutraceuticals, which provide essential nutrients along with prevention and treatment of diseases (Table 3).

Table 2. Pearson correlation analysis of phenolics, flavonoids and antioxidant activity in free and bound fractions of almond varieties (without skin).

Variables	FP(WE)	FP(AE)	BP(WE)	BP(AE)	FF(WE)	FF(AE)	BF(WE)	BF(AE)	DPPH (FF-WE)	DPPH (FF-AE)	DPPH (BF-WE)	DPPH (BF-AE)	FRAP (FF-WE)	FRAP (FF-AE)	FRAP (BF-WE)	FRAP (BF-AE)
FP(WE)	1															
FP(AE)	-0.34	1.00														
BP(WE)	-0.80	0.69	1.00													
BP(AE)	-0.53	0.82*	0.56	1.00												
FF(WE)	-0.36	-0.73	-0.07	-0.46	1.00											
FF(AE)	0.09	0.50	-0.03	0.69	-0.66	1.00										
BF(WE)	-0.66	**0.90****	**0.84***	**0.85***	-0.37	0.28	1.00									
BF(AE)	-0.42	0.30	0.27	0.74	-0.05	0.67	0.42	1.00								
DPPH (FF-WE)	-0.40	**0.91****	**0.82***	0.69	-0.59	0.37	**0.85***	0.35	1.00							
DPPH (FF-AE)	0.53	-0.18	-0.18	-0.68	-0.09	-0.64	-0.31	-0.87	-0.11	1.00						
DPPH (BF-WE)	-0.32	0.38	0.18	0.58	-0.33	0.75	0.27	0.49	0.25	-0.75	1.00					
DPPH (BF-AE)	-0.65	0.36	0.80	0.24	0.04	0.00	0.42	0.18	0.59	-0.21	0.41	1.00				
FRAP (FF-WE)	-0.15	-0.21	0.24	-0.49	0.47	-0.94*	0.00	-0.61	-0.08	0.69	-0.80	0.05	1.00			
FRAP (FF-AE)	-0.11	-0.61	-0.24	-0.16	0.67	-0.04	-0.38	0.52	-0.41	-0.43	-0.12	-0.10	0.12	1.00		
FRAP (BF-WE)	-0.32	0.87	0.58	0.68	-0.55	0.17	0.87*	0.08	0.71	0.04	0.02	0.06	0.13	-0.64	1.00	
FRAP(BF-AE)	-0.63	0.71	0.68	0.76	-0.16	0.10	**0.91***	0.39	0.62	-0.26	0.01	0.12	0.16	-0.22	0.87	1.00

** Correlation is significant at the 0.01 level (2-tailed).
* Correlation is significant at the 0.05 level (2-tailed).

Table 3. Pearson correlation analysis of phenolics, flavonoids and antioxidant activity in free and bound fractions of almond varieties (with skin).

Variables	FP(WE)	FP(AE)	BP(WE)	BP(AE)	FF(WE)	FF(AE)	BF(WE)	BF(AE)	DPPH (FF-WE)	DPPH (FF-AE)	DPPH (BF-WE)	DPPH (BF-AE)	FRAP (FF-WE)	FRAP (FF-AE)	FRAP (BF-WE)	FRAP (BF-AE)
FP(WE)	1.00	0.57	0.69	0.40	0.36	0.60	-0.11	-0.11	-0.58	-0.14	-0.74	-0.23	0.38	0.60	0.35	0.05
FP(AE)		1.00	**0.94****	0.47	-0.47	**0.93****	-0.57	-0.53	-0.20	0.48	-0.96	-0.13	-0.11	**0.98****	**0.81***	-0.51
BP(WE)			1.00	0.70	-0.21	**0.99****	-0.29	-0.24	-0.23	0.18	-0.94**	-0.08	-0.18	**0.98****	**0.80***	-0.26
BP(AE)				1.00	0.21	0.75	0.36	0.48	0.06	-0.33	-0.44	0.38	-0.43	0.61	0.53	0.29
FF(WE)					1.00	-0.23	0.78	0.74	-0.45	-0.74	0.32	0.17	0.42	-0.33	-0.25	0.89
FF(AE)						1.00	-0.25	-0.19	-0.18	0.21	-0.89*	0.05	-0.23	**0.98****	0.84*	-0.22
BF(WE)							1.00	0.97	-0.07	-0.82	0.51	0.24	-0.11	-0.41	-0.17	**0.92****
BF(AE)								1.00	0.11	-0.84*	0.49	0.37	-0.24	-0.37	-0.23	**0.85***
DPPH (FF-WE)									1.00	-0.09	0.27	0.18	-0.80	-0.25	-0.43	-0.38
DPPH (FF-AE)										1.00	-0.29	0.11	0.23	0.38	0.39	-0.65
DPPH (BF-WE)											1.00	0.32	0.05	-0.93**	-0.69	0.47
DPPH (BF-AE)												1.00	0.01	-0.02	0.02	0.35
FRAP (FF-WE)													1.00	-0.12	-0.05	0.25
FRAP (FF-AE)														1.00	0.87*	-0.34
FRAP (BF-WE)															1.00	-0.07
FRAP(BF-AE)																1.00

** Correlation is significant at the 0.01 level (2-tailed).

* Correlation is significant at the 0.05 level (2-tailed).

Conclusion

It can be concluded from the current study that tested almond varieties accumulated heavy metals at different level. Overall analysis showed that Surkh talwar accumulated higher contents of Zn, Cd and Cu. During analysis for free and bound phenolics, flavonoids and antioxidant activity; Patasa was ranked high as it proved to be the best source of health beneficial phytochemicals and possess significant antioxidant potential. In comparison the almonds with skin depicted highest total phenolics and flavonoids, therefore they may contribute as valued ingredient in food products. Strong positive correlations between phytochemicals and antioxidant activity proved that phenolics and flavonoids either in free or in bound form are good scavengers of free radicals and help in the prevention of diseases that are caused by oxidative stress.

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