Research Article

Spectrophotometric determination of phenolic antioxidants in four varieties of apples (*Pyrus malus*) from Balochistan, Pakistan

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Abstract

Apples (*Malus domestica* Borkh) are the good source of phenolic compounds and antioxidants, which are not only potential nutrients but also effective in chronic diseases. Four famous varieties of apple namely Shin Kulu, Amri, Kaja, and Tor Kulu from different areas of Balochistan province were collected and analyzed for phenolic contents and antioxidant activities in methanol-water (80:20% (v/v)) extract by using standard spectrophotometric methods. The results of average extract yields of the antioxidant component obtained in dry weights (dw) of both peel and pulp were found 27.3 ± 0.52 g/100g and 19.1 ± 0.21 g/100g respectively. The different varieties of apples contain quantity of entire phenolics content on apple peel and pulp ranged from 13.12 ± 0.22 mg to 17.87 ± 0.10 mg GAE/g dw for peel and 7.21 ± 0.10 mg to 11.57 ± 0.08 mg GAE/g dw for pulp and total flavonoids content were found from 12.22 ± 0.11 mg to 15.30 ± 0.18 mg CE/g dw for peel and 6.01 ± 0.03 mg to 9.69 ± 0.14 mg CE/g dw for pulp. The variations in reducing power of the 10 mg/mL were ranged in peel from 2.139 to 2.725 and pulp from 1.181 to 1.724. The variations in the inhibition of DPPH scavenging activity of the 80% methanolic extracts were seen from 79.6% to 67.6% in peel and 57.8% to 41.2% in pulp. The variations were also found on the basis of genotype, soil fertility, care of watering and climate of the area. The results reveal high concentration of phenolics content, flavonoids content and antioxidant capacity in peel than pulp, so the consumption of peel with fruit might be strongly recommended to achieve better nutritional benefits.

Keywords: Antioxidant activity; Apple peel; DPPH; Flavonoids; Phenolics; Pulp

Introduction

During the normal cellular metabolism molecular species with unpaired electrons are

formed, which are unstable, short lived and highly reactive inside body of living organism, are called free radicals [1], which

behave like oxidants or reductants. These chemical species usually trigger chain reactions, which damage the body cells of the living organism. Reactive Oxygen Species (ROS) and Reactive Nitrogen Species (RNS) are the most important pro-oxidant/oxidants (free radicals) which are formed during metabolic reactions, due to their harmful effects. Several harmful chemical compounds are formed in a human body [2]. The ROS results in the production of abnormal cells and tissues due to the oxidation of poly-unsaturated fatty acids of the cell membranes. Excess of free radical formation (ROS/RNS) and having less antioxidant defenses causes imbalance, so due to high concentration and high reactive nature of free radical oxidative stresses are produced, which further react with other important bio-molecules such as proteins, DNA, cell membrane, nucleic acids and lipids [3]. These stresses initiate diseases like cancer, cardiovascular, arthritis, diabetes, emphysema, cirrhosis and ageing [4, 5]. Free radicals in the form of ROS are either generated endogenously in internal cellular organs like mitochondria, endoplasmic reticulum and peroxisomes [6] or exogenously due to alcohol, cigarette smoking, transition metals like Cd, Hg, Pb, As, Co etc, pesticides, UV rays, high temperature, smoked or fried fatty meat and drugs [7].

Antioxidants are the chemical compounds that quench the reactions of free radicals by reducing their oxidation processes [8] and providing universal defensive mechanism for cells against many chronic diseases [9, 10]. Dietary source of antioxidants participates an essential job for a person's general health because the fact that the living bodies require a large number of antioxidants than having the capability to generate them [11]. On the basis of activity, antioxidants are categorized into two types, enzymatic antioxidants and non-enzymatic antioxidants. The former, in

the presence of cofactors such as zinc, copper, iron, manganese etc, break down and remove the free radical by changing them into hydrogen peroxide and last into water through a series of reactions. The later, include vitamin C, Vitamin E and plant phenolics, carotenoids, glutathione and other phytonutrients present in vegetables and fruits, which interrupt the chain reactions of the free radicals [12, 13]. Fruits have the excess amounts of flavonoids, phenolic acids, vitamins, minerals and valuable strong antioxidants [14]. The presence of antioxidants in fruit depend on the types of fruits, gardening condition and climate of the area and as well as in the different parts of fruit such as peel or pulp. Peels of most of fruits contain much phenolic contents and show higher antioxidants activity than the pulp [16]. The fruits contain a greater number of antioxidants like vitamin C, E, polyphenols, β -carotene and lycopene [17]. Apple (*Malus domestica* Borkh) is rich source of many types of phytonutrients such as phenolic compounds which are effective antioxidants [18]. The most interesting feature is that apple contains the biggest portion of free phenolics as compared to other fruits. So, these bioactive substances are more easily absorbed [19]. About 80% of polyphenolic compounds and 3 to 6-fold of total flavonoids are distributed in the peel of apples [20]. The apple flesh contains catechin, procyanidin, chlorogenic acid, phloridzin and caffeic acid. The peel of apple contains additional compounds, like quercetin and cyanidin, which are not present in the pulp [21].

Apples are the best sources of vitamin C and fibers [22] and therefore may decrease weight if utilize before meals, support heart health in several ways and lower the cholesterol level [23]. The epicatechin a type of polyphenols, present in apples, linked to decrease blood pressure hence stroke risk [24]. Apple fiber pectin can speed up the

movement of the stool through the intestines, decrease the symptoms of constipation and increase the number of useful bacteria in the gut and also protects from colon cancer [25]. The consumption of apple flavonoids may decrease the cardiovascular diseases (CVD) risk, obesity and diabetes [26]. Apples peel contain quercetin a type of flavonoids which work as antioxidant and anti-inflammatory and helps to normalize immune system and give protection against disease like asthma [27]. Medical science studies proved that the usual consumption of apple with peel decreases the cancer risk of colon [28], regulates the amounts of calcium mineral which helps to support bone health [29] and acetylcholine, a neurotransmitter, which prevents Alzheimer's disease [30].

In Pakistan, Balochistan province is the largest producer of fruits, therefore, known as "Fruit basket of Pakistan" with annual apples production of 224000 tons. Apple contributing over 23% of total production of the country [31]. Apple producing areas in the highland of Balochistan comprising Mastung, Kalat, Quetta, Pishin, Ziarat, Killa Abdullah, Killa Saifullah, Loralai and Zhob districts. Several varieties of apples are grown in these districts and the commonly grown commercial varieties include Kaja, Tor Kulu, Shin Kulu, Amri, Mashadi and Kashmiri [32]. Tor Kulu apple accounts for about 35% of apple produced in Balochistan. It is fragrant, juicy and crispy with rational size. Shin Kulu apple fruit is popular due to its juicy flavor with acidic sweet taste and accounts for about 40% of apple produced in Balochistan [33].

Materials and methods

All analytical grade chemicals and reagents were used. Methanol, potassium ferricyanide, trichloroacetic acid (ACS reagent), FeCl_3 , Na_2CO_3 , AlCl_3 , NaNO_2 and NaOH which were purchased from Merck (Darmstadt, Germany) Folin-Ciocalteu Reagent (FCR), gallic acid, 2,2-diphenyl-1-picrylhydrazyl

(DPPH) radical, catechin and ascorbic acid were obtained from Sigma Aldrich (Buchs, Switzerland).

A homogenizer used for homogenizing sample (Westpoint-1844-Juicer-Blender-Mincer), A magnet stirrer used for extraction (JENWAY-1000 Hot Plate Magnetic stirrer), a vacuum rotatory evaporator used for the removal of methanol solvent from extract (vacuum rotary evaporator, KARL KOLB, Germany), a table top centrifuge (EBA 20, Hettich Zentrifugen) is used for phases separation, a double beam UV-Vis spectrophotometer (Shimadzu, Model UV-1700, Japan) was employed for the measurement of absorbency throughout the experimental studies.

Sample collection

Apple fruits of different varieties in fresh condition, named: Kaja, Tor Kulu, Amri and Shin Kulu were collected from different areas of different districts of Balochistan (Ziarat, Quetta, Pishin and Kalat) based on different climates. The apple varieties were further identified by Horticulture Research Department, Sariyab Road, Quetta.

Sample preparation

Firstly, samples of apple fruit were washed properly with tap water followed by peeling off with the help of a knife. After removing of seeds, the pulp and peel of the fruit were homogenized separately with the help of a juicer (Westpoint-1844-Juicer-Blender-Mincer).

As the level of water content in apple fruit varieties is different, all calculations were made on the basis of dry matter for dry matter determination according to a procedure reported previously [34]. The extraction and extraction yield are explained in the following paragraphs briefly.

20g homogenized fruit sample (pulp or peel) of each apple's variety was extracted with 200mL of 80:20% methanol-water mixture (v/v) at normal room temperature by using the magnetic stirrer for about 8 hours. Then

the residue was separated from extract by filtration process through Whatman filter paper No 1. After three extractions (3×200 mL), the excess of the methanol solvent was removed by distillation process using a vacuum rotatory evaporator at 45°C . The resulting crude extracts were further dried with the help of a freeze drier and stored at 4°C for further experimentation.

Following equation was used for the calculation of yield of extracts on the basis of dry weight of pulp and peel.

$$\text{Yield} \left(\frac{\text{g}}{100 \text{ g}} \right) = \frac{W_1}{W_2} \times 100$$

Where,

W_1 = weight of extracted residue after solvent removal

W_2 = weight of peel or pulp taken for extraction

Determination of total phenolic contents

Total phenolics contents (TPC) in pulp and peel of apples were determined by using a well-known colorimetric technique with some modifications [35] in which FCR involves as an oxidizing agent and antioxidants in plant/fruit extracts act as reducing agent. The reduction of FCR by phenolic compounds with simultaneous formation of blue colored complex with a wavelength of maximum absorbance (λ_{max}) of 760 nm. FCR measures the reducing capacity in a given sample. FCR contains hetero polyphosphotungstates-molybdates, as it gains one or two electrons from antioxidants during a redox reaction leading to the formation of a blue colored reduced complex ($\text{PMoW}_{11}\text{O}_{40}$). It is believed that the Mo(VI) is easily reduced in the complex and the transfer of electrons occurs from reductants (phenolic compounds) as shown in the reaction given below:

The apple's peel or pulp extract (30mg/mL) mixed well with 5mL FCR (which had already been diluted 10 times with deionized water). Then 4mL of 7.5% (w/v) of Na_2CO_3

solution was added to the above mixture and incubated at 25°C for about 20 minutes. The absorbance of the resulted product was measured at λ_{max} of 760 nm against a reagent blank. The total phenolic contents in the samples were estimated from the regression equation obtained by the subject procedure from a calibration curve between the absorbance and a series of standards (60–300 $\mu\text{g/mL}$) of gallic acid. The final results for phenolic contents in samples were calculated as mg/g dry weight of samples.

Determination of total flavonoid contents

For the determination of total flavonoid contents (TFC) in the methanolic extract of apple's peel and pulp, a reported method with slight modifications was employed [36]. In this method, Al^{3+} makes a complex with hydroxyl functionality present in flavonoids which absorbs visible radiation at a λ_{max} of 510 nm. For the estimation of TFC in extracts (mg/g dry weight), a regression equation obtained from the calibration curve between the absorbance intensity on the ordinate and catechin concentration (20–100 $\mu\text{g/mL}$; $R^2 = 0.9978$) on abscissa was employed.

In brief, the experimental procedure for TFC estimation is as: 1mL of catechin standard or methanolic extract (0.2mg/mL) diluted to 3mL with methanol in a test tube followed by the addition of 5% (w/v) NaNO_2 aqueous solution, after 5 min, 0.3mL of 10% (w/v) AlCl_3 aqueous solution and after 6 min, 2mL of 1.0 M NaOH . Finally, the mixture was diluted to 10mL with distilled water and after incubation for 30 min at room temperature; the absorbance was measured spectrophotometrically at λ_{max} of 510 nm against a reagent blank.

Reducing power capacity assay

A reported method [37] with minor modification was employed for assessing the ferric reducing power of the apple's pulp and peel extracts. The generated ferrous was then coupled with ferricyanide to make a Prussian

blue colored charge transfer complex with a λ_{max} of 700 nm. The reducing activity of a substance may serve as a significant indicator for its potential antioxidants capacity.

The procedure in brief is as: 1 mL of either extract or ascorbic acid standards (2–10mg/mL) was taken in a test tube followed by the addition of 3mL of 0.2 M sodium phosphate buffer (pH = 6.6), 3mL of 1% (w/v) potassium ferricyanide solution, incubation at 50°C in a water bath for about 20 min and addition of 3mL of trichloro acetic acid. The reaction mixture was then centrifuged at 3000 rpm for 15 min and 3mL of supernatant was recovered and added 3mL of distilled water followed by the addition of 1mL of 0.1% (w/v) ferric chloride resulting in the appearance of Prussian blue color whose intensity was measured at 700 nm against a reagent blank.

DPPH scavenging assay

DPPH (2,2- Diphenyl-1-picrylHydrazyl) is a dark violet color crystalline organic compound composed of stable free radical molecules. The mechanistic approach of the method is based on the scavenging of DPPH radicals by antioxidants present either in the working standards or extract of apple and thus inhibiting the absorbance intensity at λ_{max} of 517 nm. During the redox reaction, a hydrogen is donated from an antioxidant to DPPH with the simultaneous generation of reduced DPPH and its dark violet color turns into dark red to light yellow color indicating the direct antioxidant activity of either standards or samples. The reaction scheme for the redox reaction is as follows:

The DPPH radical scavenging capacity of the extracts and standards were measured according to a reported procedure [38] with few modifications. In brief, the determination procedure is as: 2mL (0.4mg/ml) of fresh methanolic extract was added to a test tube containing 3mL methanolic solution of DPPH (0.004 % w/v) followed by incubation

in dark at room temperature for almost 30 min. Absorbance intensity of the test solution was measured at λ_{max} of 517 nm against a reagent blank. The % inhibition activity (%I) was calculated employing the following equation:

$$\%I = \{(A_0 - A_1)/A_0\} \times 100$$

Where, A_0 is the absorbance of the control and A_1 is the absorbance of extract or standard.

Statistical analysis

Four different samples of each fruit cultivar peel and pulp separately were assayed randomly. Each sample was analyzed individually in triplicate and data are reported as Mean ($n = 3$) $\pm$ SD.

Results and discussion

Extraction yield

On the basis of dry weight, the peel and pulp of the studied varieties of apple yielded an appreciable amount of extractable matter with aqueous methanol. The mean extraction yield of antioxidant components was found to be 27.3g/100g for peel and 19.1g/100g for pulp, which showed higher extraction yield for peel than pulp. The relative major differences in the yield of peel and pulp in same fruits might be due to the variation in the chemical composition of different tissues of apple [39].

Total phenolics and flavonoids contents

The table 1 indicates the total phenolic content, calculated as Gallic Acid Equivalent (GAE), and the total flavonoids contents which was expressed as Catechin Equivalent (CE), of 80% methanolic extracts of apple's peel and pulp separately. The calibration curves obtained between the absorbencies and concentrations of gallic acid over the range of 60–300 μ g/mL at λ_{max} of 760 nm and catechin over the range of 20–100 μ g/mL at λ_{max} of 510 nm are shown in the figures 1 and 2 with regression equations of $y = 0.007x + 0.0011$ ($R^2 = 0.9983$) and $y = 0.0036x + 0.005$ ($R^2 = 0.9978$) respectively (where y is

absorbance intensity and x is the concentration in mg/L).

Total phenolic contents ranged from 13.12mg to 17.87mg GAE/g in peel extracts and 7.21 to 11.57mg GAE/g in the pulp extracts of the different tested varieties. Tor Kulu peel's extracts showed significantly high contents of total phenolic contents i.e. 17.87mg GAE/g as reported previously [40]. The quantity of nutrients and highly valuable components such as phenolic compounds in fruits depends upon genotypes, fruit tissues, and the maturity level of fruits, soil fertility and climatic or environmental aspects. The fruits which are not peeled have higher quantity of phenolic contents and their consumption is good for health [41].

The table 1 also shows the total flavonoid contents determined in different varieties of apple's peel and pulp. In peel extract, it was ranged from 12.22 to 15.30mg CE/g and the highest value was obtained for Tor Kulu apples i.e. 15.30mg CE/g followed by Kaja, Amri and Shin Kulu whose phenolic contents

were obtained as 14.67, 13.65 and 12.89mg CE/g respectively. Pulp extracts were found to have very less amount of flavonoid contents in comparison to peel. In pulp extracts the flavonoid contents ranged from 9.69mg CE/g for Tor Kulu to 6.01mg CE/g for Shin Kulu.

The considerable variation in the amounts of TPC and TFC among the four apple varieties between the peel and pulp and the results obtained in this research project are in agreement with those reported by various researchers previously [42].

These results also show that TPC and TFC variations in the same variety of apple can also be correlated with the climate variation of different areas of Balochistan. Among the four Districts of Balochistan (Ziarat, Pishin, Quetta and Kalat), the highest amount of TPC and TFC were obtained in peel and pulp extracts of all varieties of apples collected from District Ziarat (one of the coldest district of Balochistan) followed by District Pishin.

Figure 1. Standard calibration curve for gallic acid

Figure 2. Standard calibration curve for catechin

Table 1. TPC & TFC of pulp and peel extracts of different varieties of apple (*Malus domestica* Borkh) and sampling areas

Sampling area	Apple variety	TPC (mg GAE*/g dw±SD)		TFC (mg CE*/g dw±SD)	
		Pulp	Peel	Pulp	Peel
Ziarat	Shin Kulu	7.83±0.02	13.76±0.14	6.17±0.15	12.89±0.11
Pishin	Shin Kulu	7.65±0.06	13.58±0.15	6.06±0.15	12.70±0.17
Quetta	Shin Kulu	7.21±0.10	13.12±0.22	6.01±0.03	12.22±0.11
Kalat	Shin Kulu	7.60±0.09	13.28±0.23	6.00±0.10	12.46±0.16
Ziarat	Amri	9.05±0.21	14.26±0.01	6.63±0.17	13.65±0.08
Pishin	Amri	8.50±0.17	14.15±0.11	6.52±0.17	13.39±0.06
Quetta	Amri	8.38±0.16	13.01±1.83	6.11±0.11	12.78±0.06
Kalat	Amri	8.43±0.17	14.05±0.07	6.3±0.13	13.09±0.08
Ziarat	Kaja	10.12±0.39	16.32±0.44	7.39±0.25	14.67±0.28
Pishin	Kaja	9.72±0.22	16.24±0.50	7.11±0.33	14.41±0.34
Quetta	Kaja	9.33±0.44	16.40±0.75	6.37±0.38	13.83±0.40
Kalat	Kaja	9.52±0.37	16.13±0.59	6.69±0.42	14.09±0.37
Ziarat	Tor Kulu	11.57±0.08	17.87±0.10	9.69±0.14	15.30±0.18
Pishin	Tor Kulu	10.55±0.10	17.83±0.10	9.50±0.11	15.15±0.17
Quetta	Tor Kulu	10.27±0.11	17.66±0.07	9.07±0.26	14.78±0.22
Kalat	Tor Kulu	10.35±0.10	17.75±0.07	9.35±0.12	15.02±0.17

*GAE = Gallic acid equivalent, CE = Catechin equivalent, Data are mean ± SD (n = 3 × 3, P < 0.05)

Measurement of reducing power

In this assay, the generated amount of Fe²⁺ as a result of the reducing power of apple extract

is monitored by the measurement of the intensity of blue coloration at 700 nm. A sample of higher absorbance value indicated

high reducing power, thus high antioxidant activity [43].

In this procedure of reducing power measurement of the extract of apple (peel or pulp), various quantities (2, 4, 6, 8 and 10mg/mL) of the dried residue were taken for the measurement of antioxidant activities. As the amount of the residue was increased, the increase in the absorbance intensity was also observed. Figure 3 shows the reducing power of the different extracts of different cultivars of apple. The variations in reducing power of

the 10mg/mL were ranged in peel from 2.139 to 2.725 and pulp from 1.181 to 1.724. The reducing power capability of extracts of apple fruit (peel or pulp) of the given analysis was comparatively near to those reported in extracts of Logan fruits [44]. The reducing power capability of apple's peel extracts was higher than the pulp extracts. The reason might be the presence of higher concentration of reductants in the form of phenolic compounds present in the outer tissues (peel) of the fruits than the inner tissues (pulp) [45].

Figure 3. Reducing powers of different apple's extracts (bars indicate means $\pm$ SD ($n = 3 \times 3$))

DPPH radical scavenging activity

DPPH free radical assay is used to evaluate the scavenging capacity of antioxidants present in the plants/fruits extracts. It is a time saving assay and can be applied to analyze many samples in less time.

Moreover, this method is incredibly sensitive even at low concentration of antioxidants.

Scavenging capacity of apple peels and pulps extracts were analyzed by DPPH free radicals and compared with ascorbic acid (standards). The absorbances were recorded after 30 minutes when highest variation in scavenging

capacity was found then that record is used for calculation. Figure 4 shows the DPPH radical scavenging capacities of different extracts of different apple varieties. The scavenging activity in all apple peel extracts were found higher, ranging from 79.6 to 67.6% compared to the apple pulp extracts, ranging from 41.2 to 57.8%. Among the peel extracts of different varieties of apple, Tor Kulu exhibited the highest scavenging activity i.e. 79.6%, while the Amri showed the lowest scavenging activity i.e. 67.6%. In pulp extract of different varieties, the highest scavenging activity 57.8% showed by Tor Kulu and the lowest 41.2% was recorded for Amri and the data showed close

agreement with the previously reported data. In the previously reported study, 1mg/mL residue of peel and pulp extracts have shown 78 and 55% scavenging activity respectively. It is clear from the observed results that the high DPPH radical scavenging activity in peel extracts than pulp extracts might be related to the presence of the largest quantity of antioxidants in the form of phenolic compounds and flavonoids [46]. So, the consumption of apple fruit without peel may induce loss of these valuable phytonutrients, which scavenge the free radicals and delay the cellular damage inside the bodies of living organisms.

Figure 4. DPPH radical scavenging capacities of different extracts of different apple varieties (bars indicate means $\pm$ SD ($n = 3 \times 3$))

Conclusions

In this study, the TPC, TFC, reducing power of antioxidants and DPPH %inhibition of peels and pulps extracts of four varieties of apple collected from the different areas of some districts of Balochistan were analyzed. The peels of all varieties were found rich in phenolic and flavonoid contents, reducing power of antioxidants and DPPH scavenging activity than pulp. Among four apple varieties analyzed, Tor Kulu was found to be rich in all these measured variables comparing to other varieties. Based on the obtained results, it is concluded that apple peel removal may provoke for loss of considerable nutrients. As with many other fruits and vegetables, apple consumption along with peel could provide more nutritional and health benefits.

Authors' contributions

Conceived and designed the experiments: A Muhammad, AU Rehman & Samiullah, Performed the experiments: A Muhammad, AU Rehman, A Baqi & Hayatullah, Analyzed the data: AU Rehman, N Khan & Samiullah, Contributed materials/ analysis/ tools: M Asghar, AJ Zeerak & M Hussain, Wrote the paper: A Muhammad, AU Rehman, Samiullah & A Baqi.

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