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RESEARCH ARTICLE

Phenolic and antioxidant activity in two selected apple (Malus domestica Borkh.) cultivars

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Abstract

Apple (*Malus domestica* Borkh., Family Rosaceae) has been identified as one of the main dietary source of polyphenols and antioxidants, however, the concentrations and composition of the phytochemicals varied among the growing locations and type of cultivars. The present study focused on two apple cultivars one is traditional cultivar (Benoni) and another is commercial one (Red delicious). No significant variation in phenolic content and antioxidant activity between the cultivars was observed, however, variation among growing locations was prevalent. While developing relationship between phytochemical contents and altitude, a significant positive correlation was observed, which indicate that the higher altitude is most suitable for apple plantation. The present study revealed that since both the species have almost equal amount of total phenolics and antioxidant activity, therefore, both the cultivars can be promoted for their plantation at suitable locations. Also, there is need to promote traditional cultivar such as Benoni, which is declining from the region as this is one important genepool of apple, therefore, should receive more attention.

Introduction

Fruits are considered rich sources of polyphenols, and widely known for their antioxidant properties. It is extensively reported that dietary intake of fruits, particularly those containing functional bioactive such as phenolic acids, tannins, flavonoids and nutrients is associated to reduce risk of several chronic diseases (Abrosca et al., 2007; He and Liu, 2007). Among others, Apple (*Malus domestica* Borkh.; Family-Rosaceae) is one of the most important fruit crop of the temperate region and most frequently consumed in many regions of the world. Apple fruit has been reported as a potential source of polyphenolics, carbohydrates and antioxidants (Knekt et al., 2002; Wolf et al., 2003) and represent a major source of dietary antioxidants. Several epidemiological studies reported that regular consumption of fresh apple and processed products prevents several degenerative and neurodegenerative diseases such as aging, diabetes, lower risk of cardiovascular diseases, lung dysfunction, asthma, thrombotic stroke, liver, colon and lung cancer (Block et al., 1992; Knekt et al., 1996; Eberhardt et al., 2000; Knekt et al., 2002). However, composition and distribution of nutrients and high value components such as phenolics and antioxidants depends upon genotypes, fruit tissue and environmental aspects (Podesedeic et al., 2000; Lata et al., 2007).

Variation in microclimatic conditions, wide altitudinal range and geographical diversification in Uttarakhand state has been considered one of the suitable areas for apple cultivation and is the third largest apple producer state after Jammu & Kashmir and Himachal Pradesh. It has been well known that apples are one of the important sources of polyphenolics, which are receiving attention all across the globe. Reports indicate that apples are the third largest contributors of flavonoids in the Dutch diet beside tea and onions (Hertog et al., 1993) and over twenty two percent of the fruit phenolics consumed in the United States are from apples (Vinson et al., 2001). Such types of studies are poorly known for the apple growing in the Indian Himalayan region (IHR). Therefore, the present study attempted to quantify nutritive content (Carbohydrate and protein), phenolics and antioxidant activity in two cultivars i.e. Benoni and Red delicious growing in the Uttarakhand Himalaya.

Materials and Methods

Fruit sample collection

Fresh fruits (fully ripen) of 2 different apple cultivars i.e., Benoni and Red delicious were collected from different locations of Uttarakhand Himalaya for the detail analysis of nutritive, phytochemical and antioxidant analysis. Details of the locations are provided in Table 1.

Chemicals and reagents

2, 2-Diphenyl-1-picryhydrazyl (DPPH) radical, gallic acid, ascorbic acid, quercetin and catechin were purchased from Sigma–Aldrich (Steinheim, Germany). Sodium carbonate, 2-(N-morpholino) potassium persulphate, ferric chloride, sodium acetate, potassium acetate, aluminium chloride, acetic acid and hydrochloric acid from Qualigens (Mumbai, India), and 2,2-Azinobis-3-ethylbenzthiazoline-6-sulphonic acid (ABTS), 2,4,6-tri-2-pyridyl-1,3,5-triazin (TPTZ), methanol and ethanol from Merck Co., (Darmstadt, Germany). All the chemicals purchased were of analytical grade.

Extraction

The experiments were performed in the Biodiversity Conservation and Management Laboratory of G. B. Pant Institute of Himalayan Environment & Development (GBPIHED) Almora, Uttarakhand, India. The samples were cut into thin slices and then grounded in to a mortar and pestle to a fine texture, and precisely 20 mg of each sample was weighed into a test tube and extracted with 200 ml methanol (80%) containing 0.1% (v/v) of hydrochloric acid to obtain phenolics. The mixture was homogenized for 1 min using an Ultra sonicator (Toshiba - India). The homogenized mixture was kept on 45º C at water bath for 1 h. The homogenate was stored in tightly capped bottles for 24 h at 4º C. Homogenate centrifuged at room temperature for 20 min at 10000 rpm. Supernatant was removed from each tube and filtered by whatman filter paper No. 2 and stored in separate amber screw - cap glass vials at -20º C prior to analysis of phenolic and antioxidant activity.

Total phenolic content estimation

Total phenolic content in the methanolic extract was determined by Folin-Ciocalteu's calorimetric method with minor modification (Singleton et al., 1999; Bahukhandi et al., 2013). Methanolic extract (0.25 ml) were diluted with distilled water (2.25 ml). Folin-Ciocalteu's reagent (0.25 ml) was added and allowed to stand for reaction up to 5 min. This mixture was neutralized by 2.50 ml of 7% sodium carbonate (w/v) and kept in dark at room temperature for 90 min. The absorbance of resulting blue colour was measured at 765 nm using UV-VIS spectrophotometer (Hitachi U-2001). Quantification was done on the basis of standard curve of gallic acid prepared in 80% methanol (v/v) and results were expressed in mg gallic acid equivalent (GAE) per gram fresh weight (fw).

Determination of total tannin content

Total tannin content measured by Folin's Dennis method described by Nwinuka et al. (2005). Briefly, 0.25 ml of methanolic extract were diluted with 2.25 ml distilled water, 0.50 ml Folin's Dennis reagent was added and allowed to stand for reaction up to 1 min. This mixture was neutralized by 1.0 ml of 7% sodium carbonate (w/v) and kept in water bath in 25ºC for 20 min. The absorbance of resulting blue colour was measured at 700 nm using UV-VIS spectrophotometer (Hitachi U-2001). Quantification was done on the basis of standard curve of tannic acid prepared in 80% methanol (v/v) and results were expressed in mg tannic acid equivalent (TAE) per gram fresh weight (fw).

Total flavonol content estimation

The total flavonol content was estimated by adapting the method described by Miliauskas et al. (2004) with slight modification. The quercetin curve is prepared by mixing 1 ml of 0.150-0.05 mg ml^{-1} quercetin methanol solution with 1 ml of 2% aluminum trichloride and 3 ml of 5% sodium acetate. The absorption at 440 nm was read after 150 min at 20 $^{\circ}$C. The sample procedure was carried out with 1 ml of fruit extract (1 mg/mL^{-1}) instead of quercetin solution. Total flavonol were expressed as mg quercetin (QAE) equivalent per gram fresh weight (fw).

Total carbohydrate and protein content estimation

The analysis of total carbohydrate content was carried out by method given by Hedge and Hofreiter (1962) with slight modification. The sample extract was prepared by hydrolyzing the test sample in 2.5N HCL for 3 h in boiling water bath, followed by neutralizing with sodium carbonate. It was then centrifuged and the supernatant was collected for analysis. Similarly, protein was estimated with slight modification (Lowery et al., 1951; Sawhney and Randhir, 2006). The supernatant solution (10.0 ml) and alkaline copper sulphate reagent (5.0 ml) were mixed thoroughly and allowed to stand for 10 min and thereafter Folin's reagent (0.5 ml) was added to develop the color.

After 30 min, absorbance measurements were performed at 660 nm against a blank (1.0 ml of 0.5 NaOH in place of sample). Bovine serum albumin was used to construct a standard curve and the amount of protein in different samples was estimated.

Determination of antioxidant activity

The antioxidant activity was determined using different *in vitro* methods such as ABTS [2, 2'-azinobis (3-ethylbenzothiazoline-6-sulphonic acid) diammonium salts], DPPH (2, 2-diphenyl-1-picrylhydrazyl) and FRAP (ferric reducing antioxidant power) (Bhatt et al., 2012; Bahukhandi et al., 2013). All the assays were carried out in six replicates and average values were considered.

Statistical analysis

All determinations of carbohydrate, proteins, total phenolic, tannin, flavonol content and antioxidant activity by ABTS, DPPH, FRAP assay were conducted in six replicates. The value for each sample was calculated as means of all replicates with ± standard error. Analysis of variance and significant difference among means were tested as Duncan's multiple range test on mean values by one way ANOVA using SPSS programme (10 versions).

Results

A significant ($p<0.05$) variation was recorded in nutritive content, phenolics, flavonol and antioxidant activity among the locations in selected apple cultivars (Table 2 & 3). Benoni exhibited higher total phenolic (2.40 mg GAE/g fw), tannin (24.70 mg TAE/g fw) and protein (6.01 mg/g fw) content in Mukhwa. However, highest antioxidant properties (FRAP-21.19; DPPH-14.35 mM AAE/100g fw) and carbohydrate (5.61 mg/g fw) content was observed in the fruits collected from Khbarar (Table 2). Red delicious showed maximum total phenolic (2.71 mg GAE/g fw) and protein (5.99 mg/g fw) content in Mukhwa, however, highest antioxidant activity (FRAP- 18.18; ABTS-27.98 mM AAE/100g fw) and carbohydrate (5.54 mg/g fw) in Satbunga. Fruits collected from Chaubatiya locations exhibited lowest antioxidant activity in both Benoni and Red delicious cultivars as compared to other locations (Table 3).

Correlation analysis showed a significant ($p<0.05$) increase in polypheolic content with increasing altitude. A significant positive relationship was exhibited in total phenolics ($r=0.595$) and protein content ($r=0.685$) with altitude (Table 4). Similarly, total protein content showed a significant positive relationship with total phenolic content ($r= 0.655$, $p<0.05$) and DPPH assay ($r =0.600$, $p<0.05$). A positive correlation ($r= 0.637$, $p<0.05$) also exhibited between ABTS assay and total tannin content. However, altitude showed a negative relationship with total tannin, flavonol and carbohydrate content respectively.

Table 1: Collection of apple genotypes from different locations of Uttarakhand,(West Himalaya)

S.No	Location	Altitude (m asl)	Latitude (N)	Longitude (E)
1	Naugown	1771 m	30º46'0.89"	37 º88'51.9"
2	Chaubatiya	2040 m	29º25'12.8"	79 º33'25.4"
3	Shitlakhet	2100 m	29º26'36.91"	79 º32'41.52"
4	Khbarar	2200 m	29º25'12.9"	79 º36'32.33"
5	Satbunga	2400 m	29º26'22.9"	79 º36'38.7"
6	Mukhwa	2780 m	31º25'76.0"	78 º46'45.2"

Table 2: Polyphenolics and antioxidant activity in Benoni cultivar in different locations of Uttarakhand, (West Himalaya)

	Chaubatiya	Shitlakhet	Khbarar	Satbunga	Mukhwa	Naugown
Total phenol	1.71±0.13^{d}	0.98±0.61^{f}	2.02±0.92^{c}	2.16±0.40^{b}	2.40±0.42^{a}	1.14±1.08^{e}
Total tannin	22.1±0.35^{d}	22.84±6.78^{c}	16.29±4.04^{f}	23.51±4.43^{b}	24.70±5.94^{a}	18.33±5.88^{e}
Total flavonol	0.40±0.49^{b}	0.39±0.21^{c}	0.96±0.22^{a}	0.16±0.13^{e}	0.75±0.20^{d}	0.96±0.25^{a}
ABTS assay	7.74±3.84^{d}	24.34±8.63^{a}	2.70±1.07^{f}	23.38±8.53^{b}	10.85±3.33^{c}	3.63±1.90^{e}
FRAP assay	16.97±3.50^{c}	5.12±0.53^{f}	21.19±12.23^{a}	18.35±3.09^{b}	11.85±0.55^{d}	8.36±0.08^{e}
DPPH assay	7.23±3.92^{f}	9.27±5.45^{d}	14.35±9.31^{a}	10.40±5.56^{c}	13.67±8.67b	7.62±2.90^{e}
Carbohydrate content	1.67±0.64^{f}	5.32±0.56^{c}	5.61±0.47^{a}	5.52±1.03^{b}	2.36±0.75^{e}	5.08±0.15^{d}
Protein content	1.50±0.34^{f}	2.19±0.48^{d}	5.21±0.19^{c}	5.56±0.80^{b}	6.01±0.38^{a}	2.02±0.56^{e}

Data are presented as the mean ± SE (n=6); DMRT- Duncan multiple range test.
Dissimilar letter in the row show significant difference (* Significant at $p<0.05$)

Table 3: Polyphenolics and antioxidant activity in Red delicious in different locations of Uttarakhand, (West Himalaya)

	Chaubatiya	Shitlakhet	Khbarar	Satbunga	Mukhwa	Naugown
Total phenol	2.00±0.21^{c}	2.61±0.40^{b}	1.57±0.90^{e}	1.42±0.52^{f}	2.71±0.29^{a}	1.66±1.24^{d}
Total tannin	22.53±13.91^{b}	28.63±8.49^{a}	12.05±0.82^{f}	21.54±3.92^{c}	15.12±3.28^{e}	20.67±2.94^{d}
Total flavonol	0.29±0.12^{cd}	0.31±0.08^{c}	1.00±0.32^{b}	0.32±0.19^{c}	0.24±0.20^{d}	1.08±0.62^{a}
ABTS assay	8.57±2.18^{c}	23.00±7.48^{b}	1.63±0.04^{e}	27.98±9.20^{a}	7.84±5.05^{d}	1.64±0.80^{de}
FRAP assay	8.28±5.06^{e}	13.06±0.77^{c}	13.12±7.67^{c}	18.18±7.29^{a}	10.30±0.32^{d}	14.12±7.59^{b}
DPPH assay	6.78±5.13^{e}	12.24±5.60^{b}	16.71±9.07^{a}	11.90±1.57^{c}	7.54±5.49^{d}	12.17±3.94^{b}
Carbohydrate content	1.82±0.55^{f}	5.13±0.73^{c}	5.18±0.55^{b}	5.54±0.83^{a}	2.99±0.29^{e}	5.04±0.45^{d}
Protein content	4.41±0.48^{f}	4.73±0.90^{d}	5.11±0.72^{c}	5.51±1.26^{b}	5.99±0.62^{a}	4.48±0.70^{e}

Data are presented as the mean ± SE (n=6); DMRT- Duncan multiple range test.
Dissimilar letter in the row show significant difference (* Significant at $p<0.05$)

Table 4: Relationship between polyphenolic content and antioxidant activity across altitude

	Altitude	Total phenol	Total tannin	Total flavonol	ABTS assay	FRAP assay	DPPH assay	Carbohydrate	Protein
Altitude	1.000								
Total phenol	0.595*	1.000							
Total tannin	-0.041	0.200	1.000						
Total flavonol	-0.389	-0.328	-0.481	1.000					
ABTS assay	0.261	-0.010	0.637*	-0.727	1.000				
FRAP assay	0.129	0.224	-0.066	0.068	0.017	1.000			
DPPH assay	0.297	0.219	-0.109	0.295	0.026	0.468	1.000		
Carbohydrate	-0.249	-0.350	-0.136	0.265	0.277	0.244	0.334	1.000	
Protein	0.685*	0.655*	-0.106	-0.074	0.088	0.351	0.600*	0.113	1.000

*Significant at $p<0.05$

Discussion

The present study, demonstrated a marked intraspecific variability in the concentration of polyphenols in selected apple cultivars. Total phenolics, flavonol, tannins, carbohydrate, protein and antioxidant activities varied considerably among the growing locations. Among different locations, both the cultivars showed higher phenolics, tannins and nutrient contents in Mukhwa, however, only Benoni cultivar showed higher antioxidant activity in Khbarar, and Red delicious in Satbunga. The results of this study are in agreement with a number of earlier studies, which showed that apples possessed strong antioxidant capacity but the amount may vary between the cultivars (Wolf et al., 2003; McGhie et al., 2005). Also, it has been reported that various factors such as microclimatic condition of the growing sites, type of cultivars, agronomic practices, etc. influenced the chemical content in the fruits. These chemicals content not only increases the quality of fruit but also has a major impact on shelf life and susceptibility to diseases (Khanizadesh et al., 2007). Glevitzky et al (2008) reported that higher amount of

antioxidant phytochemicals and activity act advantageously on increasing shelf life of fruits as well as quality of apples and their products in appearance, flavor and nutritional properties (Zardo et al., 2009).

Variations in concentration and composition of phenolic compounds among the cultivars are also reported (Alonso-Salces et al., 2004; Markowski and Plocharski, 2006). This hold true in the present study, where phenolic and antioxidant activity varied significantly among the locations and cultivars. Mukhwa exhibited best for harnessing carbohydrate, protein, phenolics while Satbunga and Khbarar showed potential for antioxidant activities. Reports indicated that genetic background, developmental stage and environmental factors such as nutrient availability, temperature and particularly light, influences the synthesis of polyphenols (Saure 1990). Treutter, (2001) reported that sunlight induce many enzymes involved in flavonoids synthesis. Similarly, Mc Ghie et al. (2005) also reported that impact of geographical location and altitude affected the concentration of phenolics and antioxidant activity in the fruit. Presence of higher amount of phenolic content in the fruits collected from Mukhwa location might be due to the fact that this location is more suitable for apple growing as it has optimum altitude (2780 m), open and sunny site, and may fulfill complete requirements as compared to other geographical location. Reports are also available which showed that cultivars grown on cooler agro climatic region have higher phenolic and antioxidant compounds.

Conclusion

The present study concluded that the altitude is one of the major factors responsible for accumulations of phenolic content and antioxidant activity as revealed in the present study. This suggest that plantation of these cultivars should be promoted on the higher elevation. As such, apples is widely been produced in Uttarakhand but due to poor transportation, distance from road head and other natural calamities in the region halt the full potential of the apples. As these cultivars have optimum level of phytochemicals, therefore, development of some value added products such as apple juice, jellies, jam, sauces, canned apple, beverages, etc. may be promoted to harness the full potential. The results of this study also suggest that regular consumption of apple can maximize the dietary intake of phenolic-antioxidant compounds that may have health benefits.

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