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TOTAL PHENOLIC, CONDENSED TANNINS, ASCORBIC ACID CONTENTS AND FREE RADICAL SCAVENGING ACTIVITY IN SOME OF THE UNDERUTILIZED HORTICULTURAL CROPS FROM NORTH-WESTERN INDIAN HIMALAYAS

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ABSTRACT
Mature fruits of *F. roxburghii* and *F. daltoniana,* areal bulbils of *D. kumaunensis* and roots of *P. acinosa* were collected from Uttarakhand, India and were evaluated for their total phenolics, condensed tannins, ascorbic acid (Vita C) and Free radical scavenging activities against DPPH$^{\bullet}$, ABTS$^{\bullet+}$ and Hydrogen peroxide (H_2O_2) free radicals. Total phenolics varied between 9.24-16.92 mg TA/g, catechins between 1.31-5.03 mg/g, ascorbic acid between 35.80-63.69 mg/100g. Methanolic extracts of *F. roxburghii* showed maximum percent scavenging activities on DPPH and ABTS radical while H_2O_2 scavenging activity was observed highest in *D. kumaunensis.* IC_{50} values for radical scavenging assays of methanolic extracts were calculated and found that in terms of antioxidant activities, *F. roxburghii* showed maximum followed by *D. kumaunensis* and minimum was by *P. acinosa*. Multifactorial comparisons using principal component analysis (PCA) was done and found that *F. daltoniana* possessed higher ascorbic acid content, while *F. roxburghii* possessed higher total phenolics, condensed tannin and free radical scavenging activities then others studied plant samples. Results of the finding provides evidence that the crude methanolic extract of the studied underutilized horticultural crops are valuable source of natural antioxidant, which can be applied in both healthy medicine and food industry.

INTRODUCTION

Polyphenolic compounds occur virtually in all plant parts, particularly the photosynthesizing plant cells, and are an integral part of both human and animal diets (Bravo, 1998). Consequently, there is an increasing interest in food polyphenolic compounds, due to their possible beneficial roles in human health as antioxidants, in the prevention of cancer, cardiovascular diseases and of many other pathological disorders, such as gastric and duodenal ulcers, allergies, vascular fragility, viral and bacterial infections (Huang *et al*., 1992; Bravo, 1998). Polyphenols from fruits and vegetables have been reported to be the most potent nutraceuticals: therapeutic plant-derived chemicals (Bravo, 1998). Condensed tannins are a group of water soluble phenolic compounds with molecular weight ranging between 0.5-3.0 KDa. Positive correlation between total phenolics, condensed tannins and antioxidant properties were observed by various workers (Zhiping *et al.,* 2011; Kunyanga *et al.,* 2011).

In recent decades, wild horticultural crops have become an important source of income for local people, due to their high nutritional value and special aroma. The fruits of *Ficus roxburghii carica* had high phenolics and used to protect oxidation of lipoproteins and produced significant increase in plasma antioxidant capacity after consumption (Vinson *et al.,* 2005). North western Himalayan regions are rich in plant diversity and also recorded with very large number of non-traditional or underutilized horticultural crops with high medicinal and nutritive value which has not yet been fully exploited due to lack of awareness among the farming community. These fruits and vegetables contained various kinds of anti-oxidant compounds, *viz*., flavonoids, phenolics, carotenoids and vitamins, which are considered to be beneficial for human health (Prior *et al.,* 2003; Rangkadilok *et al.,* 2007).

Different plant parts of timul (*Ficus roxburghii)* and Wild strawberry (*Fragaria daltoniana),* areal bulbils of gethi (*Dioscoria kumaunensis* kunth) and roots of Himalayan pokeberry (*Phytolacca acinosa)* are traditionally consumed by local people of north western Himalayan states of India**.** These crops are very rich in antioxidant but they have not been studied for antioxidant properties. Therefore, the phytochemicals (phenolic acids, condensed tannins, vitamin C) and free radical scavenging activity of methanolic extracts from *F. daltoniana* and *F. roxburghii* fruits, *D. kumaunensis* areal bulbils and *P. acinosa* roots were evaluated.

MATERIALS AND METHODS

Samples

Mature fruits of timul (*Ficus roxburghii)* and wild strawberry (*Fragaria daltoniana),* areal bulbils of gethi (*Dioscoria kumaunensis* kunth) and roots of himalayan pokeberry (*Phytolacca acinosa)* were collected from Uttarakhand regions of north western Himalaya of India. These fruits are identified by Local taxonomist and herbariums are kept at Vivekananda Institute of Hill Agriculture (Indian Council of Agricultural Research), Almora, Uttarakhand. After collection the plant samples were cut down into small pieces, air dried at 50ºC, powdered and stored at room temperature in desiccator before analysis.

Preparation of extracts

A fine dried plant powder (5g) was continuously extracted with 500mL of methanol at 30ºC (200 rpm) for 24h and filtered with Whatman Grade No. 1 Filter Paper. The methanolic extract was then evaporated at 40ºC to dryness, redissolved in methanol to a concentration of 10 mg/mL and stored at 4ºC for further use. The plant extracts of different concentrations (0.5, 1.0 1.5 and 2 mg/mL) were prepared by using distilled methanol and kept for further analysis. Methanolic extract of above plant materials were taken separately to measure Vitamin C, free radical scavenging activity.

A Fine dried plant powder (100 mg) of each samples were dissolved in 20mL of pre-boiled double distilled water, kept in an orbital shaker at 60ºC for 20 min and allowed to cool at ambient temperature and finally filtered through Whatman Grade No. 1 Filter Paper. The filtered aqueous extract of above plant materials were taken for analysis of total polyphenols and condensed tannins.

Determination of total phenolic content (TPC)

The total phenolic compounds were determined by Folin-Ciocalteau reagent (Singleton and Rossi, 1965). To the freshly prepared aqueous extract (0.1 mL), 0.9 mL distilled water, 0.5mL Folin-Ciocalteau reagent and 2.5 mL of sodium carbonate solution were added sequentially and the final solution was mixed thoroughly in vortex shaker. The reaction was kept for 40 min at 30ºC, after which the absorbance was read at 725nm. TPC was calculated from standard calibration curve based on tannic acid.

Determination of condensed tannins (CT)

Condensed tannins were estimated using the method of Sun *et al*. (1998) with some modifications. To the freshly prepared aqueous extract (0.1 mL), 0.9 mL methanol, 2.5 mL of 1% vanillin reagent and 2.5 mL of 9M HCl was added. The solution was mixed thoroughly and absorbance at 500 nm was recorded after 20 min of incubation at 30ºC. Condensed tannins content was calculated from the standard calibration curve based on catechins.

Determination of ascorbic acid (ASA)

Ascorbic acid was determined according to the volumetric method (Thimmaiah, 1999). Ten milliliter of 4% oxalic acid was added to standard solution of vitamin C (100μg/ml) and the resulting solution was titrated against 2, 6-dichloroindophenol dye until a pink colour end point was obtained and the titer value was noted as V_1. Again, dried methanolic extract of each samples (100 mg) were extracted with 4% oxalic acid and volume was made to 100 mL. The filtered extract (5 mL) was mixed with 10 mL of 4% oxalic acid and titrated against 2, 6-dichloroindophenol dye until a pink colour end point was obtained and the titer value was noted as V_2. Ascorbic acid content was calculated based on the following equation: Amount of ascorbic acid (mg/100 g sample) = $[(0.5 \text{ mg} \times V_2 \times 100 \text{ mL}) / (V_1 \times 15 \text{ mL} \times \text{Wt. of samples})] \times 100$, where V_1 is and V_2 were the volume of the dye used to titrate vitamin C and sample extract respectively. The result was expressed as mg ascorbic acid /100g dry weight (DW) of the plant material.

Determination of scavenging effects on DPPH$^\bullet$ radicals

The DPPH assay was done by measuring the decrease in absorbance of methanolic DPPH solution at 515 nm in the presence of the extract (Brand-Williams *et al.,* 1995) with some modifications. The stock solution was prepared by dissolving 24 mg of DPPH with 100mL methanol and stored at -20ºC and the working solution was obtained by mixing 10mL stock solution with 45mL methanol to get an absorbance of 1.17 $\pm$ 0.02 units at 515 nm. Plant extracts (150 μL) of different concentrations (0.1, 0.25 0.5, 1.0 1.5 and 2 mg/mL) were allowed to react with 2850 μL of DPPH working solution for 24h in the dark after which the absorbance was read at 515 nm. Vitamin C and Trolox were employed as a reference and the radical scavenging activity was calculated as a percentage of DPPH$^\bullet$ discolouration by the equation: DPPH$^\bullet$ radical scavenging (%) = $[(A_{control} - A_{sample})/ (A_{control})] \times 100$, where A_{sample} is the absorbance of the solution recorded during addition of extract/reference at a particular level, and $A_{control}$ is the absorbance of the DPPH solution without addition of extract.

Determination of scavenging effect on ABTS$^{\bullet+}$ radicals

The ABTS assay was done by measuring the decrease in absorbance of methanolic ABTS solution at 745 nm in the presence of the extract (Arnao *et al.,* 2001). The stock solutions 7.0 mM ABTS and 2.3 mM ammonium persulfate were prepared and the working solution was prepared by mixing two stock solutions in equal quantities and allowing them to react for 12h at room temperature in the dark. The solution was then diluted by mixing 1mL ABTS solution with 3mL methanol to obtain an absorbance of 0.9 $\pm$ 0.02 units at 745nm. Fruit extracts (200μL) of different concentrations (0.25, 0.50, 0.75 and 1.00mg/mL) were allowed to react with 2000μL of the freshly prepared ABTS solution for 30min in dark condition and absorbance was taken at 745 nm.Vitamin C and Trolox were employed as a reference and the percentage inhibition was calculated by the equation: ABTS$^{\bullet+}$ radical scavenging (%) = $[(A_{control} - A_{sample})/ (A_{control})] \times 100$, where A_{sample} is the absorbance of the solution recorded during addition of extract/reference at a particular level and $A_{control}$ is the absorbance of the ABTS solution without addition of extract.

Determination of scavenging effect on hydrogen peroxide

The ability of the plant extracts to scavenge hydrogen peroxide was measured spectrophotometrically (Ebrahimzadeh *et al.,* 2009). A solution of hydrogen peroxide (40mM) was prepared in phosphate buffer (pH 7.4). The extracts (0.5, 1.0 1.5 and 2.0 mg/mL) were added to a hydrogen peroxide solution (0.6mL, 40 mM) and after 10 min the absorbance was read at 230nm against a blank solution containing phosphate buffer

without hydrogen peroxide. The percentage of hydrogen peroxide scavenged by the extracts and standard was calculated according to the following equation: % scavenged (H_2O_2) = [($A_{control}$ – A_{sample})/ ($A_{control}$)] × 100, where A_{sample} is the absorbance of the solution recorded during addition of extract/ reference at a particular level and $A_{control}$ is the absorbance of the hydrogen peroxide solution without addition of extract.

Statistical analysis

The statistical analyses were performed using the statistical package SPSS (Statistical Package for Social Science, SPSS Inc., Chicago, IL). Analyses of variance were performed by ANOVA and significance of each group was verified with one-way analysis of variance followed by Duncan's multiple range test ($p < 0.05$). The 50% inhibitory concentration (IC_{50}) was calculated according to Concentration-Effect regression line. For multifactorial comparison, principal component analyses (PCA) was used to display the correlations between the various antioxidant and related parameters and their relationship with different underutilized plant species. Multifactorial analysis was carried out using the using the XLStat-Pro 7.5 (Addinsoft, New York, USA) software.

RESULTS AND DISCUSSION

Total phenolics, condensed tannin and ascorbic acid

The amount of total phenolics content (TPC), condensed tannins (CT) and ascorbic acid (ASA) of plant samples were shown in Table 1. The TPC was determined from the regression equation of the calibration curve obtained from tannic acid ($y = 0.0210x$, $r > 0.99$). The result showed that the *F. roxburghii* possess the highest levels of TPC (16.93 ± 0.494 mg /g), while the lowest amount of TPC was obtained in *P. acinosa* (9.25 ± 0.197 mg /g). TPC was found highest in *F. roxburghii* which is comparable to those of the leaf extract of *F. roxburghii* species (Yin-Xian *et al.,* 2011). The key role of phenolic compounds as scavengers of free radicals is emphasized by several workers (Smirnoff and Cumbes, 1989; Komali *et al.,* 1999).

The total CT content was assayed by vanillin-HCl colorimetric assay from the regression equation of calibration curve ($y = 0.0022x + 0.013536$; $r = 0.99$) and expressed in catechin equivalents. The result showed that *Ficus roxburghii* possessed the highest levels of CT (5.03 ± 0.012 mg/g), while the lowest amounts of CT was found in *P. acinosa* root (1.13 ± 0.014 mg /g). TPC and CT was recorded in decreased order as *F. roxburghii* > *D. kumaunensis* > *F. daltoniana* > *P. acinosa*. Ascorbic acid (ASA) content was recorded highest in *F. daltoniana* (63.69 ± 0.603 mg/100 g), while lowest ASA was obtained in *P. acinosa* (35.80 ± 0.421 mg/100g). Content of ASA showed a decreasing order of *F. daltoniana* > *F. roxburghii* > *D. kumaunensis* > *P. acinosa*. Phenols and polyphenolic compounds such as condensed tannins are widely found in food products derived from plant sources and they have been shown to possess significant antioxidant activities (Nabavi *et al.,* 2009).

Radical scavenging activity

The abilities for each plant sample extract concentration to scavenge DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals are shown in Fig.1 and 2, respectively.

The results showed that these extracts significantly inhibited the activities of DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ radicals in dose-dependent manner ($p < 0.05$). The percent scavenging activities of methanolic extracts of *F. roxburghii, P. acinosa, F. daltoniana and D. kumaunensis* on DPPH$^{\bullet}$ radical ranged from 72.30 to 100, 35.23 to 62.80, 21.5 to 91.50, and 61.90 to 99.20, respectively. However, at 100 ppm Trolox and L-ascorbic acid showed excellent scavenging activities of 74.19% and 76.05%, respectively. DPPH$^{\bullet}$ is a stable nitrogen-centered free radical which get reduced either by the process of hydrogen or electron donation. Substances which are able to perform this reaction can be considered as antioxidants and therefore radical scavengers.

Table 1: Total Polyphenols, Condensed tannins, Ascorbic acid contents of Underutilized Horticultural Crops

Crop Name	Total Polyphenols [A]	Condensed tannins [B]	Ascorbic acid [C]
F. roxburghii	16.92 ± 0.494a	5.03 ± 0.012a	37.82 ± 0.766b
P. acinosa	9.24 ± 0.197d	1.31 ± 0.014d	35.62 ± 0.421c
F. daltoniana	11.13 ± 0.144c	2.57 ± 0.027c	63.69 ± 0.603a
D. kumaunensis	14.34 ± 0.196b	3.25 ± 0.092b	35.89 ± 0.683bc

Values are expressed as means + S.E. of triplicate measurements. Values with different letters indicate significant difference ($P < 0.05$); [A]Total Polyphenols expressed in mg Tannic acid equivalents /g dry weight (DW); [B]Condenced Tannins content expressed in mg Catechins /g dry weight (DW); [C]Ascorbic acid expressed in mg ascorbic acid /100g dry weight (DW).

Table 2: Comparison of the IC_{50} values for radical scavenging assays of methanolic extracts of the Underutilized Horticultural Crop as well as Different Standard

Crop Name	IC_{50} values of each free radical scavenging assay (mg/mL)		
	DPPH[a]	ABTS[b]	HPSA[c]
F. roxburghii	0.19	0.45	0.95
P. acinosa	1.04	1.53	1.6
F. daltoniana	1.24	0.58	1.2
D. kumaunensis	0.3	0.7	0.46
Trolox	0.089	0.05	0.45
Vita C	0.073	0.26	0.39

[a]IC_{50} (mg/mL): eûective concentration at which 50% of DPPH$^{\bullet}$ radicals are scavenged; [b]IC_{50} (mg/mL): eûective concentration at which 50% of ABTS$^{\bullet+}$ radicals are scavenged; [c]IC_{50} (mg/mL): eûective concentration at which 50% of H_2O_2 radicals are scavenged

ABTS$^{\bullet+}$ radicals scavenging effect of the methanolic extracts increased with increase in concentration in a dose-dependent manner and almost complete inhibition of ABTS$^{\bullet+}$ radicals (98.91%) was observed for 1.0 mg/mL of fruit extract of *F. roxburghii.* The scavenging activities of methanolic extracts of *F. roxburghii, P. acinosa, F. daltoniana and D. kumaunensis* on ABTS$^{\bullet+}$ radical ranged from 24.13 to 98.91%, 11.70 to 37.39%, 34.14 to 92.40% and 24.13 to 94.57%, respectively (Fig. 2). Comparable ABTS$^{\bullet+}$ and DPPH$^{\bullet}$ free radical scavenging activities have previously been documented for the ethanol extract of *F. roxburghii carica* L. fruits (Ao *et al.,* 2008; Yang *et al.,* 2009) and methanol extract of *P. Americana* (Nabavi *et al.,* 2009).

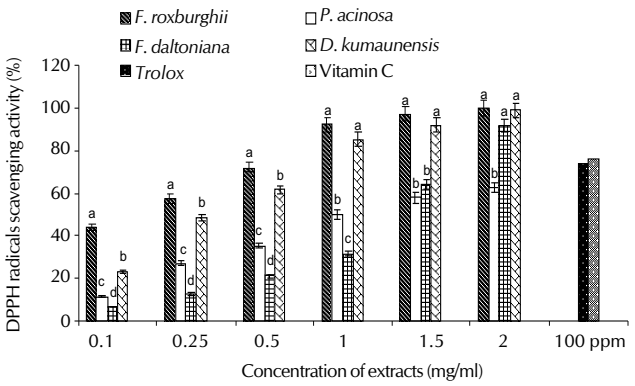

Figure 1: Scavenging activity (%) on DPPH• radicals of four crops, *F. roxburghii, P. acinosa, F. daltoniana, D. kumaunensis*. Each value is expressed as mean ± standard error (n = 3). Values with different letters (a, b, c, d, e) were significantly different (P < 0.05, ANOVA)

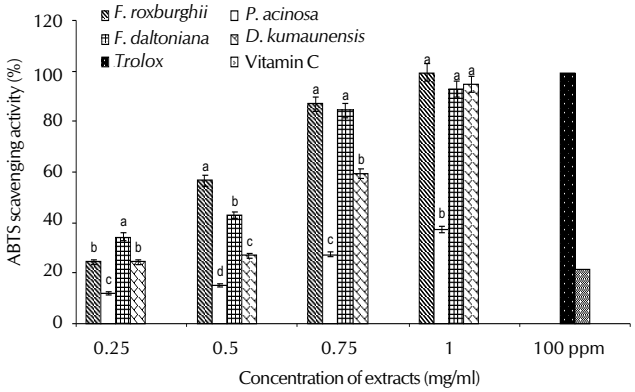

Figure 2: Scavenging activity (%) on ABTS•+ radicals of four crops, *F. roxburghii, P. acinosa, F. daltoniana , D. kumaunensis*. Each value is expressed as mean + standard error (n = 3). Values with different letters (a, b, c, d, e) were significantly different (P < 0.05, ANOVA)

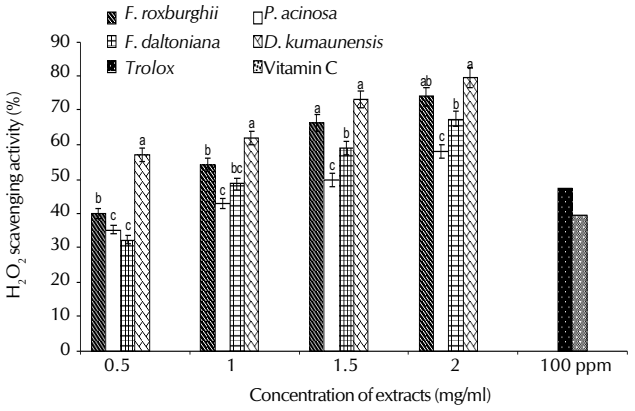

Figure 3: Hydrogen Peroxide (H_2O_2) Scavenging Activity (%) of four crops, *F. roxburghii, P. acinosa, F. daltoniana, D. kumaunensis*. Each value is expressed as mean + standard error (n = 3). Values with different letters (a, b, c, d, e) were significantly different (P < 0.05, ANOVA)

H_2O_2 scavenging activity of methanolic extracts increased with increase in concentration and for all the crops it was excellent at 2.0 mg/mL. H_2O_2 scavenging activity was observed in a decreasing order as Vitamin C > Trolox > *D. kumaunensis* > *F. roxburghii* > *F. daltoniana* > *P. acinosa*. The scavenging activities of methanolic extracts of *D. kumaunensis, F. roxburghii, F. daltoniana and P. acinosa* on hydrogen peroxide radical ranged from 69.46 to 92.89%, 57.32 to 79.92%, 57.32 to 79.50%, 40.17 to 74.06%, respectively (Fig. 3). However, at 100ppm Trolox and L-ascorbic acid showed excellent scavenging activities of 74.19% and 76.05% respectively. It is well established that hydrogen peroxide is not dangerous as it is, but it can sometimes cause cytotoxicity by giving rise to hydroxyl radicals in the cell. Thus, removing H_2O_2 is very important throughout food systems and scavenging of H_2O_2 by plant extracts may be attributed by phenolics, which can donate electrons to H_2O_2, thus neutralizing it to water (Ebrahimzadeh *et al.,* 2009).

The IC_{50} value for each fruit extract is defined as the concentration of extract causing 50% inhibition of free radical ware calculated, since IC_{50} is a measure of inhibitory concentration, a lower IC_{50} value would reflect greater scavenging activity of the sample.

The scavenging ability on DPPH and ABTS radicals was higher in methanolic extract of *F. roxburghii* whereas Hydrogen Peroxide (H_2O_2) scavenging ability was found maximum in methanolic extract of *D. kumaunensis* followed by *F. roxburghii*.

Multivariate analysis

Multifactorial comparisons using principal component analysis clearly indicated correlation between various antioxidant and related parameters and their relationship in different under-utilized plant species. The principal component analysis (PCA) and their correlation were shown in Fig. 4. Among the data first factor F1 represents 46.65 per cent of variability, while the second factor F2 represents 43.50 per cent of variability. Almost all parameters were occupied on the right side of the biplot and among the parameters the *ASA*-ascorbic acid, *ABTS*- $ABTS^{\bullet+}$ radicals scavenging activity (0.5, 1.0, 1.5 and 2.0 mg/mL), DPPH- $DPPH^{\bullet}$ radicals scavenging activity (0.5, 1.0, 1.5 and 2.0 mg/mL), CT- condensed tannin, TPP- total polyphenol, *HPSA*- hydrogen peroxide scavenging activity (0.5, 1.0, 1.5 and 2.0 mg/mL).

This suggested that condensed tannin, total polyphenol, HPSA, DPPH and ABTS showed positive correlation with one another. PCA revealed that four underutilized plant species were found in three separate positions in biplots (Fig. 4). The *F. daltoniana*, *F. roxburghii*, *D. kumaunensis* were observed at right side of biplot showing high positive loadings to F1, while *P. acinosa* was observed at left side of biplot showing high negative loadings to F2. *F. roxburghii* and *D. kumaunensis* showed higher condensed tannin, total polyphenols, DPPH, HPSA than other crop. *P. acinosa* was observed with significantly lesser antioxidant and related parameters.

It may be thus concluded that for the first time, antioxidant activities of the edible plant parts of *D. kumaunensis*, *F. roxburghii*, *P. acinosa* and *F. daltoniana* along with their total phenolics and condensed tannins were evaluated. Our results showed that the edible plant parts possessed abundant free radical scavenging activity at various concentrations and the methanol extracts of *F. roxburghii* showed considerable higher antioxidant potential compared with other studied plant

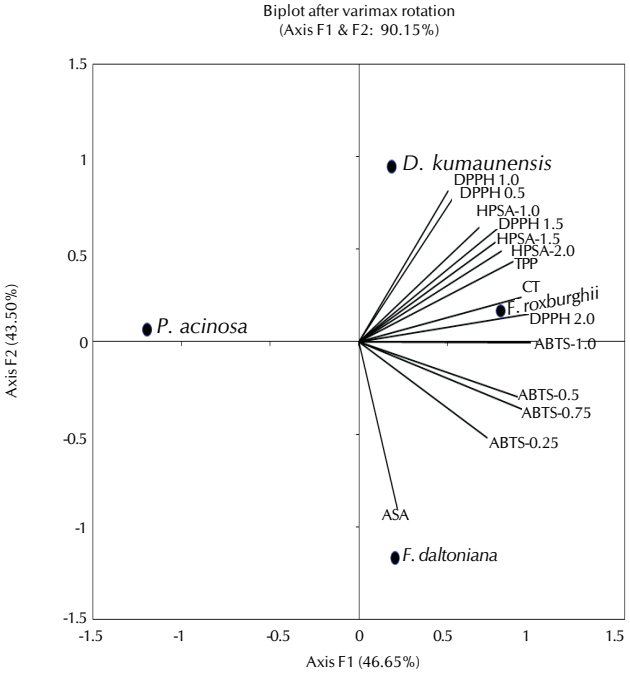

Figure 4: Multifactorial comparison of total phenolic (TPP), condensed tannins (CT), ascorbic acid (ASA) contents and free radical scavenging activity against DPPH, ABTS, H_2O_2 at four concentrations (0.5, 1.0, 1.5 and 2.0 mg/mL), obtained from underutilized plant species using principal component analysis (PCA)

materials. In terms of antioxidant capacity and phenolic compounds the following trend was observed *F. roxburghii* > *D. kumaunensis* > *F. daltoniana* > *P. acinosa*. Positive correlation between higher antioxidant activity and larger amount of total phenolics was found in all the four plant extract. Though other antioxidants were probably present in these crops, the phenolic compounds could make a significant contribution to the antioxidant activities. Further studies on chemical characterization of antioxidative components, which may have pharmacological or dietetic applications, may yield some more information regarding certain phytochemicals present in these crops in future.

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