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Genotypes, geographical regions and solvents dependent antioxidant activity of *Rumex patientia* L. in cold desert of trans-Himalaya Ladakh, India

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Abstract

Indus, Nubra and Suru are three geographically different Indian trans-Himalayan regions. Ten plants from each region were studied to find out the variations in total phenolic content (TPC), total antioxidant capacity (TAC) in Rumex leaves. The Folin-Ciocalteu reagent assay was used to determine the TPC, 1,1-diphenyl-2-picrylhydrazyl (DPPH) and 2,2-azino-bis-3-ethylbenzothiazoline-6-sulfonic acid (ABTS) for free radical scavenging activity. Total antioxidant power was determined by ferric reducing/antioxidant power (FRAP) assay. Among the solvents used, 70% acetone has higher TPC (803.43 mg GAE /100 g FW), IC50 DPPH (0.99 mg mL^{-1}), ABTS (1.37 mg mL^{-1}) and FRAP (0.261 mg mL^{-1}). Genotypes belonging to Indus valley show highest TPC (797.59 mg GAE /100 g FW), DPPH (1.06 mg mL^{-1}), ABTS (1.54 mg mL^{-1}) and FRAP (0.248 mg mL^{-1}) among the regions studied. Cluster analysis demonstrated that genotypes belong to Nubra valley shaping distinct cluster. The results of principle component analysis were comparable to cluster analysis. The TPC and TAC content were influenced by the environment of studied regions, genetic background of population and the solvent used. The harsh climatic conditions of Indian trans-Himalaya cold desert lead to the scarcity of fresh fruits and vegetables. Therefore, Rumex leaves could be a potential food for high altitude cold desert with high value nutritional supplement provide great health benefits in stressful condition of Ladakh.

Keywords: Functional food, high altitude, inhibitory concentration, leaves, *Rumex patientia* L., total antioxidant capacity.
Abbreviations: ABTS_ 2,2'-azino-bis-(3-ethylbenzothiazoline-6-sulfonic acid); ANOVA_ analysis of variance; DPPH_ 1,1-diphenyl-2-picrylhydrazyl; FRAP_Ferric reducing/antioxidant power assay; GAE_ Gallic acid equivalent; GLM_ Generalized linear model; IC_{50} _Inhibitory concentration; pH_ Potential of hydrogen ion; PCA_ Principal component analysis; RBD_Randomized block design; TAC_ Total antioxidant capacity; TPC_ Total polyphenol content.

Introduction

Rumex patientia, commonly known as Patience doc which belongs to the family polygonaceae, is a perennial, herbaceous, glabrous, naturally growing plant and is widely distributed in the North, Northeast and Northwest provinces of India. More than 250 species of *Rumex* have been found worldwide. In Ladakh, it is popularly known as Soma. It thrives in acidic and sour soils. Owing to its strong adaptability, the plant can grow in different environmental conditions. In Ladakh, *Rumex patientia* is the most dominant species growing abundantly in the wild. It can tolerate abiotic stress and grows successfully in both arid and water logging conditions and withstands extreme temperatures (-35°C to + 35°C) (Chaurasia et al., 2007). This plant species has immense medicinal and nutraceutical properties (Rao et al., 2011). The plant has several bioactive compounds like, anthraquinones, napthalenes, flavonoids and other polyphenols, carotenoids, tocopherols and ascorbic acid (Mei et al., 2009; Zhang et al., 2009; Alfawaz et al., 2006; Filho et al., 2008). Only a few reports have been published on antioxidant and antimicrobial activity of *Rumex* species (Guerra et al., 2008; Mostafa et al., 2011). The chronic dose of *Rumex patientia* seeds improved the retention and recall capacity in passive avoidance test in STZ-diabetic rats (Roghani et al., 2009). Oxygen radicals have been implicated as a risk factor in several diseases, ageing etc. (Halliwell and Gutteridge, 1999). Thus increasing the antioxidant intake in the human diet is considered crucial and one can achieve this by enriching food with antioxidants. As some synthetic antioxidants may require high manufacturing costs but show lower efficiency than natural antioxidants, there is a need to identify natural and possibly more economical and effective antioxidants with potential to be incorporated into food. Polyphenols are an important class of defense antioxidant. Phenolics have received considerable attention because of their physiological functions, including antioxidant, antimutagenic and antitumour activities. Rumex is a wild edible plant used as leafy vegetable by native people of trans-Himalaya. In Tibetan traditional medicine system Rumex is used by local Amchis (local traditional practitioner) for treating diseases like headache, piles, itching of foot, diabetes, stomach problems and angina (Chaursia et al., 2007). However, quantification of the health-promoting antioxidant component of the species, especially the population growing in trans-Himalaya, has not been investigated so far. This is the first hand information on effect of geographical regions, genotypes and solvents on total phenolic content (TPC) and antioxidant capacity of *Rumex patientia*. Extraction of phenolics and determination of antioxidant capacity of leaves using various solvent extracts have also not been investigated. It is a fact that TPC and antioxidant content depends on various factors including variety, location and environmental conditions (Klepacka et

al., 2011; Yu et al., 2011). Importance of distribution on TPC and antioxidant activity is vital to recognize the potential health benefits of Rumex, spinach of high-altitude cold desert (Singh et al., 2013). Therefore, the objective of the present study was to investigate TPC and antioxidant capacity of various solvent extracts from leaves of Rumex of various geographical locations for their potential as a natural source of antioxidant.

Results and Discussion

Total phenolic contents in the Patience doc leaf extract

Rumex leaves were found to be rich in TPC ranging from 323.10 to 983.59 mg GAE/100 g among population studied in different geographical regions (Table 2). At regional level, the lowest TPC (714.22 mg GAE/100g FW) was observed in Nubra valley, with the highest in Indus valley (797.59 mg GAE/100g FW). The overall mean TPC was 746.14 mg GAE/100g FW. These results may be due to the interaction among the environmental conditions because low temperature may increase production of phenolics by enhancing the synthesis of phenylalanine ammonia lyase (PAL) in plants, while high altitude and long sunlight hours with higher UV radiation positively affect the activity of phenolics synthase (Kishore et al.,2010). However, small amount of precipitation could enhance the defence system of plant against stress leading to an increase in phenolic content (Suzuki et al., 2005). Among the three solvents, 70% acetone (803.43 mg GAE/100g FW) had higher TPC, while the 50% acidic methanol had low TPC (665.51 mg GAE/100g FW). Similar results were reported by Korekar et al., (2011b) in *Prunus armeniaca* L. kernel 79.6 mg GAE/100g DW while Kchaou et al., (2013) reported, the level of phenolic compound to be in the range 199.43 to 57.48 mg/100g FW. Among all the extracts, 70% acetone was found to be the most efficient solvent for extracting phenolic compounds as compared to other solvents. The recovery of phenolic content in different samples is influenced by the polarity of extracting solvents and solubility of the compound in the solvent used for extraction (Alothman et al., 2009; Sulaiman et al., 2011).

Evaluation of antioxidant capacity by DPPH and ABTS-radical scavenging activity

The free radical-scavenging activity values were measured using DPPH and ABTS in terms of IC_{50} (mg/ml extract) of leaves (Table 2). A large range of DPPH (0.35 to 2.20 mg mL^{-1}) was observed. The large variations in IC_{50DPPH} values highlight unexploited variability among the *Rumex* genotypes. At population level, the mean IC_{50DPPH} value was 1.27 mg mL^{-1}, while the highest and lowest free radical scavenging activity was 0.35 mg mL^{-1} and 2.20 mg mL^{-1} in Suru (Table 2) respectively. The result might be due to genotypic potential of the population. Capocasa et al., (2008) reported that effect of genotype is stronger than that of cultivation conditions for TPC and antioxidant in Strawberry. Similar results have been reported by Scalzo et al., (2005), Drogoudi et al.,(2008) and Kalyonku et al., (2009). Among the three solvents used, 70% acetone has significantly lower IC_{50DPPH} (0.99 mg mL^{-1}) while 50% acidic methanol has high (1.80 mg mL^{-1}). This suggests that the 70% acetone has higher DPPH free radical scavenging activity. Similar trend was found for the ABTS radical. Many previous studies have measured the effect of different solvents in antioxidant activity using different methods. Our results are in agreement with those of previous report of Al-Farsi et al., (2005b) who reported highest antioxidant activity in acetone/H_2O extraction (70:30 v/v), whereas it was minimum in methanol/H_2O (50:50 v/v). This significant variation indicated that change in solvent polarity might significantly influence antioxidant activity. Indus region has lower IC_{50DPPH} (1.06 mg mL^{-1}) and IC_{50ABTS} (1.54 mg mL^{-1}) among the three regions (Table 2). Fruits and vegetables mainly contain antioxidant compound like vitamins A, C, E, β-carotene and polyphenolics compounds (Sies et al., 1992). The higher total content of polyphenols in the present study may be due to the cold and dry environmental conditions prevailing in trans-Himalaya which lies more than 2,500 m above mean sea level. It also lies open to high UV-radiation.

Ferric reducing antioxidant power (FRAP) assay in the Patience doc leaf extract

A high range of values was obtained for ferric reducing activity (Table 2). Among the regions, the ferric reducing activity ranged from 0.242 (Nubra) to 0.248 (Indus) mg mL^{-1} $FeSO_4.7H_2O$. At population level, the highest value 0.346 mg/ml $FeSO_4.7H_2O$ as well as the lowest 0.195 mg mL^{-1} $FeSO_4.7H_2O$ was in Indus. The solvents have shown significant difference in FRAP activity, 70% acetone has the highest (0.261 mg mL^{-1}) followed by 50% acidic methanol (0.244 mg mL^{-1}) (Table 2). Korekar et al., (2011a) found similar results in plant Seabuckthorn leaves. The order of population in terms of TAC by two different antioxidant assays was different which could be due to presence of compounds having different affinity to react with DPPH, ABTS and FRAP.

Interaction of Genotype, geographical region and solvent on TPC and TAC of Patience doc leaves

We conducted a GLM model based analysis to determine the significant effect of genotypes, regions, different solvents and their interaction on the TPC and TAC of *Rumex patientia* L. in the trans-Himalaya. We found that the geographical regions, genotypes, solvents and their interactions significantly affect the TPC and TAC in the present study (Table 3). This underlines the importance of genetic background, location of sampling and different solvents for determining health promoting compounds in *Rumex patientia* L. Significant variation among plants within a population could be because *Rumex patientia* L. is a wind pollinated species and therefore high genetic variation is expected in natural populations with different geographical conditions. We hypothesize that concentration of phenolic compounds, which are produced by plants for different functions, might be the major contributor to the antioxidant activity. Unidentified stresses and other conditions such as altitudinal variation, UV radiation, cold/heat stress, drought stress and infections may have increased the production of phenolic compounds in plant population present in different locations. Emmons and Peterson (2001) reported significant interaction effect of cultivar and location on concentration of total free phenolic contents in oat. Similar results were reported by Connor et al.,(2002) for blueberry (Vaccinium L.), who reported cultivar and location interaction for TPH and anthocyanins and cultivar × year within location interactions for AA, TPH and anthocyanins among nine genotypes.

The first two PCA's explaining the total 76.03 % of variations out of which first PC1 explained 38.988 % of variation while PC2 37.044 % (Table 4). The PCA variable loading score represented in table 5. Most of the variability in PC1 is correlated (0.912) with TPC's of 70% acetone

Table 1. Geographical distribution of *Rumex patientia* L. in trans-Himalaya (Fig 1).

Sr. no.	Valleys	Number of Samples	Latitude(°N)	Longitude(°E)	Elevation amsl(m)
1	Nubra	10 (1 to 10)	34°33'07'83"N	77°33'10.26"E	3000
			34°39'24.95"N	27°21'53.77"E	3116
2	Indus	10 (11 to 20)	34º02'32.69"N	77°40'20.69"E	3144
			34°11'43.31"N	77°20'06.39"E	3261
3	Suru	10 (21 to 30)	34°32'30.25"N	76°08'19.28"E	2765
			34°22'42.21"N	76°22'04.68"E	3288

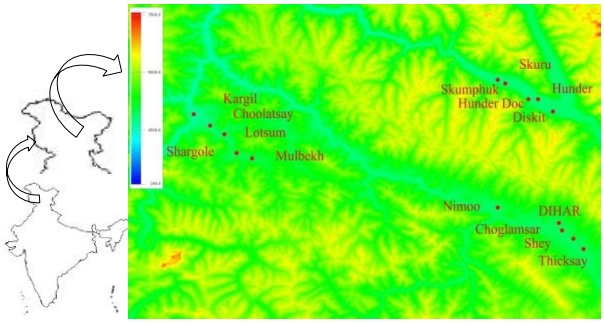

Fig 1. Location Map of sampling areas in Ladakh (Nubra valley 1 to 10 samples, Indus valley 11 to 20 and 21 to 30 from Suru valley, from each location two samples were collected).

Table 2. TPC and TAC of *Rumex patientia* L. leaves with respect to solvents and geographical locations.

Solvents	TPC (mg GAE/100g FW)	IC_{50DPPH} ($mgmL^{-1}$)	IC_{5ABTS} ($mgmL^{-}$)	FRAP ($mgmL^{-1}$)
70% Acetone	803.43±44.06^{b}	0.99±0.12^{a}	1.37±0.78^{a}	0.261±0.033^{c}
50% Acidic Methanol	665.51±26.82a	1.80±0.33^{b}	1.74±0.95^{b}	0.244±0.021^{b}
70%Aceton + 50% Acidic Methanol	769.48±41.50^{b}	1.01±0.41^{a}	1.72±0.78^{b}	0.232±0.035^{a}
Total	746.14±129.46	1.27±0.43	1.61±0.86	0.246±0.033
Valley				
Nubra	714.22±124.08^{p}	1.39±2.05^{p}	1.71±0.75^{p}	0.242±0.002^{p}
Min	348.44±29.27	0.55±0.09	0.65±0.07	0.195±0.001
Max	850.10±40.83	1.54±0.35	2.90±0.35	0.346±0.030
Indus	797.59±72.80^{q}	1.06±0.62^{p}	1.54±0.84^{p}	0.248±0.037^{p}
Min	323.10±20.14	0.43±0.02	0.48±0.08	0.195±0.005
Max	975.77±27.40	1.66±0.36	3.33±0.31	0.316±0.035
Suru	726.61±160.83^{p}	1.34±1.23^{p}	1.57±0.97^{p}	0.247±0.031^{p}
Min	398.34±36.43	0.35±0.06	0.53±0.09	0.195±0.001
Max	983.59±25.16	2.20±0.30	2.63±0.17	0.346±0.009
Total	746.14±129.46	1.27±1.43	1.61±0.86	0.246±0.033

Values represented as mean ± SD; for each column, different lowercase letters indicate significantly different at $p<0.05$, as measured by 2-sided Tukey's HSD between solvents (a, b and c) and valleys (p, q and r).

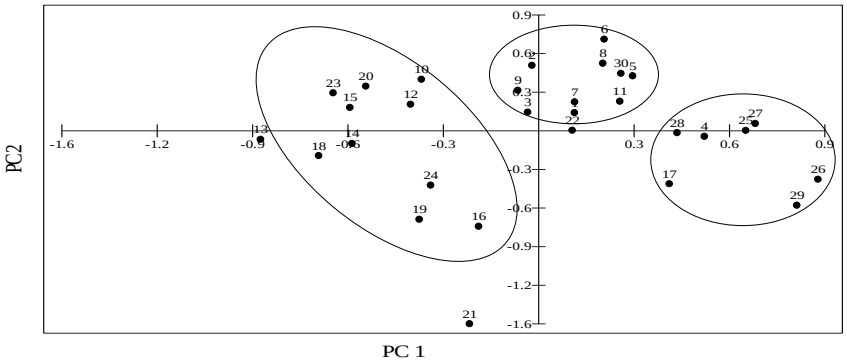

Fig 2. Principal Component analysis of biochemical data with respect to genotypes of Rumex (1 to 10 Nubra valley; 11 to 20 Indus valley and 21 to 30 Suru valley).

Table 3. Two-way ANOVA to test the effect of geographical regions, genotypes, solvents and their interaction on TPC and TAC of *Rumex patientia* L.

Source	Dependent Variable	Type III Sum of Squares	df	Mean Square	F	P ≤ 0.05
Solvents	TPC	929605.323	2	464802.662	617.524	0.000
	DPPH	38.384	2	19.192	313.590	0.000
	ABTS	7.601	2	3.801	44.935	0.000
	FRAP	39569.730	2	19784.865	4434.269	0.000
Valleys	TPC	364239.417	2	182119.709	241.959	0.000
	DPPH	5.671	2	2.836	46.332	0.000
	ABTS	1.396	2	.698	8.250	0.000
	FRAP	1896.391	2	948.196	212.514	0.000
Genotypes	TPC	232094.794	9	25788.310	34.262	0.000
	DPPH	38.299	9	4.255	69.532	0.000
	ABTS	26.594	9	2.955	34.935	0.000
	FRAP	43902.681	9	4878.076	1093.295	0.000
Solvents * Valleys	TPC	598286.417	4	149571.604	198.717	0.000
	DPPH	10.432	4	2.608	42.612	0.000
	ABTS	5.464	4	1.366	16.151	0.000
	FRAP	10999.152	4	2749.788	616.294	0.000
Solvents * Genotypes	TPC	766082.014	18	42560.112	56.544	0.000
	DPPH	85.929	18	4.774	78.002	0.000
	ABTS	44.358	18	2.464	29.135	0.000
	FRAP	21939.726	18	1218.874	273.179	0.000
Valleys * Genotypes	TPC	605718.920	18	33651.051	44.708	0.000
	DPPH	151.345	18	8.408	137.384	0.000
	ABTS	46.093	18	2.561	30.275	0.000
	FRAP	64983.870	18	3610.215	809.137	0.000
Solvents * Valleys * Genotypes	TPC	876859.251	36	24357.201	32.360	0.000
	DPPH	210.598	36	5.850	95.585	0.000
	ABTS	50.434	36	1.401	16.563	0.000
	FRAP	109021.714	36	3028.381	678.734	0.000

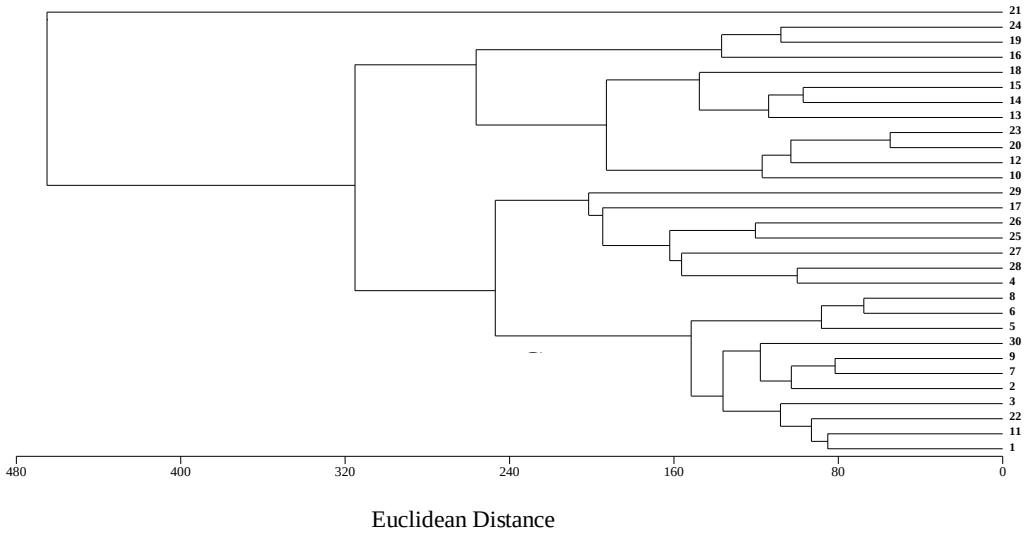

Fig 3. Dendrogram showing the genotypic relationship among 3 populations based on euclidean distances from biochemical data matrix (1 to 10 Nubra valley; 11 to 20 Indus valley and 21 to 30 Suru valley).

extraction followed by 50 % acidic methanol while PC2 strongly correlated with TPC's of 70 % acetone extract (table 5).Fig-2 shows the biochemical characters values plotted against the first two principal component variates from PCA. However, two patterns could be seen regarding the position of mean scores and euclidean distance. Firstly, biochemical traits are positioned relatively close to each other in the axis with respect to their population and secondly, genotype population forms three groups. The most closely related genotype belongs to group-1 which contains Nubra valley population, while group-2 contains Indus valley and group-3 contains Suru valley. A Dendrogram was drawn to display the biochemical relationship among different populations of Ladakh region based on euclidean distances from the biochemical data matrix. All genotypes were represented in three clusters (Fig-3). Dendrogram based on UPGMA method analysis grouped the genotype into population groups with main clusters 1, 2 and 3. Group-1 represents the genotypes of Nubra valley, group-2 represents the genotypes of Indus valley while group-3 represents the genotypes of Suru valley. The results of PCA were comparable to cluster analysis. Nubra valley, geographically restricted by high mountains, having closely related populations, climatic condition, and altitude which might be responsible for synthesis of TPC and antioxidant content that may play an important role for shaping a distinct cluster.

Table 4. PCA eigenvalues for discrimination analysis of three valleys based on different biochemicals of *Rumex patientia* L.

	PC 1	PC 2
Eigenvalues	7.039	6.688
Percentage	38.988	37.043
Cum. Percentage	38.988	76.03

Table 5. PCA variable loadings for discrimination analysis of three valleys based on different biochemicals of *Rumex patientia* L.

	PC 1	PC 2
TPCs1	-0.352	0.919
DPPHs1	0.003	-0.005
ABTSs1	-0.006	0.029
FRAPs1	-0.057	-0.049
TPCs2	-0.083	0.137
DPPHs2	-0.022	0.007
ABTSs2	-0.009	-0.016
FRAPs2	-0.025	-0.011
TPCs3	0.912	0.365
DPPHs3	-0.019	0.009
ABTSs3	0.004	-0.004
FRAPs3	0.18	-0.002

Materials and Methods

Leaf Sample collection

Thirty individual plants; ten plants from each valley separated by 200-250 m from each other; were sampled across three geographically distinct valleys (Fig. 1, Eye4 software) of Indian trans-Himalaya.

Collection sites

The altitude of collection sites ranged from 2765 m to 3336 m above mean sea level (Table 1). The mean maximum and minimum temperature of the study site was 18.9±9.5ºC and -5.8±9.8ºC, respectively while the mean maximum and minimum relative humidity were 35.54±7.3 and 25.0±3.7, respectively during 2001-2011. Mean maximum temperature during cropping season (May-September) was 28.4ºC while the minimum was 4.5ºC during the last decade (Korekar et al., 2013). Ten samples were collected from each valley as a whole plant and transplanted in agricultural fields of Defence Institute of High Altitude Research in 2011 in RBD model with 1×1m spacing. All plants had the same age and standard cultural practices were performed. The soil texture of the experimental site was silty loam with pH 7.1±0.2. Organic carbon and organic matter content were 1.2.±0.4% and 4.2±0.5%, respectively. The leaf samples were collected in July 2012. Leaves were lyophilized in a Laboratory freeze dryer (ALPHA 2-4 LD plus, Fisher Bioblock Scientific, France).

Preparation of Extract

Extracts of samples were prepared as per the method of Korekar et al. (2011a) with some modifications. Lyophilized powder of each sample (0.5 g) was extracted separately with 25 mL solvent for 18 h. The sample was centrifuged at 12,000 rpm for 20 min and the supernatant was recovered. The residue was mixed with 25 ml of the solvent and the process was repeated. TPC and antioxidant capacity were measured directly in extract fractions.

Total phenolic contents in the Patience doc leaf extract

The Folin-Ciocalteu reagent assay was used to determine the TPC (Singelton and Rossi, 1965) with minor modifications. Plates were vortexed, covered with parafilm and allowed to stand for 20 min in an Elisa reader. Absorbance at 765 nm was recorded in the Elisa reader (SpectroMax M2 e, Molecular Devices, Sunnyvale, CA, United States). TPC was expressed in gallic acid equivalents (GAE mg per 100 g). The calibration equation for gallic acid was y=0.010x-0.059 (R^2=0.998) where y is the absorbance at 765 nm and x is the concentration of gallic acid in mgL^{-1}.

Evaluation of antioxidant capacity by DPPH- radical scavenging activity

Free radical scavenging method by DPPH developed by Brand-Williams et al. (1995) was followed with minor modifications, (Korekar et al., 2011a).

Evaluation of antioxidant capacity by ABTS- radical scavenging activity

ABTS radical scavenging assay was determined using a previously reported method Re et al. (1999) with minor modifications. A 0.1 mM solution of ABTS was prepared and 4 ml of the solution was treated with 0.2 ml of the extracted sample. A control was treated with 0.2 ml of solvent as a substitute of the extract. The mixture was left to stand at room temperature for 30 min before the decrease in absorbance at 734 nm was recorded with reader (SpectroMax M2 e, Molecular Devices, Sunnyvale, CA, United States).

Ferric reducing antioxidant power (FRAP) assay in the Patience doc leaf extract

Ferric reducing antioxidant potential (FRAP) assay was conducted using the method previously described (Ikram et

al., 2009) with minor modifications (Korekar et al., 2011a). The calibration equation for $FeSO_4.7H_2O$ was y=0.0017x-0.029 (R^2=0.991) where y is the absorbance at 593 nm and x is the concentration of $FeSO_4.7H_2O$ in mg mL^{-1}.

Statistical analysis

All the experiments were performed in triplicate. Effects of geographical locations, genotypes and solvents on studied parameters were analyzed in General Linear Model (GLM). One way ANOVA with 2-sided Tukey's Honestly Significant Difference (HSD) at $p \leq 0.05$ was carried out using SPSS 17.0. Principal component analysis (PCA) was used to ordinate population means considering variance and covariance among genotypes within and among populations (kim, 1975). Average Euclidean distance was calculated for each population and the resulting distance matrix was used to construct a dendrogram using UPGMA method (Mohammadi and Prasanna 2003)

Conclusion

The influence of plant geographical locations, genetic background and solvent on TPC and TAC in *Rumex patientia* was demonstrated. Significant variation was found within and among the geographical locations, genotypes and solvents, which underline the role of genetic background and topography of regions for determining the health promoting compounds in *Rumex*. High variation in TPC, ferric reducing activity, free radical scavenging activity were observed within the 187 female plants studied. Genotypes have significant effect on the health promoting compounds studied. Among all three valleys, Indus valley shows high TPC and antioxidant activity. Cluster analysis demonstrated that geographical effect is more pronounced towards TPC and antioxidant content. PCA is comparable to cluster analysis. Results obtained in this study can be considered for selection of genotype for breeding purpose to improve health promoting compounds in the *Rumex* which may be a valuable vegetable for cold desert conditions in trans-Himalaya.

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