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Investigation of antioxidant properties of *Withania somnifera* (L.) Dunal and influence of physico-chemical properties of soil along the topographic gradients in sub-tropical region of the Indian Himalaya

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Abstract

A high value medicinal plant *W. somnifera* L. Indian Ginseng; Ashwagandha investigated for the antioxidant and influence of physico-chemical properties of soil on antioxidant along the topographical gradients in Kullu Valley, North Western Indian Himalaya. Total phenolic and flavonoid contents in methanol extracts of fruits, leaves, stems and roots were quantified and their antioxidant activities were evaluated using the *in-vitro* assays such as DPPH, ABTS and FRAP. The study revealed that fruits, leaves, stems and roots of *W. somnifera* were potent source of natural phenolics and flavonoids and possesses antioxidant activities, which were significantly affected by the changes in altitude (p 0.05). Total phenolic and flavonoid contents in different parts of *W. somnifera* showed significant correlations with soil properties. The study conclude that changes in topographical gradients particularly altitude influenced antioxidant activities of *W. somnifera* and suggest that antioxidant rich population of a desired medicinal plant should be identified for their conservation management and sustainable utilization in pharmaceutical and food industries.

Keywords: *Withania somnifera*, total phenolics, total flavonoids, *In-vitro* assays, Population, Soil properties

Introduction

Withania sominifera Dunal (Solanaceae), also known as Indian Ginseng, Ashwagandha or Winter Cherry, is a small, erect, evergreen, woody shrub that grows up to a height of one meter. It *is* one of the most valuable plants in Traditional Indian Systems of Medicine. The different parts of the plant are used in > 100 formulations in Ayurveda, Unani and Siddha, and it is believed to be therapeutically equivalent to Ginseng [1], [2], [3] and [4]. The crude extracts of the plant has been found to have strong free radical scavenging potential. Indian Himalayan Region (IHR) is the rich source of medicinal plants and may have a strong antioxidant potential due to rich in phenolic and flavonoid compounds [5], [6], [7] and [8].

The entire plant is used for the treatment of tuberculosis, rheumatism, inflammatory conditions, and cardiac diseases. In addition, it is used as an antitumor, antibiotic, anticonvulsant and CNS-depressant agent [9]. The pharmacological activities of *W. somnifera* like reverses Alzheimer's disease pathology increased by enhancing low-density lipoprotein receptor-related protein in liver [10] inhibits amyloid beta fibril formation *in vitro* [11]. There are a number of reports elucidating the chemical and pharmacological properties of *W. somnifera* [12]. The numbers of reports indicated antioxidant properties and secondary metabolite production of medicinal plants [13], [14] and [15]. The presence of catechin in *W. somnifera* was reported by [16].

Keeping the medicinal importance of *W. somnifera* species in view, the present study was carried out in Kullu Valley of Himachal Pradesh, India to assess the spatial patterns of total phenolic contents, total flavonoid contents and antioxidant properties of different parts of *W. somnifera* plants. The generated data were also correlated with soil properties to assess the influence of soil properties on antioxidant properties of *W. somnifera* plants.

Materials and Methods

Study area

The study was carried out in Kullu Valley of the Kullu district in Himachal Pradesh, India. The latitude, longitude and altitude of different sampling sites varied from $31^0$45'40" to $32^0$55'59"N, $77^0$05'26" to $77^0$22'54"E, and 1000 m to 1250 m amsl, respectively.

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The site-1, Banala was represented by the S-E aspect with 5 ° slope, near the settlement at 1000m amsl; site-2, Larji by the S-E aspect with 25 ° slope, near the dumping site at 1050m amsl; site-3, Maitrana by the S-W aspect with 55 ° slope, dry forest at 1100m amsl; the site-4, Mohal by the N-E aspect with 5 ° slope, sandy site at 1150m amsl; the site-5, Thalaut by the S-E aspect with 5 ° slope, dumping site at 1200m amsl; and the site-6, Koliberh by N aspect with 5 ° slope, sandy dry site at 1250m amsl. The variations in the climatic conditions of Himachal Pradesh are ascribed to extreme variations in topography and elevation. The geographical characteristics of different study sites are given in Figure 1 and Table 1.

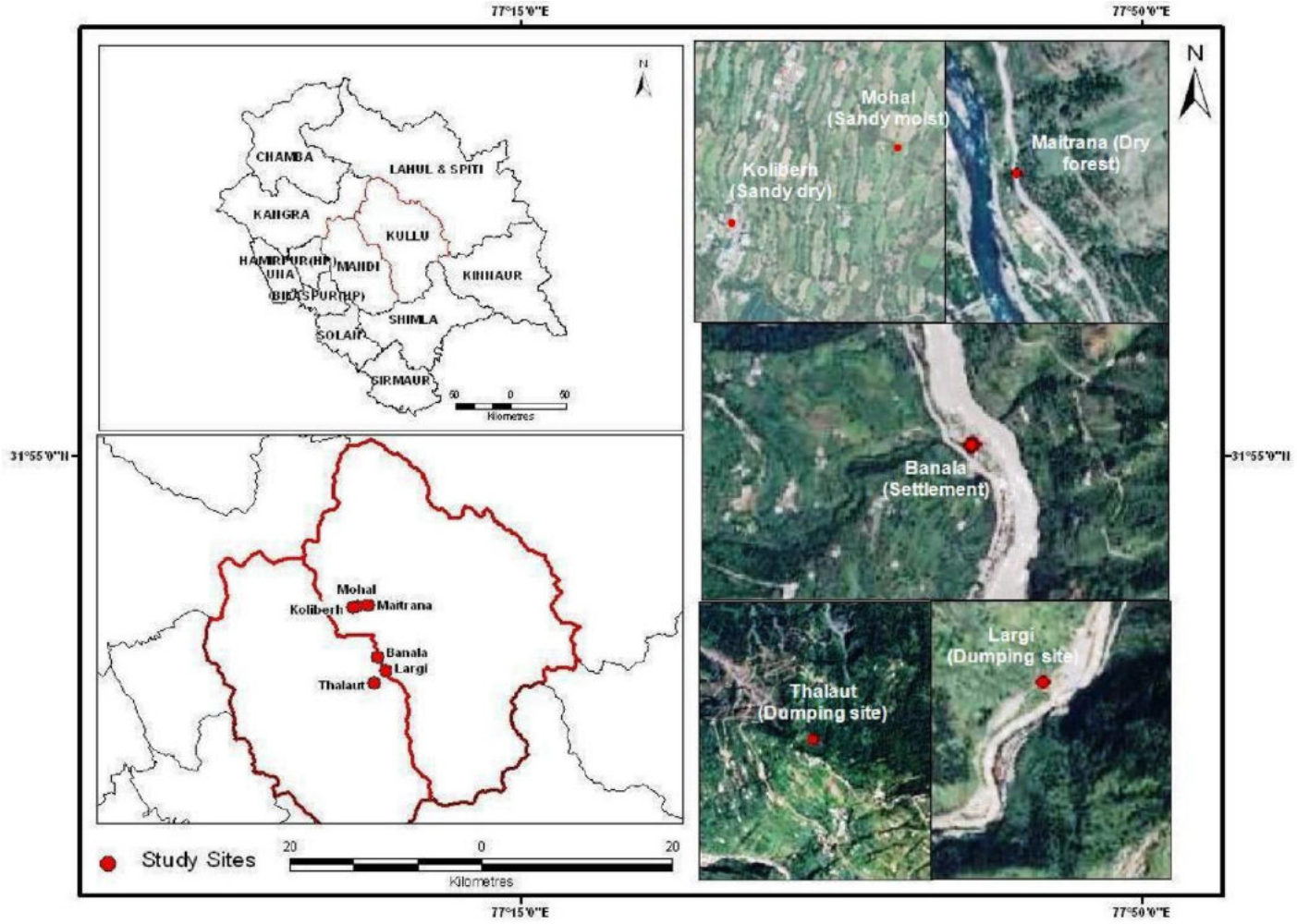

Figure 1. Geographical locations of different study sites

Table 1: Geographical characteristics of different study sites

Sites	Sample I.D	Habitats	Latitude	Longitude	Altitude (m amsl)	Aspect	Slope
Banala	BAN	Settlement	31 46'19"	77 12'02"	1000	S-E	5
Largi	LAR	Dumping site	31 43'55"	77 13'47"	1050	S-E	25
Maitrana	MAI	Dry forest	31 55'08"	77 07'40"	1100	S-W	55
Mohal	MOH	Sandy moist	31 44'34"	77 12'29"	1150	N-E	5
Thalaut	THA	Dumping site	31 41'31"	77 11'25"	1200	S-E	5
Koliberh	KOL	Sandy dry	31 54'50"	77 06'48"	1250	N	5

Samplings and Processing

The whole plants of *W. somnifera* along with top soil (0-15cm) were collected in triplicates from each site in a pre-washed polyethylene bags. The five soil samples, one from center and four from corner were mixed together and a composite sample weighing 200 gm of the homogenized soil was collected in air-tight pre-washed polyethylene bags from each site. Soil was air dried at room temperature till a constant weight was achieved. The dried soil samples were powdered using a mortar and pestle and then passed through a 2 mm size sieve. The powdered soil samples were stored at 25 ^{o}C ambient temperature till analysis. The soil samples were weighed in triplicate for the analysis. The plant samples were separated into roots, stems, leaves and fruits and washed properly under running tap water to remove the adhered soil particles and other materials, chopped into small pieces and oven dried at 40^{o} C till a constant weight was obtained. The oven dried plant material was powdered using a stainless steel grinder, then passed through a 2 mm size sieve and stored at room temperature before analysis.

Soil analysis

Soil pH was measured in suspension of soil to water in a ratio of 1:5 (w/v) using a glass electrode attached to a Multi-Parameter System (Eutech, PC 700). Organic carbon content was determined using a method of Walkley and Black's rapid titration [17]. Total nitrogen content was determined following the micro- KJELDAHAL technique through the Gerhardt Automatic Analyzer (KES 12 L, KEL PLUS, India). The digested samples were used for the determination of total Phosphorus using the method of [18]. Total Potassium was measured in digested solution using a Flame Photometer (MJ 272, Rescholar, India).

Preparation of methanol extracts and analysis

One gram of leaf, stem, fruit and root each was weighted accurately and then crushed using 10ml of 80% (v/v) methanol using mortar and pestle, the extract was kept at 4^{o} C in a refrigerator for 72 hours and then centrifuged at 5000rpm for 5 minutes (R-8 CDX, Remi). The supernatant of extracts was used for the quantifications of total phenolic and total flavonoid contents. The antioxidant activities of supernatant were further assessed using *in-vitro* assays.

Determination of total phenolic contents

Total phenolic contents in methanol extracts of plant sample were quantified using the method of [19]. In 1 ml of extract, 1ml of Folin-Ciocalteu Phenol Reagent and 2ml of 2% (w/v) sodium carbonate were added and then the final volume was made up to 10ml with double distilled water. The whole mixture was heated at 80 C for 1 hour. The absorbance of the cooled solution was measured at 650nm using a UV-Visible Spectrophotometer (Ultra spec 2100 Pro, Healthcare Biosciences AB, Uppsala, Sweden). The total phenolic contents in methanol extracts were quantified using standard curve prepared with different concentrations of Gallic acid. The results were expressed as mg Gallic acid equivalent (GAE)/100g fw of leaves, stems, fruits and roots of *W. somnifera* L. species.

Determination of total flavonoid contents

Total flavonoid contents in methanol extracts of roots, stems, leaves and fruits of *W. somnifera* plants were determined using the method of [20]. The 1 ml of 2% $AlCl_3$ was mixed with 1ml of extract and then kept at room temperature for 1 hour. The absorbance of the yellow colored assay was measured at 420 nm using a UV-Visible Spectrophotometer (Ultra Spec 2100 Pro, Healthcare Biosciences AB, Uppsala, Sweden). A standard curve was plotted between different concentrations of quercetin and absorbance to quantify the total flavonoid contents in extracts. The results were expressed as mg quercetin equivalent (QE)/100g fw.

Measurement of antioxidant activities

The antioxidant activities in the methanol extracts of leaves, stems, fruits and roots were evaluated using *in-vitro* assays, namely ABTS, DPPH and FRAP as per methods described by [21], [22] and [23]; respectively.

DPPH assay

To determine the DPPH radical scavenging potential of methanol extract of test plants, 1 ml of extract was mixed with 4ml of 0.004% DPPH (prepared in methanol) and then kept the reaction mixture in dark at room temperature for one hour. Then, the absorbance of reaction mixture was measured at 517 nm using a UV-Visible Spectrophotometer (Ultra Spec 2100 Pro, Healthcare Biosciences AB, Sweden). DPPH radical inhibition potential of the extract was calculated using the following equation.

$$\text{DPPH inhibition (\%)} = [A_s - A_c] \ 100/ A_c$$

Where As and Ac are the absorbance of samples with and without plant extracts, respectively.

ABTS assay

To carry out the ABTS assay, an equal volume of ABTS salt (7µM) and potassium persulphate (2.45µM) were added and kept in dark at room temperature for 16 hours for the production of ABTS cation. After 16 hours, the above solution was diluted with 80% (v/v) methanol till an absorbance of 0.707±0.001 was obtained at

734 nm. The 1 ml of diluted ABTS cation was properly mixed with 1 ml of extract and kept in dark for 7 minutes at room temperature. The absorbance of resulting mixture was measured at 734 nm against a methanol blank using a UV-Visible Spectrophotometer (Ultra Spec 2100 Pro, Healthcare Biosciences AB, Sweden). The percentage of ABTS radical inhibition was calculated as ABTS inhibition (%) = [As – Ac] 100/Ac, where As and Ac are the absorbance of samples with and without plant extracts, respectively.

FRAP assay

The FRAP assay was achieved using fresh working reagents of 3ml containing 300 mM Acetate buffer (3.1g of Sodium Acetate and 16 ml Glacial Acetic Acid per liter), 10 mM TPTZ (2,4,6-tri-2pyridyl-1,3,5-triazine) in 40 mM HCl and 20 mM Ferric Chloride ($FeCl_3$) and mixed with 1ml of plant extract. The reaction mixture was kept in the dark room for 30minutes to complete the reaction. The absorbance of reaction mixture was then measured at 593nm against a methanol blank using a UV-Visible Spectrophotometer (Ultra Spec 2100 Pro, Healthcare Biosciences AB, Sweden). The FRAP activity in methanol extracts was measured using a standard curve prepared between different concentrations of 0-100 µgFe(II)/ ml and absorbance. The results were expressed in µg Fe (II) /g fw.

Statistical analysis

The generated data was presented as mean ± standard errors (S.E) of three independent measurements. The 1-way ANNOVA test and Duncan Multiple Range's Test were also performed using SPSS software, (version 16.0) to separate the treatment means and relationships. A significant level of 95% (p 0.05) was considered statistically different.

Results and Discussion

Physico-chemical properties of soil

Soil pH was found slightly alkaline, varied significantly between sites and ranged from 7.8-8 (Table 2). Organic carbon (OC), total nitrogen (N), phosphorus (P) and potassium (K) in soil ranged between 0.72% - 1.43%, 0.42% - 0.90%, 0.36 µg g^{-1} dw - 1.43 µg g^{-1} dw and 646.67 mg kg^{-1} dw -1592.50 mg kg^{-1} dw, respectively (Table 2). The highest concentration of K in soil was found at 1250m amsl, pH and OC in soil at 1100m amsl, total nitrogen in soil at 1000m amsl and total phosphorus in soil at 1050m amsl (Table 2). The highest OC in the soil at 1100m amsl may be ascribed to dense forest vegetation as compared to the other study sites. The results of the present study clearly indicated that the changes/variations in tested physico-chemical properties of the soil among the sites may be ascribed to local environmental conditions, settlement, vehicular emissions, etc. The physico-chemical properties of growth media i.e., soils influenced the growth and development of plants by altering their production capacity of enzymatic and non-enzymatic antioxidants [24]. The physico-chemical properties of soil collected from different study sites have been given in Table 2.

Table 2: Physico-chemical properties of the soil collected from different study sites

Physicochemical properties of soil	Altitudes (m amsl)						p-value
	1000	1050	1100	1150	1200	1250	
pH (soil: water)	7.90±0.01	7.89±0.00	8.02±0.00	7.82±0.01	7.90±0.01	7.92±0.04	0.001
Organic carbon (%)	0.72±0.00	1.28±0.07	1.43±0.05	1.34±0.02	1.12±0.10	1.26±0.03	0.000
Total N (%)	0.90±0.01	0.65±0.01	0.42±0.00	0.42±0.01	0.42±0.01	0.76±0.00	0.000
Total P (mg g^{-1} dw)	7.32±0.30	15.14±0.24	6.63±0.16	8.73±0.07	7.49±0.18	12.32±0.13	0.000
Total K (mg kg^{-1})	1373.33±9.16	646.67±5.06	1592.50±18.08	1313.33±15.16	1125.8±1.04	1500.00±16.06	0.000

Values are mean ± 1S.E. of three replicates.

Total phenolic and flavonoid contents

A number of studies have proved that high total phenolic contents are associated with greater antioxidant activity [5], [25], [26] and [27]. In the present study, total phenolic contents showed significant variations between the sites as well as within the plant parts (Table 3). The contents of total phenolics among different study sites ranged between 13.6 mg GAE/100g fw- 25.7mg GAE/100g fw, 10.8 mg GAE/100g fw-16.2 mg GAE/100g fw, 8.8 mg GAE/100g fw-16.6 mg GAE/100g fw and 8.8 mg GAE/100g fw-16.6 mg GAE/100g fw in roots, stems, leaves and fruits, respectively (Table 3). The minimum total phenolic contents in roots and stems were found at 1050m amsl and 1200m amsl, respectively, in leaf and fruits at 1250m amsl. Similarly, the maximum total phenolic contents were found in the stem at 1000m amsl and 1050m amsl, in the root, leaf and fruit at 1000m amsl [5], [28] have reported that leaves of *W. somnifera* growing near the roadside had higher total phenolics than those in the forests. Significant variations in the total phenolic contents have been reported in fruits of a wild edible plant, *Myrica esculenta,* which was ascribed due to altitudinal variations [29].

Data pertaining to total flavonoid contents in different parts of *W. somnifera* are presented in Table 3. The total flavonoid contents

expressed as mg QE/100g dw ranged between 2.84-9.43, 7.67-11.56, 2.25-5.38 and 23.62-53.86 in roots, stems, leaves and fruits, respectively (Table 4). The results further showed that minimum and maximum total flavonoid contents were found at 1000m amsl and 1250m amsl in root, 1200m amsl and 1000m amsl in stem, 1150m amsl and 1100m amsl in leaf, 1050m amsl and 1100m amsl in fruits, respectively (Table 4). The average total phenolic and total flavonoid contents in *W. somnifera* plant was found in order of maximum to minimum as 1150m > 1200m > 1250m > 1050m > 1100m > 1000m and 1100m > 1250m > 1000m > 1200m > 1150m > 1050m, respectively (Tables 3 and 4). The variations in total phenolic and total flavonoid contents among the parts may be ascribed to local climatic conditions and soil physico-chemical properties of soil. The study revealed that the medicinal potential of *W. somnifera* is controlled by multiple environmental factors such as soil, water, climate, natural vegetation, landforms etc.

Table 3: Spatial and intra-specific patterns of total phenolic compounds in methanol extracts of *W. somnifera*

Altitudes (m amsl)	Total Phenolic Contents (mg GAE/100g dw)				
	Roots	Stems	Leaves	Fruits	Average
1000	13.58c±0.01	13.55b±0.21	8.81c±0.07	8.81d±0.07	11.18
1050	25.71a±0.87	10.77c±0.02	13.77b±0.43	13.77c±0.42	16.00
1100	15.15c±0.13	14.12ba±0.61	14.29b±0.58	14.29cb±0.58	14.46
1150	24.62ba±0.70	14.78a±0.05	16.29a±0.14	15.64ba±0.08	17.83
1200	20.61 b ±0.30	16.17c±0.16	16.06a±0.19	16.06a±0.19	17.22
1250	20.85b±0.23	11.48c±0.19	16.56a±0.15	16.56a±0.15	16.36

Values are mean ± 1S.E. of three replicates.
Values within a column followed by different letter are significantly different at p 0.05 (Duncan's Multiple Range test).

Table 4: Spatial and intra-specific patterns of total flavonoid compounds in methanol extracts of *W. somnifera*

Altitudes (m amsl)	Total flavonoid compounds (mg QE/100g dw)				
	Roots	Stems	Leaves	Fruits	Average
1000	2.84c±0.02	11.56a±0.08	4.80b±0.73	48.04a±0.07	16.81
1050	3.10c±0.23	9.76c±0.07	2.36d±0.49	23.62c±0.04	9.71
1100	5.39b±0.14	8.65d±0.05	5.38a±3.18	53.86a±0.31	18.32
1150	5.77b±0.13	10.42b±0.06	2.25d±0.40	26.25c±0.34	11.17
1200	5.50a±0.38	7.67f±0.09	3.75c±0.34	37.57b±0.03	13.62
1250	9.43a±0.47	8.14e±0.21	5.06ab±1.16	50.64a±0.11	18.31

Values are mean ± 1S.E. of three replicates.
Values within a column followed by different letter are significantly different at p 0.05 (Duncan's Multiple Range test)

Antioxidant activities

Antioxidant compounds in plant extracts have potential to donate hydrogen which react with DPPH radical and consequently decrease its content in the reaction mixture. Among the tested populations, per cent DPPH inhibition potential of methanol extracts of roots, stems, leaves and fruits of *W. somnifera* varied from minimum to maximum as 76.9%-88.1%, 82.3%-87.4%, 22%-73.6% and 26.4%-53.7% in roots, stems, leaves and fruits, respectively (Figure. 2 and Table 5). The minimum and maximum per cent DPPH inhibition potential of roots, stems, leaves and fruits extract were recorded at 1150m amsl and 1000m amsl, 1150m amsl and 1050m amsl, 1150m amsl and 1050m amsl and 1100m amsl and 1000m amsl, respectively (Figure. 2 and Table 5). The antioxidant capacity of plants was also determined in terms of scavenging of $ABTS^{\bullet+}$ radical cation generated during the application method. In the present study, $ABTS^{\bullet+}$ inhibition ranged between 19.2%-99.6%, 7.8%-88%, 4.7%-73.5% and 24.5%-89.8% by root, stem, leaf and fruit extracts, respectively (Figure.2 and Table 5). The maximum ABTS inhibition potential of root, stem, leaf and fruit was found at 1050m amsl, 1100m amsl, 1250m amsl and 1200m amsl, respectively. The FRAP activity of extracts of different parts of test plant (expressed as µg Fe (Π)/g dw) was found minimum to maximum as 0.6-5.5 in roots at 1250m amsl and 1000m amsl, 3.8-6.0 in stems at 1050m amsl and 1150m amsl, 6.2-7.2 in leaves at 1200m amsl and 1250m amsl and 1.24-2.48 at 1200m amsl and 1250m amsl in fruits, respectively (Figure. 2 and Table 5). The antioxidant properties of methanol extracts of roots, stems, leaves and fruits were varied significantly due to diverse habitat ($P< 0.05$) [30].

The FRAP and ABTS activities were found maximum in fruits, whereas [29] have also reported significant variation in antioxidant

activities in the methanol extracts of fruits of *Myrica esculenta* populations growing at different altitudes i.e; 1775-2100m amsl. [31] Pointed out that the antioxidant activity of phenolic compounds may depend on factors such as growing conditions, quality and origin i.e., geographical location of plants as well as the extraction and purification methods used to determine the antioxidant activity.

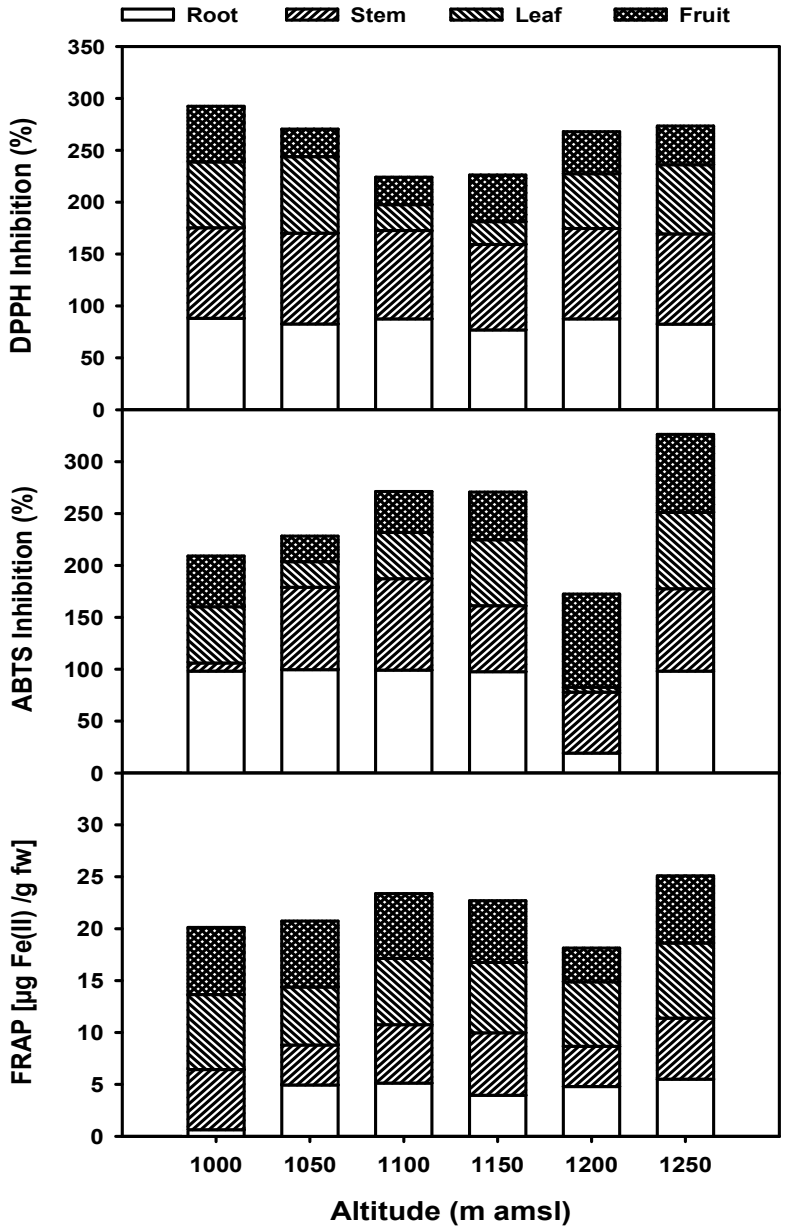

Figure 2. Spatial trend in DPPH, ABTS free radical scavenging activities and FRAP activities of methanol extracts of fruits, stems, leaves and roots of *W. somnifera* plants.

Table 5: Mean, median, minimum and maximum DPPH, ABTS and FRAP activities in methanol extracts of fruits, leaves, stems and roots of *W. somnifera* plants collected from different sites

Properties	Plant parts	Values (n*=6)			
		Mean	Median	Minimum	Maximum
DPPH (%)	Roots	84.24	85.15	75.50	89.11
	Stems	86.00	86.68	81.66	88.26
	Leaves	50.58	59.11	21.39	74.55
	Fruits	38.20	39.00	25.50	54.09
ABTS (%)	Roots	85.34	98.23	18.39	99.72
	Stems	62.83	71.29	5.80	88.26
	Leaves	44.19	49.86	4.24	75.11
	Fruits	54.01	48.52	15.28	90.95
FRAP	Roots	4.16	4.39	0.54	6.18
[µgFe (Π)/g dw]	Stems	5.17	5.39	3.48	6.82
	Leaves	6.57	6.64	5.57	7.38
	Fruits	5.79	6.20	2.81	6.91

*N is numbers of study sites.

Relationships

Soil properties are playing an important role in plant uptake and accumulation of mineral nutrients and consequently affecting their growth and development by altering free radical scavenging potential [24], [32]. Therefore, in the present study the correlations between total phenolic and flavonoid contents in methanol extracts from different parts of *W. somnifera* with physico-chemical properties of soil was developed and outputs are given in Table 6. Results showed significant and both positive and negative correlations between the soil properties and total phenolic and flavonoid contents in methanol extracts from different parts of *W. somnifera.* Soil K showed significant positive correlation with total flavonoid contents in leaf (R= 0.78, P<0.001) and root (R =0.56, P<0.01) and significant negative correlation with total phenolic contents of root (R = 0.60, P<0.001). Soil pH had significant positive correlation with the total flavonoid contents in fruit (R = 0.68, P<0.001) and leaf (R =0.62, P<0.001) and significant negative correlation with total phenolic contents of root (R= 0.47, P<0.001). Soil OC showed significantly and positive relationship with total phenolics contents in leaf (R= 0.69, P<0.001) and root (R =0.52, P<0.01). Soil P showed significant positive correlation with the total phenolic contents in roots (R =0.64, P<0.001) of *W. somnifera.* Soil N did not show any positive and negative correlation with total phenolic and total flavonoid contents in different parts of *W. somnifera.* From the present results, it is clear that the total phenolic and total flavonoid contents in the different parts of *W. somnifera* and its antioxidant properties are significantly affected by physico-chemical properties of the soil.

Table 6: Correlation between total phenolic and flavonoid contents in methanol extracts of different parts of *W. somnifera* and physico-chemical properties of soil

Correlation	Fruits	Leaves	Stems	Roots
TPC vs pH	-0.39ns	-0.04ns	-0.19ns	-0.47*
TFC vs pH	+0.68**	+0.62**	-0.32ns	+0.32ns
TPC vs OC	-0.19ns	+0.69**	+0.10ns	+0.52*
TFC vs OC	-0.03ns	-0.14ns	-0.43ns	+0.37ns
TPC vs N	+0.13ns	-0.36ns	-0.44ns	-0.27ns
TFC vs N	-0.06ns	+0.19ns	-0.01ns	-0.23ns
TPC vs P	-0.21ns	+0.23ns	-0.37ns	+0.64**
TFC vs P	-0.40ns	-0.46ns	+0.006ns	-0.04ns
TPC vs K	+0.26ns	+0.02ns	+0.22ns	-0.60**
TFC vs K	+0.06ns	+0.78**	-0.14ns	+0.56*

TPC = Total Phenolic Contents ,TFC = Total Flavonoid Contents
P = Total phosphorus , OC = Organic carbon
N = Total nitrogen , K = Potassium
Levels of significance: * = p<0.01, ** = p <0.001 and ns = Not significant

Conclusions

The present study reports the spatial trends and intra specific variations in total phenolic and flavonoid contents in menthol extracts from different parts of *W. somnifera* and their antioxidant activities commonly found in the mid hill region of Himachal Pradesh. The results further showed that total phenolic and flavonoid contents and antioxidant properties of *W. somnifera* varied significantly (p 0.05) with their location and growing habitat which may be ascribed to variations in microclimatic and soil conditions. The relationship between soil properties and total phenolic and flavonoid contents in methanol extracts of *W. somnifera* was further studied and results showed that soil properties have significant influence on total phenolic and flavonoid contents in different parts of *W. somnifera*. More investigations are still required to strengthen the present findings and to develop a sustainable plan for the conservation of *W. somnifera* plant under mountain ecosystem and sustainable utilization for commercial purposes in pharmaceutical and food industries. Since, *W. somnifera* plants growing in Kullu Valley are found as rich sources of natural antioxidants, therefore, can be used for consumption by the local mountain people.

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