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The present study was undertaken on the ethno-medicinal plants of Sangla valley, Kinnaur (Himachal Pradesh) and the characterization of the chemical constituents and antioxidant properties of selected plants. This section deals with the details of collection of various material and the methods followed.

Description of study site and ethnic community

North-western Himalayan region, with its wide range of altitudes, topography and climatic conditions, is a rich repository of medicinal wealth, which occupies an important place in Vedic treatise. More than 800 valuable medicinal species found in this part of India are extensively used by the locals since times immemorial for curing various diseases of human kind (Pushpangadan, 1995b).

Himachal Pradesh is mostly a mountainous state with altitude ranging from 247 m to 7000 m above mean sea level and covers an area of 55,673 km² (Chowdhery, 1999). It is situated in the centre of the Western Himalayas, having diverse agro-climatic conditions ranging from semi-tropical to temperate, alpine, and culminating in the cold desert region. It is bestowed with a vast wealth of medicinal and aromatic plants due to its unique location (Chauhan, 2003).

Sangla valley, the untouched, unpolluted and non-commercialized valley with its charming beauty, is nested in the Kinnaur district of Himachal Pradesh in India. It starts from Kupa and ends in Chitkul which is the last tribal inhabited village. It is a part of the Greater Himalayas situated at a latitudinal range from 31° 6' to 31° 27' N and longitudinal range from 78° 10' to 79° E (Fig. 1). Its altitude varies from 1500 m to 7000 m. The length of the valley is about 65 km, which covers an area of nearly 300 km² and accommodating approximately 9500 inhabitants. The valley comprises a
number of small watersheds which find their way into the Baspa river. The hamlets are scattered all along the valley. North facing slope is relatively gentle and has adequate vegetation cover, farm fields, soil cover and sporadic settlements. The uppermost parts of the mountain peaks are usually covered with snow for 4–8 months and are remarkable for a variety of beautifully colored flowers forming a rich storehouse of medicinal and aromatic plants. The area is inhabited by the tribal population known as Kanaurs and people here depend on agriculture for their livelihood. Apple is the only cash crop grown in this area. They also grow a number of other crops like potato, maize, wheat, pulses and buckwheat to fulfill their needs. People of the area follow both Hinduism and Buddhism and have constructed a number of monasteries and wooden carved temples. The beauty of the temples and monasteries should be seen firsthand to feel its serenity which gives peace to soul and body.

Fig. 1. Map of study site.

Though the area is very unique with blend of different things, but the only problem is the harsh climatic conditions. Along with this, it remains cut off from the
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rest of the state for nearly six months due to heavy snow fall. These conditions led or forced the inhabitants to develop an indigenous system of medicine to treat various ailments. Since, the area is relatively unexplored, thus little work has been done to inventorize, evaluate and investigate the medicinal and aromatic plants that are used by the locals in the region. Keeping this thing in mind, a study was conducted in Sangla valley. Earlier, only the local people used these plant resources for their own consumption. In recent years, there has been a sudden rise in the demand of herbal products and plant based drugs across the world to cure the sufferings and ailments of the people. There are also a number of plant species which are exclusive to this region. Thus, it is pertinent to explore and validate the medicinal and aromatic plants of this region.

Methods

A. Inventorization of Ethno-medicinal plants

The study was undertaken during the year 2010 to 2013. For this, regular field trips were undertaken between June to September to different localities and areas of the Sangla Valley. Study site was surveyed on the basis of different villages (for the ease of study). Regular field trips were undertaken to become familiar with the area and flora. An exhaustive census of flowering plants, along with the study of structure and composition of vegetation, was carried out on the basis of visual observations, questionnaire survey; relevant literatures regarding medicinal plants were screened to gather more information. In addition, local people were consulted regarding the plants they frequently used for the treatment of various ailments.

For the Primary data collection, the area was visited along with the locals residing there to get familiar with the region and to identify medicinal plants. Local people were interviewed and semi-structured questionnaire was used to obtain information on medicinal plants with regards to their local names, part used, flowering time, mode of preparation and administration.

Group discussions, field observations and informal meetings were undertaken in the villages while staying with them to gather more information. Most of the interviewed persons were old people above 65 years having knowledge and strong connection with
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the medicinal usage of plant products. Besides, the traditional healers (*amchis*) were consulted because they are the local practitioners and have strong knowledge regarding administration of medicinal plants.

The information received on the uses of plant species was verified from 2 to 3 other persons apart from the primary source. The specimen of plant species were collected and dried by following conventional methods of drying and preservation. After that herbarium sheet of dry plant material was prepared. Firstly the specimens were identified from the relevant flora records and available literature and then validated from the Herbarium of Forest Research Institute, Dehradun. The identified specimens were deposited in herbarium of Panjab University, Chandigarh (India), to get voucher number. The latin names were verified and updated using Missouri Botanical Garden (*Tropicos.org*) (2015) and *The Plant List* (2015). The secondary literature, such as different flora records and various publications were also taken into account in order to learn about the different values/uses of these plant species present in the Sangla Valley.

All the gathered information was compiled and presented in the form of inventories. The selection of ethno-medicinal plants for further studies was done based on their use by the tribal community.

Data Analyses

Informant consensus factor (ICF)

ICF (also known as Informant agreement ratio, IAR) was calculated to determine consensus of informants for the treatment of a particular ailment (Heinrich *et al.*, 2009). The value of ICF varies from 0 to 1, with a high values (near one) indicating high homogeneity among informants for the medicinal use of plant species for a particular treatment, whereas low values (near 0) indicates disagreement among informants about the use of a plant species to treat an ailment. ICF was calculated as under:

\[
ICF = \frac{N_u - N_t}{N_u - 1}
\]
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where, \( N_u \) refers to the number of use reports, \( N_t \) refers to the number of taxa in each category.

**Use value (UV)**

The relative importance of a plant was calculated quantitatively employing value method (Phillips *et al.*, 1994) as per the following formula:

\[
UV = \frac{\sum U_i}{n}
\]

where, \( U_i \) is the number of uses reported by informants for a particular plant and \( n \) is the total number of informants.

High UV values imply that the numbers of use reports for a plant are high and the plant is important and broadly used for treatment of a number of ailments; whereas, UV values near 0 indicate very few use reports of the species.

**Fidelity level (FL)**

FL represents the preference of one species over the other as regards the treatment of a particular ailment (Friedman *et al.*, 1986), and calculated as under:

\[
FL = \left( \frac{I_p}{I_u} \right) \times 100
\]

where \( I_p \) is the number of informant stating the use of a plant for a particular ailment and \( I_u \) is the number of informant stating use of plant for any sort of ailment.

High FL values imply high use of plant for a particular ailment, while low values suggest wide range of use with low frequency for treatment of each ailment.

**B. Determination of chemical constituents**

**a. Aromatic Plants:** *Mentha longifolia, Heracleum candicans, Rabdosia rugosa* and *Cedrus deodara* plants were selected, followed by oil extraction and their chemical constituents were determined.
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Extraction of the essential oils

The selected plants were brought to the lab after collection and their leaves and twigs were separated. The essential oils were extracted from the chopped leaves and twigs of flowering shoots by hydro-distillation using Clevenger apparatus. Two kg of freshly chopped plant material was mixed with 2 litre of distilled water. The mixture was boiled for 3 hour and the extracted oil was collected from the nozzle of the condenser. A stable essential oil was extracted from the wood chips collected from the tree trunk of 60 year old Cedrus deodara. The wood chips were fired and resin-like dark brown coloured material was collected while the wood was burning. The water present in the essential oil was dried over by anhydrous sodium sulphate and the oil was stored at 4°C until used for analyses by Gas Chromatography Mass Spectroscopy (GC-MS) and bioassay.

Identification of chemical constituents of volatile essential oil

The identification of the various chemical constituents of the essential oils was done using Gas Chromatography (GC) coupled with Gas Chromatography and Mass Spectroscopy (MS). The analysis was done at Jawaharlal Nehru University (JNU), Delhi, India. Gas Chromatography was done using Shimadzu QP 2010 gas chromatograph equipped with a Flame Ionization Detector (FID) and an Omega SPTm column or Rtx 5MS fused silica capillary column [30 m × 0.25 mm (inside diameter), film thickness 0.25 μm].

In an Omega SPTm column, N₂/Air was used as a carrier gas at a split ratio 90:1 and the flow rate was 1.21 ml/min. The temperature of the injector and ion source was fixed at 270 °C. Initially, the oven temperature was 100 °C, held isothermally for 2 minutes, then increased to 200 °C at the rate of 6 °C per minute and finally held at 230 °C for nineteen minute.

In Rtx 5MS fused silica capillary column, oven temperature initially was programmed from 50 °C to 210 °C at the rate of 3°C/min, held isothermally for 8 min and finally raised to 260 °C at the rate of 3 °C permin. Helium (He) was used as carrier gas at the rate of 1 ml/min; temperature of injector and flame ionization
detector (FID) were fixed at 260 °C and 270 °C, respectively, and the sampling rate was 40 msec. One μL of essential oil diluted in n-hexane was injected.

GC-MS was performed on a Shimadzu QP 2010 plus mass spectrophotometer fitted with a flame ionisation detector (FID) coupled with a GC 2010 gas chromatograph and equipped with a Supelco Omega Wax or Rtx 5MS column (30 m × 0.25 mm, internal diameter; 0.25 µm film thickness). In case of Omega wax column, Helium (He) at a split ratio of 80:1 and a linear velocity of 40.9 cm sec⁻¹ was used as a carrier gas. Initially, the oven temperature was 100°C, which was held for 2 minute followed by an increase in temperature to 200°C and 230°C, respectively.

In Rtx 5MS column helium was used as a carrier gas at a split ratio of 1:50 and linear velocity of 36.3 cm/sec. Oven temperature was initially programmed to 50 °C and held for 2 min, subsequently increase in temperature to 210 °C at the rate of 3°C per min and finally to 260 °C with a rate of 30 °C per min held isothermally for 28 min. Injector port and detector temperatures were 250 °C and 270 °C, respectively, and the split ratio was 1:50. One μl of oil (diluted in n-hexane) was injected and the recording was made at 70 eV with scan time 0.5 sec, mass spectra were scanned from m/z 40-650amu.

The relative percentage of the different constituents was calculated automatically from the peak of total ion chromatograms. Constituents of the oils were identified by comparison of their retention indices relative to a homologous n-alkane (C₈-C₃₂) series and matching their mass spectra with those of reference compounds in Wiley 275 and NBS 75 K libraries (Adams, 1995; 2007)

b. Fatty Oil: Fatty oil was extracted from the seeds of *Prunus armeniaca* and *Prunus mira* plants.

**Extraction of the fatty oil**

Thirty gram seeds of *Prunus mira* and *Prunus armeniaca* were milled using pestle and mortar. The milled seeds were transferred to the conical flask. Thereafter, 250 ml of petroleum ether was put in to it, left for 24 h, followed by agitation from time to time for the proper extraction of the oil. The solvent was filtered using Whatman filter
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paper, evaporated under room temperature, and the dried crude oil was kept in vials and stored at 4°C.

**Fatty acid methyl esters (FAME) preparation**

The fatty acid of the oil were esterified in to methyl ester by adding Potassium hydroxide (KOH), then the mixture was shaken for 2 minutes and allowed to stand. The upper layer (i.e. Methyl esters of the fatty oils) was removed.

**Identification of chemical constituents of fatty oil**

The oil (as methyl esters of the fatty oils) was analyzed by GC using a Shimadzu QP 2010 plus mass spectrophotometer fitted with a flame ionisation detector (FID) and equipped with a Supelco (Omega Wax 30 m × 0.25 mm, internal diameter; 0.25 µm film thickness) column. Nitrogen (N₂) at a split ratio of 10:0 and a linear velocity of 33.9 cm sec⁻¹ was used as a carrier gas. Initially, the oven temperature was 140°C, which was held for 2 minute followed by an increase to 240°C.

GC-MS was performed on a Shimadzu QP 2010 plus mass spectrophotometer fitted with a flame ionisation detector (FID) and coupled with a GC 2010 gas chromatograph equipped with a Supelco (Omega Wax 30 m × 0.25 mm, internal diameter; 0.25 µm film thickness). N₂ with a split ratio of 10.0 and a linear velocity of 31.6 cm sec⁻¹ was used as a carrier gas. Initially, the oven temperature was 140°C which was held for 2 min followed by an increase in temperature to 240°C. The constituents were identified by comparison of their retention time with those of reference samples.

**C. Collection of Plant Material**

The plant materials were collected from the hills of Sangla valley, district Kinnaur, Himachal Pradesh, at an altitude ranging from 2800 m to 4500 m during the month of June to September. These were brought to laboratory and dried under normal conditions. Thereafter, the powder of the leaves and fruit were prepared by grinding it using pestle and mortar. The powdered material was used to determine the antioxidant activity.
D. Preparation of oil, methanolic and water extracts

Oil extracts
For estimation of antioxidant activity of oil, various concentrations of oil were prepared by dissolving oil either in methanol or acetone. The various concentrations prepared were 25, 50, 100, 200 and 400 µg/ml.

Water extracts
Water extracts were prepared from the dried powder of selected plants *R. rugosa* (PP: plant powder and RP: residue powder), *H. candicans* (PP and RP), *M. longifolia* (PP and RP), *P. armeniaca* (F: fruit, L: leaf and R: seed residue), *P. mira* (F, R and L), *B. stracheyi* (L: leaf and R: root), *S. obvallata* (L), *B. persicum* (S), *M. spicata* (L), *A. flavum* (L), *U. dioica* (L), *F. tataricum* (L: leaf and S: seed) and *F. esculentum* (L and S).

To prepare the water extract, 1g of dried plant material was dissolved in 100 ml of distilled water to make 1% solution. The contents were kept overnight for the proper extraction of the soluble water constituents from the material and it was filtered to get a pure water extract. Different concentrations were made by using dilution method ranging from 1% to 0.0625%.

Methanolic extracts
Methanolic extracts were prepared from the dried powder of selected plants *B. stracheyi* (L and R), *F. esculentum* (S and L), *F. tataricum* (S and L), *A. flavum* (L), *M. spicata* (L), *S. obvallata* (L) and *U. dioica* (L). To prepare the methanolic extract, 25 g of the dried plant material was dissolved in 250 ml of methanol and kept overnight for the proper extraction of methanol soluble constituents, followed by filtration to get a pure methanolic extract. Thereafter, the methanol was removed from the extract with the help of Soxhlet apparatus. The residue left after the removal of methanol was used for further studies. The different concentrations ranging from 25, 50, 100, 200 and 400 µg/ml were prepared by dissolving the residue in water.
E. Determination of the total phenolic and flavonoid content.

**Total phenolic content (TPC)**

The total phenolic contents were determined by Folin-Ciocalteu reagent method as given by Swain and Hillis (1959) and using gallic acid as a standard. 0.125 ml of the water extracts was mixed to 0.875 ml of distilled water and 0.5 ml of Folin-Ciocalteu reagent (FCR) was added and shaken thoroughly. After 3 min, 1 ml of 7.5% Na₂CO₃ was added and the mixture was allowed to stand for 30 min to 1 h. The absorbance of the mixture was measured at 760 nm using Shimadzu UV-190 double beam spectrophotometer. The phenolic content was expressed in terms of mg gallic acid equivalents (GAE) g⁻¹ of the plant tissue.

**Total flavonoid content (TFC)**

Total flavonoid content was determined by aluminium chloride (AlCl₃) (methanolic) method given by Meda et al. (2005) and quercetin (QE) was used as standard. About 1 ml of 2% AlCl₃ in methanol was added into 1 ml of the water extract. After 10 min, the absorbance was read at 415 nm on Shimazdu UV-190 double beam spectrophotometer. The amount of flavonoid was expressed as mg QE g⁻¹ plant tissue.

F. Determination of scavenging activity of fatty oils, essential oils, water and methanolic extracts

**Ferric ion reducing antioxidant power (FRAP) Assay**

FRAP was estimated as per the method given by Oyaizu (1986) in which the sample solutions (0.2 ml) (different concentrations of extract /oil) were mixed with 0.6 ml of 0.2 M phosphate buffer (pH 6.6) and 0.6 ml of (10 g/l) potassium ferricyanide solution. The mixtures were incubated at 50°C for 30 min. After incubation, 0.6 ml of (100 g/l) trichloroacetic acid was added and the reaction mixture was centrifuged for 10 minute at 3000 rpm. After centrifugation, 0.6 ml aliquot of the supernatant from each sample mixture was mixed with 0.6 ml of distilled water and 0.125 ml of (1.0 g/l) ferric chloride solution in a test tube. After 10 min of reaction time, the absorbance was measured at 700 nm. The higher absorbance of the reaction mixture
indicated the higher reducing power and was expressed in terms of % inhibition. Curcumin was used as a positive control

$$\% \text{ inhibition} = \left[ \frac{(A_{\text{sample}} - A_{\text{control}})}{A_{\text{sample}}} \right] \times 100$$

**Hydrogen peroxide (H$_2$O$_2$) scavenging activity**

H$_2$O$_2$ scavenging activity was examined as per the method of Ruch et al. (1989). 40 mM solution of H$_2$O$_2$ was prepared in a phosphate buffer (pH 7.4) of 0.1 M. Further, 0.6 ml of H$_2$O$_2$ was added to each sample. The concentration of H$_2$O$_2$ was determined spectrophotometrically at 230 nm after 10 min against a blank containing phosphate buffer without H$_2$O$_2$. The percent scavenging of H$_2$O$_2$ was calculated using the formula:

$$\% \text{ Scavenging of H}_2\text{O}_2 = \left[ \frac{(A_{\text{control}} - A_{\text{sample}})}{A_{\text{control}}} \right] \times 100$$

**Hydroxyl radical scavenging activity**

The hydroxyl radical scavenging activity of each fraction was determined based on the Fenton reaction as described by Yu et al. (2004) with a slight modification. The assay mixture was made up of 0.02 ml of ferrous chloride (0.02 M), 0.5 ml of 1,10-phenanthroline (0.04 M), 1 ml of phosphate buffer (0.2 M, pH 7.2), and 1ml of the sample. The reaction was initiated by the addition of 0.05 ml of hydrogen peroxide (7 mM). After 5 minute of incubation under room temperature, the absorbance was measured at 560 nm and ascorbic acid was used as a standard. The blank solution contained FeCl$_2$ + 1, 10 phenanthroline + phosphate buffer, and the control have all the reagents except sample. The percentage scavenging activity was calculated as under:

$$\% \text{ Scavenging activity} = \left[ \frac{(A_{\text{sample}} - A_{\text{control}})}{(A_{\text{blank}} - A_{\text{control}})} \right] \times 100$$

**Metal chelating activity**

The chelating activity of oil/extracts against Fe$^{2+}$ was measured as per the method given by Decker and Welch (1990). The extracts were added to a solution of 2 mM FeCl$_2$ (0.05ml). The reaction was initiated by the addition of 5 mM ferrozine (0.1ml) and then 2 ml methanol was added. The mixture was shaken vigorously and left at room temperature for 10 minute. After the mixture reached equilibrium, the
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Absorbance of the solution was determined at 562 nm and scavenging activity calculated as under.

\[
\text{Percentage of scavenging activity} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

**DPPH (2, 2-diphenyl-1-picryl hydrazyl) radical scavenging activity**

It was measured in terms of hydrogen donating or radical scavenging ability using the stable radical DPPH as per the method given by Blois (1958). For this, 200 µl of oil/extracts were taken to which 3 ml of DPPH (0.1mM in methanolic solution) was added. After this, the reaction mixture was placed in dark for incubation at 25°C for 30 minute. The absorbance of the solution including blank (without sample) and positive control (BHT, tert-butylated hydroxytoluene) was read at 517 nm on Shimadzu UV-190 double beam spectrophotometer. It was calculated in percentage by using the following formula:

\[
\text{Percentage DPPH radical scavenging} = \left( \frac{A_{\text{control}} - A_{\text{sample}}}{A_{\text{control}}} \right) \times 100
\]

**Total antioxidant activity**

The antioxidant activity of the oil/extracts was determined by the method of Prieto et al. (1999) for which 0.1ml of extract was combined with 1.0 ml of reagent solution (0.6 M sulphuric acid, 28 mM sodium phosphate and 4 mM ammonium molybdate). Then, the mixtures were incubated in boiling water bath at 95°C for 90 min. The samples were cooled to room temperature and the absorbance of solution was measured at 695 nm using Shimadzu UV-190 double beam spectrophotometer. Total antioxidant capacity of extracts/oils was expressed as µg ascorbic acid equivalent per ml (µg AAE/ml).

**Statistical Analysis**

Statistical analysis was done using SPSS ver.15. For all the estimations, minimum five replicates were maintained and the data presented is mean of five. Final values were expressed as mean ± standard errors. The significance of the values (wherever required) was checked by one way ANOVA followed by the comparison of the mean values using post hoc Tukey’s test at \( P \leq 0.05 \). For comparing two treatments 2 samples t-test was used.
Prunus mira

Prunus armeniaca
Fagopyrum tataricum

Fagopyrum esculentum
Mentha spicata

Mentha longifolia
Urtica dioica

Rabdosia rugosa
Ethnomedicinal Plants of Sangla Valley

Cedrus deodara

Saussurea obvallata
Bergenia stracheyi

Bunium persicum
Arisaema flavum

Heracleum candicans