MATERIALS AND METHODS

MATERIALS

(1). Chemicals and reagents for estimation of nutritional composition (AOAC 1990).

Sulphuric acid	1.25 %
Sodium hydroxide solution	1.25 %
Ethanol	25 ml
Potassium sulphate	0.1 g
Copper sulphate	0.9 g
Conc. sulphuric acid	25 ml
Aqueous Na2S	4%
Sodium hydroxide solution	40%
Sulphuric acid	0.1N
Methyl red indicator (I007, HiMedia). About 5 drops	
Sodium hydroxide solution	0.1N
Petroleum ether	1 ltr
Distilled water	10 ltr

(2). Chemicals and reagents for estimation of mineral using Flame Photometer (Rangana, 1986).

Potassium Chloride	1.908 g
Sodium Chloride	2.542 g

Calcium Carbonate	2.247 g
Distilled water	3 ltr
HCl (20 %)	5 ml
HCl (100 %)	10 ml
HCl (1%)	100 ml

Calcium standard solution:

2.247 g AR grade of $CaCO_3$ dissolved in distilled water. Approximately 10 ml of concentrated HCL added to effect complete solution of $CaCO_3$. Diluted to 1 litre with distilled water (1000 pp Ca). 10 ml of this solution diluted to 100 ml (100ppm Ca).

Potassium standard solution:

1.908 g of AR grade KCL dissolved in distilled water and diluted it up to 1 litre (1000 ppm K).

Sodium standard solution:

2.542 g NaCl Ar grade in 1 litre distilled water (1000 ppm Na).

(3). Chemicals and reagents for estimation of mineral following Tri-acid digestion method.

Nitric acid	9 ml

Perchloric acid	4 ml
Sulphuric acid	1 ml
Double distilled water	100 ml
Diethylene Triamine Penta Acetic Acid (0.005M)	1.967 g
Calcium chloride 0.01M	1.47 g
Tri ethanol amine (TEA) 0.1M	13.1 ml
Double distilled water	1000 ml
Zinc sulphate	0.439 g
Magnesium sulphate	10.141 g
Ammonium ferrous sulphate	0.702
Copper sulphate	0.392 g
Conc.H_2SO_4	5 ml
Neutral N NH4OAc solution	40 ppm

Extracting Solution:

For preparation of 1 litre of Diethylene Triamine Penta Acetic Acid (DTPA) extraction solution, 13.1ml reagent grade tri ethanol amine, 1.967 g DTPA (AR grade) and 1.47 g of $Cacl_2$ were dissolved in 100 ml of distilled water. The DTPA was allowed to dissolve for some time and diluted to approximately 900 ml. pH adjusted to 7.3 ± 0.5 with 1:1 HCl and diluted to 1 litre.

Zinc standard solution:

0.439 g AR grade of $ZnSO_4$. $7H_2O$ dissolved in 200 ml of distilled water in a beaker. It was then transferred to a litre volumetric flask and volume made up to the mark. 10 ml of this standard solution (100ppm Zn) was transferred to a Volumetric flask and diluted to the mark with DTPA extracting solution to have a stock solution of 10ppm Zn.

Iron standard solution:

0.702 g of AR grade ammonium ferrous sulphate dissolved in 300 ml distilled water in a beaker. 5 ml of concentrated H_2SO_4. It was then transferred to a litre measuring flask and volume made up to the mark (100ppm Fe).

Magnesium standard solution:

10.141 g $MgSO_4$. 7H2O dissolved in 200 ml distilled water and volume made to 1 litre (1000 ppm Mg). 10 ml of this solution diluted to 100 ml (100 ppm Mg).

Copper standard solution:

0.392g $CuSO_4.5H_2O$ AR grade dissolved in 400ml distilled water in a beaker. It was transferred to a litre volumetric flask and the volume made up to the mark with distilled water (100 ppm Cu).

(4). Chemicals and reagents for estimation of vitamin-C (Direct colorimetric method)

Metaphosphoric acid	2%
2, 6-dichlorophenol-indophenol	100 mg
Sodium bicarbonate	84 g
Ascorbic acid	100 mg
Distilled water	1000 ml

(5). Chemicals and reagents for antioxidant assay (Macwaan and Patel, 2010) and total phenol content estimation (Slinkard and Singleton, 1977).

(DPPH) 1, 1-diphenyl-2-picryl hydrazyl (Cat.: D9132, Sigma)

Vitamin C (Ascorbic acid) (61809405001730, Merck)

Folin-Ciocalteu's phenol reagent (10900105001730,Merck)

Sodium carbonate (61784490511730, Merck)

Potassium persulphate (RM7412-500G, HiMedia)

Gallic acid (RM233-500G, HiMedia)

Methanol (M0120, Ranbaxy)

(6). Media for analysis of occurrence of pathogenic bacteria

Bacillus cereus Agar Base (M833, HiMedia)

Baird Parker Agar Base (M043, HiMedia)

Egg Yolk Emulsion (FD045, HiMedia)

Egg Yolk Tellurite Emulsion (FD046, HiMedia)

Listeria Identification Agar Base (M 1064, HiMedia)

Listeria Selective Supplement (FD 061, HiMedia)

Salmonella–Shigella Agar (M108, HiMedia)

Violet Red Bile Glucose Agar w/o Lactose (M581, HiMedia)

METHODOLOGY

Field surveys were conducted in different regions of Sikkim covering all the four districts viz. North, South, West and East. The following villages and markets of Sikkim were surveyed for collection of information and samples. North district (Mangan, Kabi, Phensong, Phodong, Lachung and Chungthang); East district (Pangthang, Bhusuk, Rongli, Rhenock, Singtam, Aho, Pakyong, Assam Lingey, 6th mile Tadong, Lingding, Rawtey, Rumtek, 9th mile, Sang, Central Pendam, Gangtok, and Ranipool); South district (Namchi, Temi Tarku, Lingey Payoon, Ben, Rabong, Jorethang, Melli and Bermiok); West district (Pelling, Geylzing, Soreng, Reshi and Tashiding).

Based on personal observation and interviews with the key informants, village elders, farmers, vegetable vendors, consumers, vegetable middle man *Kharitey*, 26 species of wild leafy vegetables from the Sikkim Himalaya were documented, out of which, 5 less familiar and common wild leafy vegetable species, were prioritized for detail assessment. Prioritization was done, using a set of criteria, considered to be the main drivers of consumption and their availability in wild. These criteria are (a) taste, (b) frequency of occurrence, (c) ethnic consumer, (d) edibility acceptance, (e) medicinal value, (f) quantity used, (g) market demand, (h) supply volume, (i) supply source, (j) availability season and intensity and (k) preference by younger generation.

Survey was conducted in randomly selected 280 households in different villages located in all four districts of Sikkim. North (40 households), West (70 households), South (70 households) and East (100 households), representing the major ethnic communities, namely the Nepalese, Bhutias and Lepchas. Information was collected on ecological distribution, traditional knowledge, foraging, and mode of consumption, culinary and socio economy of wild leafy vegetables using questionnaire, semi-structured interviews, participant field collection and direct observation . Semi-structured interviews, participant field collection and direct observation were followed to record data on the details of WLVs local names, uses, availability season, collection, preparation and trade. Ethnobotanical information about leafy vegetables was gathered through personal observations and discussions with the villagers. Freelisting interviews with randomly selected informants were conducted. Freelists give information on salience, perception, classification and ranking of objects within a cultural domain in question, here WLVs. Ecological distribution of common and less familiar wild leafy vegetables, consumed by the ethnic people of Sikkim, were documented based on and both the primary and secondary data sources. The data were further enriched by minute details collected through scheduled field visits. Method described by Modi *et al.* (2006) was followed with some modification to collate data collection on wild leafy vegetables.

Collection of samples

The study was carried out in different villages and local periodical markets; locally called *haats*, through semi structured interviews, free listing and direct observation with, 120 Nepali, 90 Bhutia, 70 Lepcha individuals. Plant sample specimens were collected, photographed, and voucher specimens prepared for the herbarium and identified with the help of local taxonomists from Sikkim Government College, Tadong, Botanical survey of India, Gangtok and Sikkim University. Nomenclature follows Flora of Bhutan and local check list.

A total of 20 samples each of prioritized wild leafy vegetable species were collected from different natural habitats in different villages of Sikkim. Similarly, 6 market-samples each, of prioritized WLV species were collected from both rural and urban vegetables markets and periodical *haats* located in the 4 districts of Sikkim. The collected plant materials were placed in a polyethylene bag to prevent loss of moisture during transportation to the laboratory. The leafy parts of these vegetables were washed, cut and shade dried at room temperature. The dried leaves were pulverized, packed in airtight sterile bottles, labeled and stored in a dessicator until used.

Nutritional value

Moisture

Moisture content of the WLVs was determined by drying 2.0–3.0 g of well-mixed sample at 135 ± 1° C for 2 hour to constant weight (AOAC, 1990).

Ash

A sample (~2 g) was weighed into a previously dried and weighed porcelain crucible and placed in a muffle furnace, preheated to 550° C for 3 h. The crucible was transferred directly to desiccators, allowed to cool to room temperature and weighed immediately (AOAC, 1990). The process of heating for 30 min, cooling and weighing was repeated until the difference between two successive weighing was ≤ 1 mg.

Fat

Fat content of the sample was determined by ether extraction using glass soxhlet (AOAC, 1990). Flat-bottomed flask was oven dried and kept in desiccators for cooling. The weight (W_1) of the round-bottomed flask was taken. A cellulose thimble (dry and fat free) was taken and in which ~ 2 g of sample was placed and put in the soxhlet. Fat was extracted by using petroleum ether with boiling range 40-60° C, on a heating mantle at

60° C for 5 hour. The flat bottomed flask was dried for 1 h at 100° C to evaporate ether and moisture, cooled in desiccator and weighed (W_2).

Fat was calculated in percentage:

Fat (%) = W_2–W_1/Sample weight × 100

Crude fiber

Crude fiber was estimated by acid-base digestion with 1.25% H_2SO_4 (prepared by diluting 7.2 ml of 94% conc. Acid of specific gravity 1.835g/ml per 1000 ml distilled water) and 1.25% NaOH (12.5 g per 1000 ml distilled water) solutions. The residue after crude lipid extraction was put into a 600 ml beaker and 200 ml of boiling 1.25% H_2SO_4 added. The contents were boiled for 30 minutes, cooled, filtered through a filter paper and the residue was washed three times with 50 ml aliquots of boiling water. The washed residue was returned to the original beaker and further digested by boiling in 200 ml of 1.25% NAOH for 30 min. The digest was filtered to obtain the residue. This was washed three times with 50 ml aliquots of boiling water and finally with 25 ml ethanol. The washed residue was dried in an oven at 130 ^{0}C to constant weight and cooled in a dessicator. The residue was scraped into a pre-weighed porcelain crucible, weighed, ashed at 550^{0}C for two hours, cooled in a dessicator and re-weighed. Crude fiber content was expressed as

percentage loss in weight on ignition (AOAC, 1990, Nesamvuni et al., 2001).

Protein

Total nitrogen of the sample was determined following the method described in AOAC (1990). Approximately 1 g of sample was taken in a digestion flask, 0.7 g catalyst ($CuSO_4$: K_2SO_4, 1:9) and 25 ml of concentrated H_2SO_4 were added to it. The flask was heated gently until frothing ceased, boiled briskly until the solution became clear and then continued the boiling for about 1 h. The solution was transferred quantitatively to a round-bottomed flask, and mixed with approximately 100 ml of distilled water and 25 ml 4 % w/v aqueous Na_2S to precipitate mercury. A pinch of zinc granules to prevent bumping and a layer of 40 % w/v NaOH were added carefully. The flask was immediately connected to a distillation apparatus and the tip of the condenser was immersed in standard 0.1 N H_2SO_4 containing about 5 drops of methyl red indicator (I007, HiMedia). The flask was rotated to mix the contents thoroughly and heated until all the ammonia had distilled. The receiver was removed and the tip of the condenser was washed with distilled water. The remaining acid in the receiver was titrated with standard 0.1 N NaOH solutions. The blank determination on reagents was considered for correction. Nitrogen was calculated in percentage.

Total nitrogen (%) =

[(ml of standard acid × N of standard acid) - (ml of standard NaOH - C.F.) × N of standard NaOH] × 1.4007/weight of sample (g).

Correction factor (C.F.) =

(titre of standard NaOH against blank - ml of standard acid).

Protein content was determined by multiplying total nitrogen value with 6.25 (AOAC, 1990). Protein (%) = Total Nitrogen (%) × 6.25

Carbohydrate

The carbohydrate content of the samples was calculated by difference: 100 - (% protein + % fat + % ash) (Standal, 1963).

Food Value

Food value of each batter sample was determined by multiplying the protein, fat and carbohydrate contents by the factors 4, 9 and 4, respectively, and adding all the multiplication values to get kcal per 100 g (Indrayan *et al.*, 2005).

Minerals

The method of AOAC (1990) for determination of mineral was followed. The ash after heating the sample at 550° C for 3 h was dissolved in 5 ml of 20 % HCl. The solution was evaporated to dryness on a hot plate at a temperature of 100-110° C and in an oven at 110° C for 1 h. The minerals in the dried residue were dissolved in about 10 ml of 100 % HCl and the solution was heated on a hot plate at a temperature of 100-110° C for 3-4 times. The solution was made up to 100 ml with 1 % HCl. Calcium, sodium and potassium were estimated in flame-photometer Thermo Scientific Chemito (Cat. no. ER15-941-20) at 623, 589 and 766 nm, respectively following the method of Ranganna (1986).

For analysis of magnesium, zinc, copper and iron, Triacid digestion method (Prasad and Bisht, 2011) was followed with modification. It was carried out using HNO_3: $HClO_4$: $H2SO_4$ in the ratio 9:4:1. The collected leaves were dried in an oven at 60-70° c for 2-4 h. The dried leaf samples for each plant material were ground separately in Willey's mill. One gram of ground sample of each plant material was digested in 15 ml of tri-acid mixture. The volume was made up to 100 ml by adding DDW to obtain the solution ready for determination of mineral elements through Atomic Absorption Spectroscopy (AAS) (AA, Perkin-Elmer AAnalyst 200). Standard solution of each element was prepared

and calibration curves were drawn for each element using AAS (Indrayan *et al.*, 2005).

Vitamin-C (Ascorbic Acid)

Vitamin-C was estimated following direct colorimetric determination (Rangana,1986) with some modifications. The direct colorimetric method is based on measurement of the extent to which a 2,6-dichlorophenol-indophenol solution is decolorized by ascorbic acid in sample extracts and in standard ascorbic acid solutions (Loeffler and Ponting, 1942). Since interfering substances reduce the dye slowly, rapid determination would be measuring mainly the ascorbic acid.

1. 2% Metaphosphoric acid in glass distilled water.
2. Dye solution: 100 mg of 2,6-dichlorophenol-indophenol and 84 mg of sodium bicarbonate were dissolved in hot (85-95^0 C) distilled water.
3. Standard ascorbic acid solution: 100 mg of ascorbic acid was accurately weighed and volume made up to 100ml with 2% HPO_3. 4 ml of this solution was diluted to 100ml with 2% HPO_3 (1ml = 40µg of ascorbic acid).
4. Preparation of sample: 10g of sample was accurately weighed and blended with 2% HPO_3 and volume made up to 100ml with HPO_3. The solution was filtered

5. Assay of extract: 2-10 ml aliquot of extract of HPO_3 of the sample was titrated with standard dye to pink end point which persisted for about 15 sec. The titration was done rapidly and a preliminary determination of the titre was made. In the next determination most of the dye required was added and titrated accurately.
6. To the dry cuvettes, the requisite volume of standard ascorbic acid solution - 1, 2, 2.5, 3, 4 and 5 ml was pipette out and the volume made up to 5 ml with requisite amount of 2% HPO_3. With the help of a rapid delivery pipette, 10 ml of dye was added and shaken. The reading was taken within 15-20 seconds. The instrument was set to 100 % transmission using blank consisting of 5 ml of 2% HPO_3 solution and 10 ml of water. The red colour was measured at 518 nm. The absorbance against concentration was plotted.
7. Sample: 5 ml of the extract were placed in a cuvette and 10 ml of dye was added and measured as in standard.
8. Calculation: The concentration of ascorbic acid was noted from the standard curve and the ascorbic acid content in the samples was calculated with the following formula.

AA mg /100g = Ascorbic acid content x Volume made up to x 100 /ml of solution taken for estimation x 1000 x wt or volume of sample taken

Extract Preparation

The edible parts of leafy vegetables were washed in running water. The leaves were chopped into small pieces and shade dried in room temperature. The dried materials were ground to a coarse powder and extracted (20 g) successively with 200 ml methanol in a glass soxhlet extractor at 130^{0}C for 24–48 h. The extract was concentrated by using rotary evaporator. The extract was preserved in a dessicator till further use.

Total Phenolic Content

Total soluble phenolic compounds present in extracts were determined with the Folin-Ciocalteu reagent (Slinkard and Singleton, 1977) with modification. Calibration curve was prepared by mixing methanolic solution of gallic acid 1 ml, (10–100µg/ml) with 1ml Folin-Ciocalteu reagent, (diluted tenfold) and sodium carbonate (2%). The absorbance was measured at 760 nm using Lamda 25 UV Vis Spectrophotometer (Perkin Elmer/L 600-00 BB) and drew the calibration curve. 1 ml Methanol extract of the sample (100µg/ml) was also mixed with the reagents above and after 2 hrs the absorbance was measured to determine total plant phenolic contents. The total content of phenolic compounds in the extract of gallic acid equivalents (GAE) was calculated by the following formula: T= C.V/M

Where,

T= total content of phenolic compound, milligram per gram plant extract, in GAE.

C= the concentration of gallic acid established from the calibration curve, milligram per milliliter.

V= the volume of extract, milliliter.

M= the weight of methanolic plant extract, gram.

Antioxidant activity

The antioxidant activity was analyzed by estimating free radical scavenging activity following DPPH method of Macwaan and Patel (2010) with modifications.

0.1mM solution of DPPH (1, 1-diphenyl-2-picrylhydrazyl), in methanol was prepared and 1.0 ml of this solution was added to 3.0 ml of extract solution in methanol at different concentration (10-100 µg/ml). Thirty minutes later the absorbance was measured at 517 nm using Lamda 25 UV Vis Spectrophotometer (Perkin Elmer/L 600-00 BB). A blank was prepared without adding extract. Ascorbic acid at various concentrations (10-100 µg/ml) was used as standard. Lower absorbance of the reaction mixture indicates higher free radical scavenging activity. The capability to scavenge the DPPH radical was calculated using the following equation.

DPPH scavenged (%) = Abs (control) - Abs (test)/ Abs (control) x 100 Where Abs (control) is the absorbance of the control reaction and Abs (test) is the absorbance in the presence of the sample of the extracts. The antioxidant activity of the leaf extracts were expressed as IC_{50}. The IC_{50} value was defined as the concentration (in µg/ml) of extract that scavenges the DPPH radical by 50 %.

Microbiological safety analysis

Ten g of sample were homogenised with 90 ml of 0.85 % (w/v) sterile physiological saline in a stomacher lab-blender (400, Seward, UK) for 1 min. Serial dilutions (10-1 to 10-8) in the same diluents were made.

TVC: Total viable count (TVC) was determined in the plate count agar (M091A, HiMedia) plates which were incubated at 30° C for 48-72 h.