Chapter 3

MATERIALS AND METHODS

3. MATERIALS AND METHODS

3.1 STUDY AREA

Sikkim is a small hilly but naturally very beautiful northeastern state of India. It measures 7,069 sq. km. in size and ranges between 300m to 8,568m above sea level. It comprises of four districts viz. East, West, North and South. The present investigation was carried out in West district of Sikkim. West district is the second largest district of Sikkim situated in the south western part of the State between 27000'46" -28007'48" N latitude and 88000'58- 88055'25" longitude covering an area of 1166 Sq. Km.

3.1.1 Temperature

Temperature varies sharply with altitude and slope. The mean temperature varies from about 26.80°C in September to 20.70°C in the month of January. Mean daily minimum temperatures are around 7.50°C in January to 13.30°C in October. The mean monthly wind speed varies from as low as 43.52 km/day from July to September to high of 98.4 km/day in the month of April.

3.1.2 Rainfall

The area experiences a heavy rainfall during the monsoon season due to it being very close to the Bay of Bengal. The annual rainfall ranges from less than 5 mm to nearly 4000 mm. For most of the period in a year, the climate is cold and humid as rainfall occurs in each month. The river Rangit and its tributaries are the main channels of drainage, run nearly north-south. The valleys cut by this river and their chief feeders are very deep. District is mainly formed of Precambrian rock of young age constituting hard massive gneissose rocks. The total forest cover of the district is 56.31 per cent of the total geographical area (Envis).

3.1.3 Sunshine Hours

Average daylight hours are shorter during the months of November, December, January, February and March but the average sunshine hours in these months is higher than rest of the months of a year. From April daylight light hours increases, reach the highest during the month of June and July and gradually start to decrease from the month of August. However the average sunlight hours stars decreasing from the month

of April and reaches minimum in July and August. Average sunshine starts increasing again from the month of October.

3.2 SURVEY

Prior to the survey, extensive literature review was carried out including the internet search. Following steps were taken to carry out the survey:

a) Different villages were selected from all four blocks of West district of Sikkim.
b) Information was collected from the local inhabitants with informal interviews regarding their primary healthcare and existing herbal healers in their region.
c) The herbal healers were shortlisted according to the information collected from the local people on the basis of their reputation of healing the patients.
d) The herbal healers were visited and interviewed following a questionnaire regarding their knowledge of medicinal plants and their healing experiences.
e) Medicinal plants were collected from the nearby areas with the help of the healers.
f) Plants were identified from the Taxonomy and Biosystematics Laboratory, Department of Botany, University of North Bengal.
g) Available information of the identified plants was reviewed in literature. The plants on which no experimental work has been done were shortlisted. The shortlisted plants were again compared with the information related with traditional knowledge and therapeutic activity collected from healers.
h) The ethnomedicine on which there was no report of any scientific study were selected on the basis of their effectiveness and availability.

3.3 PHARMACOGNOSTIC STUDY

The fresh plants or plant parts collected from the herbal healers were washed and sun dried for few days. After the samples were completely dried, it was ground to powder form. For pharmacognostic study, powdered samples were used except for anatomy where fresh plant parts were used to cut the sections.

3.3.1 Organoleptic Tests and Powder Microscopy

The colour, odour, texture and taste of the powdered samples were noted down (Kokate *et al.*, 2002). Powder microscopy was done following the standard method of Lala (1981). The fine dried powder of sample was mounted in 80% glycerin on a glass slide to observe general structures of plant tissues. To observe starch granules, a pinch of powder sample was stained with (N/50) Iodine solution. It was then mounted on glycerin and observed under a microscope. Starch granules were stained with blue colour. For lignified tissues, powder sample was stained with phloroglucinol solution (0.1% in 90% ethanol). It was allowed to dry which was followed by addition of few drops of concentrated HCl on the slide and observed under microscope. Red to pinkish colour was taken up by the lignified tissues. To observe any fixed oil or fat globules, the powder sample was mounted in Sudan III (0.01g in 5ml 90% ethanol). The slide was warmed gently but it should not be completely dried out. A few drops of glycerin were added and orange to brownish coloured fat globules were observed under a microscope.

3.3.2 Physicochemical Study

The following physicochemical attributes were analyzed following the methods of Harborne (2005):

3.3.2a Determination of total ash

It is the method of measuring the amount of inorganic residual substance when the sample is ignited. Powder sample (2 g) was weighed and placed in silica crucible which was already ignited for removal of moisture and cooled and then weighed to take the initial weight. The silica crucible containing powder sample was ignited for 15 min after reaching 450°C with gradually increasing the heat but not exceeding 450°C. The crucible was taken out and cooled in a desiccator. After cooling it was weighed again to calculate the ash percentage. At least two consecutive constant weights were taken and the results were expressed as range of mean values ± SD.

3.3.2b Determination of acid insoluble ash

The ash obtained from total ash is boiled for 5 min in 25 ml of diluted HCl (2M). It was filtered on a filter paper and the insoluble matter was collected. Hot water was poured to wash off the remaining acid. The filtered ash on filter paper was put in a crucible and ignited. The ash was taken to weigh after cooling. The percentage of acid insoluble ash was calculated with reference to the air dried powder.

$$Ash\ \% = \left(\frac{loss\ in\ weight}{W}\right) x100$$

$$Loss\ in\ weight = final\ weight - initial\ weight$$

W= Weight of air-dried powder

3.3.2c Determination of water soluble ash

The ash obtained from total ash determination was boiled for 5 min in 25 ml of distilled water. The insoluble matter was collected after filtration and put in a crucible to be ignited for 15 min with the temperature not exceeding 450°C. The crucible was cooled down and weighed to get the final weight.

$$Ash\ \% = \left(\frac{water\ soluble\ ash}{W}\right) x100$$

$$Water\ soluble\ ash = weight\ of\ ash - weight\ of\ insoluble\ matter$$

W= weight of air dried powder

3.3.2d Determination of solvent extractive values

3.3.2d (i) Alcohol soluble extractive value

To 5 g of powder sample, 100 ml ethanol was added in a closed conical flask. It was kept for 24 hours (6h frequent shaking and 18h in rest). The mixture was filtered through a filter paper and 25 ml of filtrate was evaporated to be completely dried in a beaker (already weighed to get initial weight). The beaker with dried sample was

weighed again to get the final weight. Extractive percentage was calculated with reference to air-dried powder.

3.3.2d (ii) Water soluble extractive value

Powder sample (5g) was macerated in chloroform water in a closed conical flask for 24 hours (6 hr shaking and 18 hr standing). The mixture was filtered and 25 ml of filtrate was evaporated to dryness at 105°C and final weight was taken. The percentage extractive value was calculated with reference to air-dried powder.

3.3.2e Loss on drying

Powdered sample (1g) was put in a glass plate (weigh initially if the glass plate was already taken). It was kept in an oven at 105°C to 110°C for 5 hours. After 30 min, it was cooled at room temperature in a desiccator. The three constant weights were taken.

$$Loss\ on\ drying\ percentage = \left(\frac{Loss\ in\ weight}{W}\right) x100$$

$$Loss\ in\ weight = Final\ weight - initial\ weight$$

W = Weight of the fresh powder

3.3.2f Determination of pH values

Crude powder (1g) was put in 1% and 10% HCl w/v separately and kept at 24°C for 10 mins shaking gently. pH values were taken in a pH meter and the values were noted down.

3.3.3 Fluorescence analysis

The powder sample as such and after treatment with various chemical reagents was subjected to fluorescence analysis. Observations were made under visible light and under UV light of short (254 nm) and long wave length (365 nm) separately (Dave *et al*., 2010). The colours were identified according to the standard colour chart of RAL.

3.4 THIN LAYER CHROMATOGRAPHY (TLC)

For the identification of plant drugs on the basis of its active phytoconstituents, TLC was performed. It was done to analyze the variation in bioactive phytoconstituents present in the ethnomedicinal plants (Wagner and Bladt, 2009). The experiment was carried out in a readymade TLC plates (Silica Gel 60 F_{254} coated on aluminium sheets) purchased from Merck Germany. The powdered plant samples were extracted with different procedures which were specific to each of the active phytoconstituents i.e. alkaloids, arbutin, anthraglycosides, bitter principles, cardiac glycosides, coumarins, flavonoids and saponins. The solvent systems for mobile phase were also varied according to the phytoconstituents. For the detection of arbutin, anthraglycosides, bitter principles, cardiac glycosides and alkaloids; ethyl acetate: methanol: water (100:13.5:10) was used. Ethyl acetate: formic acid: glacial acetic acid: water (100:11:11:26) was taken for identification of flavonoids. For saponin identification, chloroform: glacial acetic acid: methanol: water (64:32:12:8) was used while for the identification of coumarins, toluene: ethyl acetate (93:7) was used. When chromatograms were developed, they were analyzed for presence of different phytochemicals by spraying with a particular group reagent/s. The various detecting reagents used were 10% ethanolic KOH for anthraglycosides and coumarin, Berlin blue reagent for arbutin, vanillin sulphuric acid reagent for bitter principles, antimony chloride ($SbCl_3$) reagent for cardiac glycoside, NP/PEG reagent for flavonoids and Dragendorff reagent for alkaloids. After detection, the chromatograms were observed under visible, UV-254 nm and UV-365 nm light. Photographs were taken with DSLR Nikon camera (D-3200) and the R_f values were calculated with the following formula.

$$Rf\ value = \frac{Distance\ travelled\ by\ solute}{Distance\ travelled\ by\ solvent}$$

3.5 QUALITATIVE PHYTOCHEMICAL ESTIMATION

The fresh plants or plant parts were sun dried and ground to powder in a mixture grinder. The powder was extracted through Soxhlet apparatus in ten different solvents according from non-polar to polar nature (Hexane< Heptane< Benzene< Ethyl acetate<

Chloroform< Acetone< Butanol< Ethanol< Methanol< Water). All the extracts were dried and dissolved in methanol except the extract obtained in water. Desired volume was measured and stored in brown bottles for further analysis.

3.5.1 Test for amino acids

The amino acids present in plants were detected with Ninhydrin test (Saxena and Saxena, 2012). With 0.5 ml of extract, equal amount of methanol was poured. Few drops of Ninhydrin reagent (30 mg ninhydrin in 10 ml n-butanol put in 0.3 ml 98% acetic acid). The mixture was heated in water bath for a minute. Appearance of purple colour indicates the presence of amino acid.

3.5.2 Test for anthraquinones

Methanolic extract (1 ml) was evaporated in a test tube to make it completely dry; 2 ml of chloroform was added to it followed by the addition of 2 ml ammonia. Red or orange colour on the bottom appears to be anthraquinones (Kumar *et al*., 2009).

3.5.3 Test for phytosterol

Methanolic extract of the sample (0.5 ml) was completely evaporated and dissolved in 2 ml chloroform. Concentrated sulphuric acid (H_2SO_4) was added to it gently from the side of the test tube. Red colour ring in the middle of the mixture indicates the presence of phytosterol in the extract (Ngbede *et al*., 2008).

3.5.4 Test for tannin

Presence of tannin in an extract can be done by lead acetate test (Kumar *et al*., 2009). In 0.5 ml of the extract, 0.5 ml of methanol was added followed by adding 1% lead acetate (dissolved in methanol). Brownish to slightly yellow coloured precipitate indicates the presence of tannin.

3.5.5 Test for triterpenoids

About 0.5 ml of the sample extract was evaporated and dissolved in 1 ml chloroform and 1 ml of acetic anhydride was added. The mixture was cooled down by keeping it in a beaker filled with ice cubes. After cooling, 2 ml concentrated H_2SO_4 was added. The reddish violet colour of the mixture indicates the presence of triterpenoids (Kumar *et al*., 2009).

3.5.6 Test for cardiac glycosides

About 0.5 ml of extract was evaporated and dissolved in 1 ml glacial acetic acid. Few drops of 10% ferric chloride were supplemented and 1 ml of concentrated H_2SO_4 was added slowly from the side of the test tube. The upper layer (acetic acid layer) appears to be green in colour and a brown ring at the interface was formed which showed the presence of cardiac glycosides in the extract (Ngbede *et al*., 2008).

3.5.7 Test for alkaloids

Alkaloids were detected in an extract with Dragendorff's reagent (Kumar *et al*., 2009). Firstly Dragendorff's reagent was prepared for which 0.6 g bismuth subnitrate in 2 ml conc. HCl and 10 ml water and 6g potassium iodide was dissolved in 10 ml water. After mixing these two solutions, 7 ml conc. HCl and 15 ml water added to the mixture. The mixture was finally diluted with 400 ml of water. The Dragendorff's reagent is prepared and stored in dark bottle for few days. For the experiment, 0.2 ml of Dragendorff's reagent was added to 0.5 ml extract. After shaking gently, if the mixture produced orange to red precipitate it indicated the presence of alkaloids in the extract.

3.5.8 Test for glycosides

To 0.5 ml methanolic extract, 2 ml of 50% HCl was added. It was hydrolysed in a water bath for 2 hours. Then 1 ml pyridine was added followed by few drops of 1% sodium nitroprusside solution and 5% sodium hydroxide solution. Appearance of pink to red colour showed the presence of glycosides (Kumar *et al*., 2009).

3.5.9 Reducing sugar

Presence of reducing sugar in an extract was determined by Fehling's test (Ibrahim and Ibrahim, 2009). For this test, Fehling's A and B solution were prepared. For Fehling's A solution, 7 g copper sulphate was dissolved in water and the total volume was made up to 10 ml. For Fehling's B, 24 g of potassium hydroxide and 34.4 g sodium potassium tartarate were dissolved in water and volume was made up to 100 ml. For the experiment, 1ml each of Fehling's A and B solutions were added to 0.5 ml of methanolic extract. The mixture was boiled in a water bath for 2 min. Brick red precipitate in the mixture indicated the presence of reducing sugar in the extract.

3.5.10 Test for flavonoids

To 1 ml of methanolic extract, few drops of 10% ferric chloride were added. Blue green colour in the mixture indicates the presence of flavonoids (Ibrahim and Ibrahim, 2009).

3.5.11 Test for resin

First of all, 0.5 ml of extract was evaporated and dissolved in 1 ml petroleum ether. After dissolving, 2 ml of 2% copper acetate was added and shaken vigorously for mixing the layers. Green colour in the lower layer indicated the presence of resin (Kumar *et al*., 2009).

3.6 QUANTITATIVE ESTIMATION OF PHYTOCHEMICALS

3.6.1 Total alkaloid content (TAC)

TAC was determined by the method of Manjunath *et al* (2012) based on the reaction between alkaloid of the sample with Bromocresol green (BCG). BCG was prepared by dissolving 0.007 g BCG in 2(N) NaOH and 5 ml water was added followed by heating until BCG was completely dissolved. The final volume was made up to 100 ml by adding distilled water. For experiment, 0.5 ml methanolic extract was evaporated and dissolved in 0.5 ml 2(N) HCl. The solution was filtered and washed with 2 ml chloroform. 2.5 ml BCG solution and 2.5 ml phosphate buffer (pH-4.7) were added to

the filtrate. The solution was thoroughly vortexed to mix uniformly. The chloroform part appeared to be yellow if alkaloids were present thus it was taken to obtain OD values at 470 nm in a UV-visible spectrophotometer. TAC was expressed as solasodine equivalents (SE).

3.6.2 Total tannin content (TTC)

TTC was determined by the method using Folin-Denis reagent (Thimmaiah, 1999). Folin-Denis reagent was prepared by mixing 10 g sodium tungstate and 2 g phosphomolybdic acid dissolved in 75 ml distilled water followed by adding 5 ml phosphoric acid. The mixture was refluxed for 2 hours and volume was made up to 100 ml. The reagent was stored in dark by covering it and stored in refrigerator. Sodium carbonate solution was also prepared by dissolving 35g sodium carbonate in 100 ml distilled water at 70°C to 80°C. The solution was allowed to stand overnight at room temperature and it was filtered before use. To 0.1 ml of extract, 5 ml distilled water and 0.5 ml of Folin-Denis reagent were added. To the mixture, 1 ml of sodium carbonate solution was added. After 30 min incubation at room temperature, the solution appeared dark blue in colour. Absorbance was taken at 700 nm for OD values. Tannic acid was used as standard and TTC was expressed as Tannic acid equivalents (TAE).

3.6.3 Total steroid content (TSC)

Steroid present in extracts was estimated by the method of Liebermann Burchard's reagent (Naik and Mishra, 2015). The reagent was prepared by adding 0.5 ml H_2SO_4 in 10 ml acetic anhydride. To 1 ml of sample extract, 4 ml of chloroform was added and mixed thoroughly. After that, 1 ml chloroform layer was taken and evaporated completely. Then 2 ml Liebermann Burchard's reagent was added and mixed thoroughly in a vortex. The mixture was kept in dark for 20 min to incubate. The formation of green colored complex in the mixture showed the presence of steroids in the extract. Absorbance was taken at 640 nm. The values were expressed as SE.

3.6.4 Total phenol content (TPC)

It was estimated by using Folin-Ciocalteau reagent (Singleton and Rossi, 1965). To 1 ml methanolic extract, 1 ml 95% ethanol and 5 ml distilled water was mixed well. To the mixture, 0.5 ml 50% Folin-Ciocalteau reagent was added. After 5 min incubation, 1 ml 5% sodium carbonate was added. The mixture was then incubated for an hour at room temperature. The green color in mixture showed the presence of phenol in the extract. OD values were taken at 725 nm. Values were expressed as quercetin equivalents (QE).

3.6.5 Total flavonoid content (TFC)

To determine TFC, 0.5 ml methanolic extract was diluted in 4 ml distilled water. Then 0.3 ml 5% sodium nitrite was added and incubated at room temperature for 5 min. After incubation, 0.3 ml 10% aluminium chloride was added and again incubated for 6 min at room temperature. Lastly, 2 ml 0.1 M sodium hydroxide was added to the mixture and diluted with 2.4 ml distilled water. The mixture was shaken well as a result pink colour appeared and absorbance was taken at 510 nm (Zhishen *et al*., 1999).

3.6.6 Total orthodihydric phenol (TOPC)

The method of Kim *et al* (2003) was used to estimate TOPC in the extracts by using Arnow's reagent. To prepare the reagent, 10 g of sodium nitrite and 10 g of sodium molybdate were dissolved in 100 ml distilled water and stored in a dark bottle. The experiment was followed by adding 0.5 ml 0.05 N HCl and 0.5 ml Arnow's reagent in 0.5 ml extract. The mixture was vortexed and 5 ml distilled water was added and vortexed again to mix well. To the mixture, 1 ml sodium hydroxide 1 (N) was added and mixed thoroughly. Appearance of pink colour indicated the presence of orthodihydric phenol. Absorbance was taken at 515 nm. The TOPC was expressed in terms of Catechol equivalents (CE).

3.7 ANTIMICROBIAL ACTIVITY

Total of five bacterial strains belonging to Gram positive and Gram negative species were used for this study. *Escherichia coli* (ATCC 14169) and *Salmonella typhi* (ATCC 51812) were Gram negative bacteria and *Bacillus subtilis* (ATCC 11774), *Bacillus megaterium* and *Staphylococcus aureus* (ATCC 11632) were Gram positive. All the strains were purchased from HiMedia. Due to their ability to survive in harsh conditions and their multiple environmental habitats, these bacterial organisms including Gram positive and Gram negative are the main source to cause severe infections in humans (Ahameethunisa and Hoper, 2010).

The method used to determine antimicrobial activity of the herbal formulation extracts was disc diffusion method. Antibacterial activity of tested plants parts was carried out by the disc diffusion method (Gulluce *et al*., 2007). First, each herbal medicine was extracted in ethanol and distilled water separately in a soxhlet apparatus. Agar media was prepared and 20 ml was poured into a petriplate. The plate was filled with 100 µl bacterial strain prepared in a broth. It was left to incubate for 24 hrs at 37°C for bacterial growth. The sterile disc of 6 mm diameter made from Whatman no.1 filter paper was dipped in an extract of different concentration. The solvent absorbed by the disc was completely evaporated in laminar airflow and placed on the surface of agar plate. The plate was incubated for 12 hrs at 37°C. One control plate was used containing streptomycin with four concentrations (25, 50, 100 and 500µg/ml). The diameters of inhibition zones around the discs were measured (Rahmoun *et al*., 2014).

3.8 *IN VITRO* CYTOTOXIC ACTIVITY

The effect of powder plant samples on human liver cell line (WRL-68) was measured by following the MTT assay method described by Denizot and Lang (1986) with some modifications. The reduction of yellow tetrazolium MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] by metabolically active cells by the action of dehydrogenase enzymes generates NADH and NADPH. The intracellular formazan (purple) thus produced can be solubilized and quantified by spectrophotometric method. MTT assay is a colorimetric, fast economical and

nonradioactive method which can be used to measure cell viability as well as proliferation of mammalian cells. ACHN, the cancerous cell line was seeded in 96 well plates containing 5×10^3 cells/well in 100 µl culture medium. It was incubated for 24 hr at 37ºC in a humified incubator with 5 % CO_2. After incubation cells were attached on the well and were washed with PBS (Phosphate-buffered saline) twice and treated with different aliquots of the selected plant extracts and kept for 24 hrs. MTT solution prepared in PBS (10 µl of 5mg/ml/well) were put in wells after 24 hrs of treatment which is again incubated for 3 hours at 37ºC. Formazan crystals were solubilized by adding 50 µl isopropanol. The plates were then gently shaken for 1 min and the absorbance was measured at 620 nm by microliter plate reader (BMG LABTECH SPECTRO star^{Nano} Germany). Absorbance values lower than control cells indicate a decrease in the rate of cell proliferation. While a higher absorbance rate shows the increase in cell proliferation.

3.9 *IN VITRO* ANTIDIABETIC ACTIVITY

In vitro antidiabetic activity of the herbal formulation extracts was determined by their ability to scavenge alpha glucosidase enzyme (Oki *et al*., 1999). 2.5 ml of sodium phosphate buffer (6.8 pH), 0.1 ml reduced glutathione (3 mM) and 0.1 ml (50 µg/ml) α-glucosidase enzyme were mixed well in a vortex and incubated for 15 min at 37°C. After incubation, 0.5 ml inhibitor (extract or standard) and 0.25 ml ρ-nitrophenyl-α-D-glucopyranoside (p-NPG) solution were added to the reaction mixture and again incubated for 15 min at room temperature. Sodium carbonate (4 ml) was added to terminate the reaction and the absorbance was taken at 405nm. Inhibition percentage was calculated by the following formula.

$$Inhibition\ percentage = \left(\frac{Control - test\ sample}{control}\right) x100$$

Control- Absorbance with enzyme and p-NPG without inhibitor

Test sample- Absorbance with enzyme, p-NPG and inhibitor

3.10 ANTIOXIDANT ACTIVITY

3.10.1 1,1-diphenyl-2- picrylhydrazyl scavenging assay (DPPH)

To observe the ability of extracts to scavenge DPPH was performed by the method of Blois (1958). 2 ml DPPH solution and 0.2 ml methanolic extract were mixed together. The purple colour of DPPH was scavenged by the extracts and the variation in the colour due to different concentration of extract was measured at 517 nm. IC_{50} values were calculated by plotting a standard curve from the inhibition percentage against concentration. Percentage inhibition was calculated by the following formula:

$$Inhibition\ percentage = \frac{(Absorbance\ \ of\ control - Absorbance\ \ of\ sample\ )}{Absorbance\ \ of\ control} x\ 100$$

3.10.2 2, 2'-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt scavenging activity ($ABTS^+$)

ABTS solution was prepared by mixing 10 ml ABTS (7 mM) and 10 ml potassium persulphate (2.4 mM) and kept in dark for 12 hr. The solution was diluted 8 times before use. $ABTS^+$ solution (2 ml) was added to 1 ml extract and incubated for 10mins. The scavenging activity was measured at 734 nm (Re *et al*, 1999). Inhibition percentage was calculated.

3.10.3 Superoxide scavenging activity (SO)

SO of extracts was determined by the reduction of nitro blue tetrazolium chloride (NBT) by the method of Nishikimi *et al* (1972). 0.1 M phosphate buffer was prepared (pH-7.4). 312 µM NBT, 936 µM Nicotinamide dinucleotide trihydrochloride (NADH) and 120 µM phenazine methosulphate (PMS) were prepared by dissolving them in buffer. A reaction mixture containing 1 ml sample and 1 ml NBT (312 µM) was kept for 5 min. NADH (1 ml) was added to the mixture and again kept for 5 min. To the reaction mixture, 1µl PMS was added and incubated for 30 min under exposure to fluorescent light. Absorbance was taken at 560 nm. Inhibition percentage was calculated.

3.10.4 Nitric oxide scavenging activity (NO)

Sodium nitroprusside produce nitric oxide which reacts with oxygen to generate nitrite ions. These ions are detected by Greiss reagent turning the reaction mixture dark purplish pink in colour (Marcocci *et al*., 1994). Nitric oxide scavengers compete with oxygen to decrease the production of nitrite ions. 2 ml of 20 mM sodium nitroprusside and 0.5 ml phosphate buffer (pH-7.4) were added to 0.5 ml of extract. The mixture was incubated for 150 min at 25°C. After incubation, 3 ml Greiss reagent was added and again incubated for 30 min at room temperature. The scavenging activity of nitric oxide was measured at 540 nm. Percentage inhibition was calculated.

3.10.5 Metal chelating activity (MC)

Chelation of ferrous ion by extracts was measured following the method of Dinis *et al* (1994). Methanolic extract (0.4 ml) was diluted with 1.6 ml of methanol. To the mixture, 0.04 ml of ferrous chloride (2 mM) and 0.80 ml of ferrozine (5 mM) were added. The reaction mixture was incubated for 10 min at room temperature. Absorbance was taken at 562 nm. Inhibition percentage was calculated.

3.10.6 Ferric reducing antioxidant power (FRAP)

Methanolic extract (1 ml) was taken to which 2.5 ml sodium phosphate buffer (0.2 M) and 2.5 ml potassium ferricyanide (1%w/v) were added followed by incubation for 20 min at 50°C. To the mixture, 2.5 ml 10% trichloroacetic acid (TCA) was added and the mixture was cooled down by keeping the test tubes on a beaker with ice cubes. It was then centrifuged at 3000 rpm for 10 min. The upper layer was collected and 2.5 ml distilled water was added to it followed by adding 0.25 ml ferric chloride (0.1%). A green colored complex was formed by the reaction among potassium ferricyanide, trichloroacetic acid and ferric chloride. Increase in colour indicates more antioxidant activity. The OD values were taken at 700 nm (Oyaizu, 1986).

3.11 ANTI-HYPERTENSIVE ACTIVITY (AHA)

AHA was determined by angiotensin converting enzyme (ACE) inhibiting activity of the extracts followed by the method of Lin *et al* (2012) with some modifications. This assay is composed of phosphate buffer (pH-8.3), 8 mM Hippuryl-histidyl-leucine (HHL), ACE from rabbit lung of 2 U/mg protein, 1 N HCl and ethyl acetate. 110 µl HHL was added in 25µl ACE (5 mU/mg) followed by adding plant extract and allowed to incubate for 1hr. After incubation, the reaction was terminated by adding 250 µl HCl. During this reaction, Gly-His bond of HHL was cleaved and the Bz-Gly (hippuric acid) was produced. The hippuric acid was extracted with 1ml ethyl acetate (upper layer). Ethyl acetate was then evaporated completely and the remains were dissolved in 1ml distilled water. The absorbance was taken at 228 nm. ACE inhibiting activity of the extracts was calculated by obtaining inhibition percentage by the following formula.

$$ACE\ inhibition\ percentage = \left(\frac{Control - reaction\ mixture}{Control - blank}\right) x100$$

Control- absorbance with HHL and enzyme without extract (no inhibition).
Reaction mixture- absorbance with HHL, enzyme and extract.
Blank- absorbance with HHL without enzyme and extract.

3.12 INFLUENCE OF EXTRACTION METHODS ON THE BIOACTIVITY OF *FRAXINUS FLORIBUNDA*

The aqueous decoction of bark of *Fraxinus floribunda* Wallich was used for treating diabetes by some herbal practitioners. This study was done to observe the influence of variation in extraction methods in the bioactivity of the same plant. Four different types of extraction methods were used as follows:

i) Normal boiling (NB) The dried powder of bark was boiled for 2 hr at room temperature through refluxing which was similar to the traditional method used by the folk healers.

ii) Soxhletion (S): The dried powder bark was extracted in a soxhlet apparatus for 8 hrs which is a conventional method of plant extraction.

iii) Autoclave pressure boiling (AB): The dried powder plant sample was kept in an autoclave for 15 min under high pressure (1.5 kg cm-2) and high temperature (121˚C).

iv) Cold percolation (CP): The bark powder was kept in water in a beaker at below -4°C for 48 hr. The sample was filtered and used for analysis.

All the extracts were filtered and volume was made for 100 mg/ml FWT (fresh weight tissue) and stored in plastic bottles in a refrigerator. The parameters used to determine the bioactivity were quantitative phytochemical estimation (TPC, TFC, TOPC) and antioxidant activity (DPPH, ABTS, SO, NO, MC, FRAP) by the methods as described before.

3.13 *IN VIVO* PHARMACOLOGICAL ACTIVITY

The herbal formulation which showed the best activity in terms of the experiments performed above was subjected to some pharmacological activities in animal model.

3.13.1 Antidiabetic activity in streptozotocin-induced diabetic rats

The animals used for this study were Wistar albino rats (150-250 g). They were bred in Animal House of Columbia Institute of Pharmacy, Raipur. Large propylene cage were used to house these animals and they were kept at 22 ± 2°C in 12 hours dark light cycle of 12:12 hours with relative humidity 55-65%. They were fed with pellet food and water *ad libitum*. The experiments were approved by Institutional Animal Ethics Committee of Columbia Institute of Pharmacy, Raipur, India (Regd. No. 1321/PO/ReBi/S/10/ CPCSEA). All the animals were acclimatized for at least 1 week before the experimental session.

3.13.1a Acute toxicity study

The acute toxicity study of the plant extract was evaluated according to the CPCSEA guideline no 420 (fixed dose method). The aqueous extracts were orally fed to the rats at the dose level of 5, 50, 300, and 1000 mg/kg, respectively. The test showed no mortality even at maximum dose of 1000 mg/kg body weight (b.w.). Hence, 200 mg/kg and 400 mg/kg doses were selected for further study.

3.13.1b Oral glucose tolerance test (OGTT)

The oral glucose tolerance test was performed in overnight fasted (18 hr) normal rats. Rats were divided into three groups (n = 6). First group were administered with drinking water, second with plant extract of 200 mg/kg and third group with 400 mg/kg extract. Glucose (0.5 mg/ kg) was given to the animals 30 min prior to the administration of extracts. At 30 and 90 min of extract administration, blood was withdrawn from the retro orbital sinus and the plasma obtained after centrifugation at 3000 rpm was taken for the estimation of fasting plasma glucose levels using a glucose oxidase-peroxidase glucose estimation kit.

3.13.1c Induction of non-insulin dependent Diabetes mellitus (NIDDM)

NIDDM was induced in overnight fasted adult Wistar albino male rats weighing 150-220 gm by a single intraperitoneal injection of 60 mg/kg streptozotocin (STZ), 15 min after the i.p. administration of 120 mg/kg of nicotinamide. STZ was dissolved in citrate buffer (pH 4.5) and nicotinamide dissolved in normal saline. Hyperglycemia in rats was confirmed by the elevated glucose levels in plasma determined at 72 hr and then on day 7 after injection. The starting value of fasting plasma glucose to identify diabetes was taken as > 126 mg/dl. Only those rats which were found to have permanent NIDDM were used for the study.

3.13.1d Bodyweight

The animals were divided into five groups with six rats each. The extract was administered for 12 days. Group I considered as normal control in which rats were

given drinking water daily for 12 days; Group II consisted of STZ induced diabetic control rats administered drinking water daily for 12 days; Group III was diabetic rats administered standard drug Glibenclamide (0.50 mg/kg) for 12 days; Group IV diabetic rats administered plant extract of 200 mg/kg b.w.; Group V had diabetic rats administered 400 mg/kg body weight extract. The fasting glucose levels were determined on day 0, 7, 14 and 28 of extract administration. Throughout the experimental period, the rats were weighed daily and the average change in body weight was calculated (Singh *et al*., 2011; Sharma *et al*., 2010).

3.13.1e Estimation of biochemical parameters

On day 12, the animals were sacrificed by cervical dislocation to determine the biochemical parameters. The tests carried out were total cholesterol (TCL), triglycerides (TGL), high-density lipoprotein (HDL), low-density lipoprotein (LDL) by glucose oxidase method using auto-analyzer (Nyarko *et al*., 1993; Barham and Trinder, 1972).

3.13.1f Histopathology

Liver were isolated from the sacrificed animals and were subjected to histopathological studies and microscopical findings were noted.

3.13.2 ANTI-INFLAMMATORY ACTIVITY

Anti-inflammatory activity of carrageenan-induced paw edema was performed according to the method described by Winter and Porter (1957). Wistar albino rats were divided into three groups (n=6). Group 1 (control) was administered with 0.9% solution of NaCl at a dose of 0.1 ml/100 g. Group 2 consisted of rats administered with extract at 100 mg/kg b.w. and group 3 with standard drug aspirin at the dose of 10 mg/kg b.w. Carrageenan solution (1%) was injected the planter surface of rat hind paw after 30 min of drug administration at the dose of 0.1 ml/100 g b.w. to induce acute inflammation. The paw edema volume was measured at the intervals of 30, 60, 120, and 180 min using plethysmometer. The paw edema among different groups was compared and percentage inhibition was calculated by the formula,

$$Percentage\ inhibition\ of\ paw\ edema = \left(\frac{Vc - Vt}{Vc}\right) x100$$

V_c = Paw edema of control animals,

V_t = Paw edema of drug-treated animals.

3.13.3 HEPATOPROTECTIVE ACTIVITY

Hepatoprotective activity of the plant extract was determined by CCl_4 (carbon tetraclroride) -induced hepatotoxicity in Wistar rats (150-200 g). Before the experiment, the animals were fasted overnight. Plant extracts were dissolved in purified water. The animals were divided into four groups with with each group consisting of 6 rats. They were kept on standard diet and water *ad libitum*. Group 1 (CONT) was fed CCL_4 (1 ml/kg) as a vehicle for 10 days by oral route; Group 2 normal control (NOR CONT) was given purified water only. Group 3 was administered with plant extract at dose of 100 mg/kg by oral route for 10 days and Group 4 (Liv-52) was administered marketed formulation, Liv-52 syrup at the dose of 2.5 ml/ kg b.w. by oral route for 10 days only (Tasaduq, 2003). Liv-52 was taken as a standard because it is a popular poly herbal formulation containing Ayurvedic herbal remedy against viral hepatitis and is commonly prescribed for infective hepatitis (Mukerjee and Dasgupta, 1970). Group 1, 3 and 4 were injected with CCl_4 at a dose of 0.75 ml/kg b.w. on 3rd, 6th, and 10th day by i.p. route. On 10th day, 1 hr after the last dose of CCl_4 injection, the blood was collected by retro-orbital route. The blood sample was centrifuged and used for the estimation of various biochemical parameters, serum glutamic oxaloacetic transaminase (SGOT), serum glutamic pyruvic transaminase (SGPT), alkaline phosphatase (ALP) and bilirubin levels which are the markers of liver damage.

3.14 BIOASSAY-GUIDED PARTIAL PURIFICATION

3.14.1 Sample preparation

Fresh bark of *Fraxinus floribunda* was washed properly in tap water and cut into small pieces. It was dried under sunlight and dried bark was ground in a mixer grinder make powder. One kg of dried bark powder of *Fraxinus floribunda* was extracted in

water under pressure in an autoclave. The extract obtained was separated through a separating funnel in six solvents from low to high polarity (hexane < diethyl ether < ethyl acetate < chloroform < butanol < water). All the separated extracts were subjected for preliminary screening.

3.14.2 Preliminary screening

For preliminary screening of the extracts, phytochemical estimation (TPC, TFC) and free radical scavenging assay (DPPH, $ABTS^{+}$) were performed. The extract which showed better activity on the basis of above assays was further subjected to column chromatography for purification.

3.14.3 Column chromatography

From the result of preliminary screening, butanol extract showed better radical scavenging activity as well as phytochemical content. Thus the butanol fraction was selected to run through column chromatography. A cylindrical column (600x40 mm) was made to stand firm on a column chromatography stand. At first, a small piece of cotton was put into the column and let it stay at the bottom of the column by slowly pushing it with a glass rod to prevent the silica gel to strain out from the column along with the solvent. For the uniform deposition of silica, a little amount of sea sand was deposited on cotton that was kept earlier. Silica gel 200-400 M was utilized as the stationary phase and mobile phase was prepared from the different combination of solvents from low to high polarity. The slurry of 100 g silica gel and 250 ml petroleum benzene was poured carefully into the column. The tap of the column was kept open while pouring the slurry to let the solvent flow freely. The solvent was drained until a clear solvent without carrying traces of silica gel or cotton wool was obtained. When the solvent was completely drained, the tap of the column was closed and allowed to stand for 24 hr for solidification. The butanol extract (7 g) was mixed with dry silica gel in a glass beaker gently heating and stirring continuously until the extract turns into fine powder after sticking with the grains of silica gel. When the silica gel in the column is solidified and stabilized, the dry powder of butanol extract prepared earlier was put into the solidified column. Firstly petroleum benzene was poured carefully into the column.

Different solvent systems used were: Petroleum benzene: ethyl acetate 100: 0, 90:10, 80:20, 70:30, 60:40, 50:50, 40:60, 30:70, 20:80, 10:90, 0:100; ethyl acetate: methanol 99:1, 98:2, 97:3, 95:5, 92.5:7.5, 90:10, 70:30; 50:50, 30:70, 10:90, 0:10; methanol: water 90:10, 70:30, 50:50, 30:70, 10:90, 0:10. 200 ml of each solvent combination was poured gently by the side of the column mouth. Tap of the column was kept open to allow the flow of eluent at the rate of 40 drops per minute. The eluted samples were collected in test tubes (10 ml in each test tube). A total of 596 sub fractions were obtained of which DPPH scavenging activity and total flavonoid content were determined. The fractions showing best free radical scavenging activity and phytochemical content were selected for TLC analysis.

3.14.4 Thin layer chromatography (TLC) and merging of fractions

TLC was performed on aluminium plates pre-coated with silica gel 60 F_{254} made in Merck, Darmstadt, Germany. The plate was cut into small strips 2×8 cm in which 20 µl of concentrated sub fraction was loaded. The sample spot on the plate was then dried by applying hot air with a drier. The plate was carefully kept in a chromatographic chamber made of glass that that contained mobile phase (ethyl acetate: methanol: water) in a ratio of 100: 13.5: 10. The jar was closed with a lid to prevent the evaporation of solvent from the chamber. After the complete run of solvent on the plate, it was removed from the chamber and dried with a drier to evaporate the remaining solvent on the plate. Dried plate was observed under UV lamp at 254 and 365nm. On the basis of TLC profiles, sub fractions showing similar bands with same R_f values were merged together consequently forming 6 main fractions viz. F1, F2, F3, F4, F5, F6. The merged fractions were subjected to *in vitro* antidiabetic activity by the inhibition of alpha-glucosidase enzyme (method mentioned earlier) by the fractions. The fraction F-1 showed the best antidiabetic activity thus it was subjected to GC-MS analysis for identification of the active compounds present in the extract.

3.14.5 Gas Chromatography - Mass Spectrometry analysis

GC-MS is a general confirmation technique to make an effective chemical analysis. It will provide a spectral output of the compounds that get separated from the

extract. The GC-MS analysis was carried out in SAIF, Madras IIT, India. The fraction F1 was subjected to GC-MS analysis. For this analysis, GC and MS JOEL GC mate equipped with secondary electron multiplier was used. It is a high resolution, double focusing instrument. The sample was dissolved in HPLC grate methanol, before injecting into the instrument. The column (HP5) was fused silica 50 m × 0.25 mm i.d. The analysis conditions were 20 min at 100°C, 3 min at 235°C for column temperature and 240°C for injector temperature. The carrier gas was helium and split ration was 5:4. The sample (1µl) was evaporated in a splitless injector at 300°C and run time was 22 min. The compounds were identified GC coupled with MS. Interpretation on mass spectrum obtained from GC-MS was done using the database of National Institute Standard and Technology (NIST). Identification was based on comparison of their mass spectra present in the library. The name, molecular weight and structure of the components separated from the extract were ascertained (Dhabhai *et al*., 2012).

3.14.6 Nuclear magnetic resonance spectroscopy (NMR)

3.14.6a Sample preparation

The bands resulted from TLC analysis of the six fractions on the TLC plates were scratched out from the plates and dissolved in methanol. After stirring for few minutes, the mixture was filtered through Whatman no1 filter paper to obtain extracts in the bands. The sample extracted from the bands on TLC plates was again analyzed to determine antidiabetic activity. There were five bands observed in F1 fraction all of which showed antidiabetic activity which were better than other fractions. The five bands were grouped into two on the basis of nearly similar R_f values and these two group of bands were scratched out from the plates for NMR analysis to identify a probable compound which might be responsible for the antidiabetic activity of the extract. Also, from the GC-MS analysis, ten different compounds were identified from the NIST library. The most abundant compound out of these ten was 2(1H)-Quinolinone, hydrazone with 25.7 %. This compound was found to possess pharmacological activities such as, anti cancer, anti-arteriosclerosis, antimicrobial, anti-malarial and antidiabetic. Thus a standard 2(1H)-Quinolinone, hydrazone was

purchased from Sigma-Aldrich and this was also subjected to NMR analysis along with the two bands of the extract to compare whether it was the same compound present in the TLC bands which was accountable for antidiabetic activity.

3.14.6b NMR analysis

1-H NMR spectra were recorded on ECZR Series 600 MHz NMR spectrophotometer (JEOL, Japan) in SAIF, Bombay IIT. Samples were prepared by dissolving in methanol d-4. Hydrogen-hydrogen correlation were analysed to observe the proton coupling with the structure of the isolated compound (Aue *et al*., 1976 and Ernst *et al*., 1987).

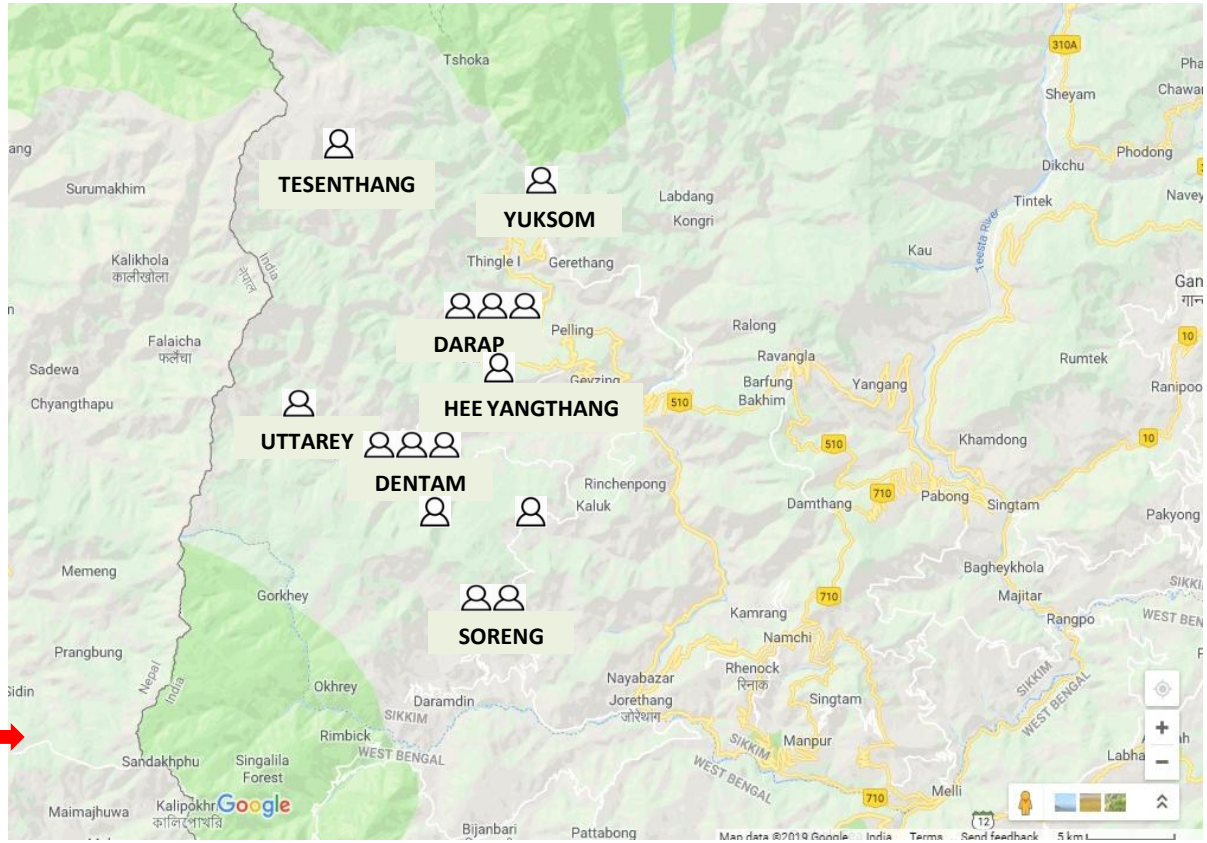

Figure 3: Study area for the documentation of traditional herbal knowledge from the herbal practitioners of West Sikkim and collection of medicinal plants.

One herbal practitioner interviewed