CHAPTER-3

MATERIALS AND METHODS

CHAPTER-3
MATERIALS AND METHODS

3.1. Study Site

The present study site is the district of Goalpara, Assam, situated in the western part of Assam. The district is bounded by the Bongaigaon district on the North; Kamrup district on the East; Meghalaya on the South and Dhubri on the West. It lies between 90º20′ - 91º20′ East longitude and 26º 0′-26º 30′ North latitude covering 1824sq. km. of geographical area. According to the Census of Assam, 2011 the total population of Goalpara district is 1,008,959. In this area, the temperature ranges from minimum about 10ºC in winter and maximum 35ºC in summer with relative humidity (RH%) ranging from 56% to 90% in different season, there is variation of different annual rainfall and also in number of rainy days in a year. The area is mainly inhabited by the ethnic tribal groups viz., Bodos, Rabhas, Hajong and Garo. Besides, Assamese, Adivasis, Bengalis, Koch Rajbongshi and Santhalis also inhabit in the district. Dhupdhara was selected as the targeted study area due to the rich diversity in flora, fauna and above all due to the presence of diverse ethnic groups with a wide array of traditional practices.

3.2. Survey Study

Field survey was carried out in the tribal dominated village of Dhupdhara, Goalpara district, Assam to collect ethno botanical information. A specially designed semi-structured questionnaire was used to do the survey on the local health practices of the study area which include most relevant questions that can give data to fulfill the objectives of the work. Questionnaire is thematic in nature and is meant to fulfill and seek very specific information. The information about ethno medicinal uses of plants, local names of plants, plant parts used, preparation and mode of administration were sought from the local healers. All these information on traditional remedies were gathered by way through both formal and informal interviews and discussion with traditional local healers, aged people and other rural folks were also consulted.

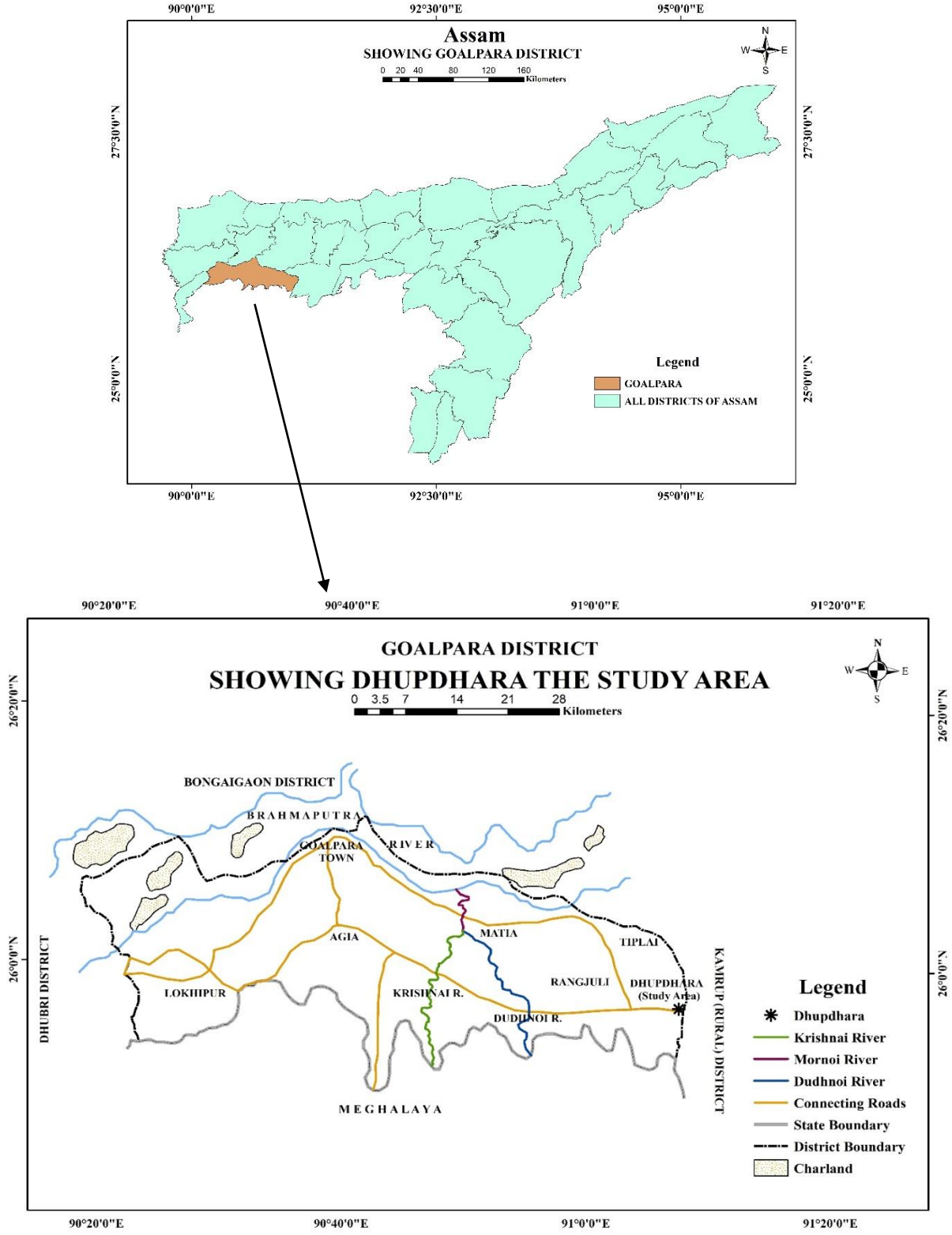

Plate 3.1: Map of Goalpara showing study area.

3.3. Collection and identification of plant material

Plant materials were collected from Dhupdhara area of Goalpara district, Assam during the month of September to October from their natural habitat. Herbarium specimens were prepared and submitted to Gauhati University Botanical Herbarium, Gauhati University and Bodoland University Botanical Herbarium, Bodoland University for taxonomic authentication. After authentication, the voucher specimens were deposited at the Department of Botany, Gauhati University and Bodoland University, Assam for future references.

3.4. MOLECULAR STUDY

3.4.1. DNA Isolation

The total genomic DNA was extracted from young leaves of collected samples using the CTAB method. Firstly, 40mg of young leaf was washed with sterile distilled water and grounded using mortar and pestle in 500µl extraction buffer (100mM Tris-HCl, 1.5M NaCl, 20mM EDTA, 2% CTAB, 1% PVP at pH-8). After mixing, the tube was thoroughly vortexed and incubated at 60^{o}C for 30mins followed by centrifugation at 14,000rpm for 5mins. The supernatant was transferred to a new tube and 10µl RNaseA was added to it and then incubated at 37^{o}C for 20mins. Then, 500µl of Chloroform Isoamyl (24:1) was added to the samples and vortexed for 5secs followed by centrifuge at 14,000rpm for 1min to separate the phases. An aqueous upper phase was transferred to a new tube and repeated this extraction phase was cleared. The upper aqueous phase was transferred to a new tube. Then, 500µl of ice-cold isopropanol was added to the tube and kept at -20^{o}C for 15minutes to precipitate the DNA. The sample was centrifuged at 14,000 rpm for 10mins. The tube containing DNA was washed with 70% ethanol and decanted the ethanol completely. The DNA was then air-dried and was then dissolved in 20µl TE (10mM Tris HCl, 1mM EDTA) and stored in -20^{o}C for long preservation.

3.4.2. PCR Amplification

PCR amplification was performed in a volume of 20µl reactions mixture contained 1X PCR buffer, 2 mM MgCl2, 2.5µM of each dNTP, 0.35 µM of each primer, 20ng of genomic DNA and 1U of Taq polymerase in a Appliedbiosystems

by Thermo Fisher Scientific with the parameter settings of 35 cycles of denaturation at 95ºC for 1min, 56ºC for annealing for 1min and extension at 72ºC for 1min. Amplification of rbcL was similar to ITS except that the denaturating temperature was 94ºC for 1min and annealing temperature at 54°C. The PCR products were visualized in 1.5% agarose gel electrophoresis. When successful, PCR products were sent for sequencing to Mr. Biologist Pvt Ltd., Pune, India using the same primer pairs. The primers used for amplification is given in Table 3.1.

Table 3.1: Primer used for PCR amplification and references used.

Sl. No	Gene	Primer Sequence	Reference
1	rbcL	Forward-ATGTCACCACAAACAGAGACTAAAGC Reverse - GAAACGGTCTCTCCAACGCAT	CBOL Plant Working Group (2009)
2	ITS1	Forward-CCTTATCATTTAGAGGAAGGAG Reverse-TCCTCCGCTTATTGATATGC	Chen *et al.*, 2010
3	ITS2	Forward-ATGCGATACTTGGTGTGAAT Reverse-GACGCTTCTCCAGACTACAAT	Chiou *et al.* (2007)

3.4.3. Sequence Analysis

The amplified sequences were analyzed using NCBI-BLAST. The nucleotide sequences were annotated based on chromatogram and searched with a reference database of all rbcL and ITS regions using the NCBI BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequence was computed to assign identity and the ID of each species was associated with the best BLAST hit and E-value cut off. This corresponds to choosing the top hit in the BLAST results (BLAST1). The Maximum likelihood phylogenetic trees based on rbcL, ITS1 and ITS2 regions were constructed with Kimura 2-parameter model using MEGA 11.00. The reliability of phylogenetic tree was evaluated by bootstrap analysis with 1000 resamplings (Felsenstein, 1985) with Mega 11.0 software.

3.5. NUTRITIONAL STUDIES

3.5.1. PROXIMATE COMPOSITION ANALYSIS

The proximate composition of the powdered plant materials were analyzed following the standard methods of food analysis as described in the Association of Official Analytical Chemists AOAC, (2005) and Food and Agriculture Organization (FAO, 2003). For proximate composition analysis, the samples were cleaned and moisture content determined. The rest of the samples were shade dried, pulverized and kept in an airtight container for further analysis.

3.5.1.1. Determination of Moisture Content

Moisture content was determined by oven-dry method in Hot Air Oven (Thermo Scientific/Heratherm-51028153) as the loss of weight due to evaporation at 105ºC from the sample. The weight loss in the samples represented the amount of moisture present in the sample. 2g of dried plant material was weighted and placed in an oven at 105ºC for 3hours. After drying it was allowed to cool in desiccators and reweighed. Moisture content was calculated using the following formula:

$$Moisture\ content\ (\%) = \frac{\text{Weight of sample} - Dry\ weight}{Weight\ of\ the\ sample\ taken} \times 100$$

3.5.1.2. Determination of Ash Content

Ash content was determined by combusting 2g of dried samples in a muffle furnace (High Temperature Furnance) at 550ºC for 8hours. Before that, the silica crucible was heated overnight in a furnace at 550ºC. Initial weight of the crucible was taken after cooling it in desiccator till constant weight was obtained. After that it was cooled in the desiccators and the final weight was measured and the ash content was calculated as per the formula given below:

$$Ash\ (\%) = \frac{\text{Weight of Ash}}{Weight\ of\ the\ sample} \times 100$$

3.5.1.3. Determination of Crude Protein Content

Crude protein content in the samples was estimated by the Micro Kjeldahl method. Here, 0.1g of sample was digested with 5g Kjedahl catalyst (0.5g $CuSO_4$ and 4.5g K_2SO_4) and 200mL of concentrated H_2SO_4. A blank was prepared with the above chemical excluding the sample. All the samples and blank were boiled

briskly until the solution appeared clear. To this solution, 60mL of distilled water was added and distilled. The condensed ammonia collected from distillation was performed against 0.1N HCl. The nitrogen concentration was calculated as per the formula given below.

$$Nitrogen\ (\%) = \frac{(A - B)\ \times\ N \text{ of HCl} \times\ 14}{Weight\ of\ the\ sample} \times 1000$$

Where, A = Volume (mL) of (0.1 N) HCl used in sample titration

B = Volume (mL) of (0.1 N) HCl used in blank titration.

14 = Atomic weight of nitrogen.

The protein content was calculated by multiplying the nitrogen content with 6.25 protein conversion factor.

Protein (%) = Nitrogen (%) × 6.25 (Conversion factor)

3.5.1.4. Determination of Crude Fat Content

To estimate the crude fat content, 2g of moisture free sample was taken in Soxhlet extractor with 250mL of petroleum ether and boiled at 40-60°C for about 6-8h. The solvent was completely evaporated by using rotary evaporator. After evaporation the dried sample was transferred to the desiccators to cool. The dried samples were reweighed and the crude fat content was calculated by the following formula:

$$Crude\ fat\ (\%) = \frac{\text{Weight of Fat}}{Weight\ of\ the\ sample} \times 100$$

3.5.1.5. Determination of Carbohydrate content

Carbohydrate content was calculated by subtracting the sum of percentage of moisture, fat, protein and ash contents from 100% (Onwuka, 2005).

Carbohydrate (%) = 100- (Moisture %+ Ash %+ Crude Protein %+ Crude Fat %)

3.5.1.6. Determination of Crude fibre content

Crude fibre content was determined by treating the fat and moisture free samples with 1.25% dilute acid and 1.25% alkali followed by washing with water and ignition of the residue. 2g of dried sample was digested with 0.25N H_2SO_4 and filtered with muslin cloth. After washing the residue with hot water for three times, it was heated again for 30mins with 0.3N NaOH untill it boiled. It was filtered

again, followed by washing with hot water, 0.5N H_2SO_4 and 50% ethanol solution. The residue was dried in a hot air oven at 130º C. The dried residue obtained was incinerated in a muffle furnance at a temperature of 600º C for 30mins. The ash so obtained was weighed and crude fibre content calculated using the formula given below:

$$Crude\ fibre\ (\%) = \frac{\text{Weight of residue} - \text{weight of ash}}{Weight\ of\ the\ sample} \times 100$$

3.5.1.7. Determination of Total Energy Content

The energy content of plant samples were determined using the method given by FAO, (2003). The energy content was calculated by multiplying the values obtained for protein, fat and carbohydrate by 4.00, 9.00 and 4.00 respectively and adding up the values.

Total Energy (kcal/100g) = 4×Protein % +9× Fat %+ 4×Carbohydrate %

1.5.2. ESTIMATION OF MINERALS

For mineral analysis, wet digestion method of Ang and Lee, (2005) was followed. Briefly, 0.2g of sample was digested in a falcon tube with HNO_3 and HCl. To the samples, 4.5mL of freshly prepared 65% HNO3 and 3mL of 37% HCl was added. The mixture was then sonicated in a water bath (95ºC) for 4hr until the samples had completely dissoloved. The mixture was further vortexed and kept in water bath for 10mins. After centrifugation, the supernatant was collected and used for analysis. Na, K, Mg, Ca, Mn, Zn, Fe, Cu, Ni and Cr contents of samples was determined by Inductively Coupled Plasma-Optical Emission Spectrometry (ICP-OES) **(Model no.** Thermo Scientific™ iCAP™ 7600)**.** Mineral content of plant sample was calculated out as mg/100g of dry weight.

3.5.3. ESTIMATION OF AMINO ACID

Amino acids and their composition were analyzed using WATERS Acquity (make) Ultra Performance Liquid Chromatography (UPLC). 3mL of 6N HCl was added to 1mg of sample in a clean glass tube and covered with paraffin. The tube was placed in a dry bath under N_2 gas for 15mins at 60ºC to maintain inertness. Then the temperature was increased at 110ºC and incubated overnight. Derivatization was accomplished by adding a combining 70µl of Borate buffer and

20µl of Accq Tag ultra-reagent to the sample and incubation for 10mins at 55ºC. After incubation 5µL of the derivatized samples was loaded to WATERS Acquity (make) Ultra Performance Liquid Chromatography. Individual amino acids were identified by comparing with the standards of amino acid run along with the samples. Peaks of individual amino acids could be visualized with the help of 260nm photodiode array detector. The pattern of amino acid requirement for preschool children for 2-5 years recommended by FAO/WHO/UNU (1985) was followed to calculate the amino acid score.

Calculation:

$$Amino\ acid\ score = \frac{\text{mg of amino acid in 1g of test protein}}{\text{mg of } amino\ acid\ in\ reference\ pattern} \times 100$$

3.5.4. ESTIMATION OF FATTY ACID

300mg of sample was taken in 1mL water and vortexed. It was then centrifuged for 10mins at 4000rpm and supernatant was collected into a fresh falcon. To the pellet 1mL methanol was added, vortexed and centrifuged for 10mins at 4000rpm. Supernatant was collected and the step was performed twice. The collected supernatant was evaporated using N_2 gas at 50°C. To the dried sample, 700 µl of 10M potassium hydroxide and 5.3mL methanol was added. Then, the sample was incubated at 55°C for 1.5hrs by mixing it for every 20mins. The samples were cooled down to room temperature and added 3mL of hexane mixed and allowed to stand for 5mins and centrifuged for 5mins at 4000rpm. The top most organic layer was collected into a separate tube and this step was performed thrice. Collected supernatant was evaporated using N_2 gas at 55°C. To the dried sample, 500µl of n-Hexane was added and injected in GCMS.

3.6. ANTINUTRITIONAL STUDY

3.6.1. Analysis of Alkaloids

Alkaloid content was estimated by the method as previously described by Oyeyinka and Afolayan (2019). 0.5g of the sample was mixed with 200mL of 10% acetic acid in ethanol. The mixture was covered and incubated at room temperature for 4 hr, filtered and concentrated to about a quarter of its original volume in a water bath. Thereafter, concentrated ammonium hydroxide was added drop wise to the

extract till complete precipitation was attained. The solution was allowed to settle and the precipitate formed was washed with dilute ammonium hydroxide and then filtered. The residue was oven dried at 40°C and weighed and the alkaloid content was calculated as:

$$\% \text{ Alkaloid} = \frac{\text{Final weight of sample}}{\text{Initial weight of sample}} \times 100$$

3.6.2. Determination of oxalate

The oxalate content was determined from the modified titration procedure described by Unuofin *et al.* (2017). About 1g of each of the pulverized plant samples was weighed in a conical flask and 75mL of 3M H_2SO_4 was added and agitated on a magnetic stirrer for one hour. The mixture was filtered and 25mL of filtrate collected was heated to about 90°C. The hot aliquot was titrated uninterruptedly against 0.05M $KMnO_4$ till a light pink colour change which lasted for 15secs was observed. This marked the endpoint of the titration. The titre value of each of the plant sample extracts was multiplied by 2.2mg of oxalate taken as the equivalence of 0.05M of $KMnO_4$ used for titration.

Oxalate content = 2.2mg x titre value.

3.6.3. Determination of tannin

Total tannin content was determined by Folin-Ciocalteu method of CI and Indira, (2016). About 0.1mL of the sample extract was taken to a volumetric flask containing 7.5mL of distilled water and 0.5mL of Folin-Ciocalteu phenol reagent, 1mL of 35% sodium carbonate solution and diluted to 10mL with distilled water. The mixture was shaken well and kept at room temperature for 30mins. A set of reference standard solutions of tannic acid (20, 40, 60, 80, 100 µg/mL) were also prepared in the same manner as described. Absorbance for test and standard solutions were measured against the blank at 700nm using the UV-VIS spectrophotometer (UV Analyst-CT8200). The estimation of the tannin content was carried out in triplicate. The tannin content was expressed in terms of mg of tannic acid equivalents/g of dried sample.

3.7. PHYTOCHEMICAL STUDIES

3.7.1. Drying and Storage

Collected leaves were cleaned, washed with water and dried under shade at room temperature for two weeks. The shade dried leaves were pulverized using a clean and sterile electric grinder. The dried powder was stored in an airtight container until further use.

3.7.2. Solvent extraction

50g of sample was defatted with petroleum ether at a temperature ranged between 60-80ºC. The defatted sample was further extracted in 500mL of hexane, chloroform, methanol and water in a Soxhlet apparatus. Four solvents were taken based on their increasing polarity to compare and find out the best solvent for extraction of the plant samples. The extraction temperature was maintained at 60ºC for 24hr. All the solvents used for extraction were evaporated in a rotary vacuum evaporator until a crude viscous semi solid extract was obtained. The extractive values of all the solvent extracts were recorded in terms of yield % as per standard method (Alebiosu and Yusuf, 2015).

$$\text{Yield percent (\%)} = a/b \times 100$$

where, a = dry weight of extract obtained.

b = initial weight of powdered material.

The extraction yield was calculated for each extract of all plants. After evaporation all the crude extracts were kept in refrigerator for further use. The hexane, chloroform, methanol and water extract of *Zanthoxylum oxyphyllum*, *Rotheca serrata* and *Blumea lanceolaria* are written as:

Zanthoxylum oxyphyllum hexane extract- ZOHE

Zanthoxylum oxyphyllum chloroform extract-ZOCE

Zanthoxylum oxyphyllum methanol extract-ZOME

Zanthoxylum oxyphyllum aqueous extract-ZOAE

Rotheca serrata hexane extract-RSHE

Rotheca serrata chloroform extract-RSCE

Rotheca serrata methanol extract-RSME

Rotheca serrata aqueous extract-RSAE

Blumea lanceolaria hexane extract-BLHE

Blumea lanceolaria chloroform extract-BLCE

Blumea lanceolaria methanol extract-BLME

Blumea lanceolaria aqueous extract-BLAE

3.7.3. PHYTOCHEMICAL SCREENING

Phytochemical investigation of plants is an interesting area of research that has resulted in the isolation of various novel compounds. Phytochemicals are secondary metabolites that plants can manufacture in large quantities. The existence of these chemical constituents such as various forms of glycosides, tannins, phenolics, flavonoids, alkaloids, lipids, fixed oils and fats and so on contributes to the drug's therapeutic value and pharmacological action. Medicinal plants are the only important natural source for development of drugs with minimum side effects. Here, the secondary metabolites are responsible for various diseases preventing activities. In this study, the biological and phytochemical screening of plant extracts from the collected ethnomedicinal plants is the first step towards this goal.

3.7.3.1. Qualitative Phytochemical Screening

The qualitative phytochemical screening of solvent extracts of the ethnomedicinal plants was carried out to detect the presence or absence of secondary metabolites. A total of ten qualitative phytochemical test of the solvent extracts of three plant samples were carried out following the standard protocols of Trease and Evans, (2009) and Harborne, (1998) which are mentioned below:

3.7.3.1.1. Test for alkaloids (Mayer's test):

For detection of alkaloids, to 2mL of extracts 2mL of concentrated HCl was added. When an aqueous layer was formed, drops of Mayer's reagent were added to it. The presence of alkaloids was indicated by the formation of white color precipitate.

3.7.3.1.2. Test for flavonoids (Alkaline reagent test):

To 2mL of extract few drops of 20% NaOH was added. Appearance of an intense yellow color indicated the presence of flavonoids.

3.7.3.1.3. Test for phenols (Ferric chloride test):

For the detection of phenols, 2 drops of 5% ferric chloride solution was added to 2mL of extract. Formation of intense color signified the presence of phenols.

3.7.3.1.4. Test for saponins (Foam test):

2mL of extract was taken and 1mL of distilled water was added to it. The solution was shaken vigorously in a graduated cylinder for 30 seconds. Development of foamy layer in the solution indicated presence of saponins.

3.7.3.1.5. Test for glycosides (Salkowski's test):

A small amount of extract was taken in 2mL of water in a test tube and a few drops of aqueous NaOH were added. Formation of yellow colour indicated the presence of glycosides.

3.7.3.1.6. Test for terpenoids (Liebermann's test):

For screening of terpenoids, 2mL of extract was taken and to it 2mL of chloroform was added and the mixture was evaporated to dryness. To the dried mixture 2mL of concentrated H_2SO_4 acid was added. A layer of yellowish green color formed at the lower portion of the solutions indicated the presence of terpenoids.

3.7.3.1.7. Test for oils and fats (Spot test):

2mg of extract was taken and pressed between two filter papers. Appearance of oil stain on the filter paper indicated the presence of fixed oil.

3.7.3.1.8. Test for steroids (Salkowski's test):

For detection of steroids, 2mL of chloroform was taken and added to 2mL of extract followed by addition of 2mL concentrated H_2SO_4. A layer of red color produced at the bottom of the test tube indicated the existence of steroids.

3.7.3.1.9. Test for tannins (Lead Acetate test):

For tannins, 10% of lead acetate was prepared. 2mL of the extract was then treated with 1 mL of 10 % lead acetate. Development of white precipitate showed the presence of tannins.

3.7.3.1.10. Test for phytosterols (Liebermann-Burchard's test):

To 2mL of extract, few drops of Chloroform, Acetic Anhydride and concentrated H_2SO_4 were added. Presences of phytosterol were indicated by the appearance of translucent green color.

3.7.3.2. Quantitative estimation of phytochemicals

3.7.3.2.1. Estimation of total phenolic content (TPC)

The total phenolic content of the plant extract was determined by using Folin-Ciocalteu reagent following a slightly modified method of Ainsworth and Gillespie, (2007). Gallic acid was used as a reference standard for plotting calibration curve. A volume of 0.5mL of the plant extract (100µg/mL) was mixed with 2mL of the Folin-Ciocalteu reagent (diluted 1:10 with de-ionized water) and were neutralized with 4mL of sodium carbonate solution (7.5%, w/v). The reaction mixture was incubated at room temperature for 30mins with intermittent shaking for color development. The absorbance of the resulting blue color was measured at 765nm using double beam UV-VIS spectrophotometer in a double beam UV-Vis spectrophotometer (UV Analyst-CT8200). The total phenolic contents were determined from the linear equation of a standard curve prepared with gallic acid. The content of total phenolic compounds expressed as mg/g gallic acid equivalent (GAE) of dry extract.

3.7.3.2.2. Estimation of total flavonoid content (TFC)

Total flavonoid content (TFC) of the plant extracts were estimated by the Aluminium chloride colorimetric method of the reported procedure of Madaan *et al.* (2011). Quercetin was used as a standard to construct the calibration curve. 1mg of quercetin and plant extracts were dissolved in 80% ethanol and then diluted to 20, 40, 60, 80 and 100µg/mL. The diluted standard solutions of quercetin and plant extracts (0.5mL) of different concentration were separately mixed with 1.5mL of 95% ethanol, 0.1mL of 10% aluminum chloride, 0.1mL of 1 mol/L potassium acetate and 2.8mL of distilled water in a test tube. The solutions were incubated for 30mins at room temperature to complete the reaction and absorbance was measured at wavelength 415nm with double beam UV-Vis spectrophotometer against blank. A typical blank solution contained all reagents except aluminium

chloride which is replaced by the same amount of distilled water. The amount of flavonoid was calculated from linear regression equation obtained from the quercetin calibration curve. The flavonoid content was calculated as mean ± SD (n=3) and expressed as mg/g of quercetin equivalent (QE) of dry extract.

3.7.3.3. INVITRO ANTIOXIDANT STUDIES

The plant leaves extracts were separately dissolved in dimethyl sulphoxide (DMSO). DPPH radical scavenging assay, H_2O_2 assay and Ferric Reducing power assay were used to evaluate the antioxidant efficacy of plant extracts. Ascorbic acid was used as a standard in the antioxidant studies.

3.7.3.3.1. 1, 1-Diphenyl-2-Picryl-Hydrazyl Assay (DPPH Method)

The free radical scavenging activities of plant extracts were evaluated by DPPH method (Shukla *et al.,* 2014). 1mL of extracts and standard ascorbic acid in different concentration (10 to 200µg/mL) was added to 3mL working DPPH solution (0.1mM DPPH in methanol). The mixture was shaken and allowed to stand for 30mins in dark. Absorbance was taken at 517nm with UV-VIS spectrophotometer (Model no. UV Analyst-CT 8200). 1mL methanol and 3mL working DPPH solution served as the blank.

The percentage inhibition was calculated as: where $A0$ is the absorbance of the control and $A1$ is the absorbance of the test sample or standard.

$$\% \text{ inhibition} = \frac{A0 - A1}{A0} \times 100$$

The concentration (µg/mL) of plant extract was plotted against the percentage inhibition. Inhibitory concentration (IC_{50}) of the extracts and ascorbic acid for DPPH free radical scavenging were calculated from linear regression equation from the graph.

3.7.3.3.2. H_2O_2 (Hydrogen Peroxide) Scavenging Assay

The scavenging ability of the plant extracts for hydrogen peroxide was evaluated according to the method described by Bhatti *et al.* (2015). The hydrogen peroxide solution was prepared by mixing the 20mM of H_2O_2 into 50mM of phosphate buffer (pH 7.4). Different concentrations of the plant extracts (50-250µg/mL) and standard ascorbic acid were taken into the test tubes and their

volume is made up by the addition of 0.4mL of phosphate buffer (50mM). This was followed by the addition of 0.6mL of hydrogen peroxide solution. The test tubes were shaken and the absorbance was recorded after 40mins of incubation at 230nm against the blank. Phosphate buffer without the involvement of hydrogen peroxide served as the blank and ascorbic acid was used as the standard. The percentage of the scavenging activity of extracts (in triplicate) was calculated by the following formula:

$$\%\ \text{H2O2 scavenging activity} = \frac{A0 - A1}{A0} \times 100$$

where *A*0 is the absorbance of the control and *A*1 is the absorbance of the test sample.

3.7.3.3.3. Ferric Reducing Power Assay

Reducing power assay was done following the method of Zhao (2008). Briefly, 50-250µg/mL of the plant extracts and standard ascorbic acid (1mg/mL) was mixed with 2.5ml of 0.2M Phosphate buffer (pH: 6.6) followed by 2.5ml of 1% potassium ferricyanide. After incubation for 20mins at 50ºC, 2.5mL of 10% trichloroacetic acid was added. The tubes were then centrifuged at 10,000 rpm for 10mins. 2.5mL of the upper layer was added with 2.5mL distilled water and 0.5mL of 0.1% ferric chloride. The absorbance of the reaction was measured at 700nm against blank. Blank was prepared with all reagents except sample and standard. Ascorbic acid was used as standard. All the tests were performed in triplicates.

3.8. GAS CHROMATOGRAPHY MASS SPECTROMETRY (GC-MS) STUDIES

GC-MS analysis was performed by using Perkin Elmer (USA) Clarus 680 GC and Clarus 600C MS comprising of a liquid autosampler. The software used in the system was Turbo Mass Ver.6.1.2. For analysis, 2µl of each plant extract was injected in GC-MS system through auto-sampler in split-less mode. The temperature of injector was fixed at 280°C and helium (99.99% purity) was used as carrier gas (i.e. mobile phase) fixed at a constant flow rate of 1mL min^{-1}. The column oven temperature was programmed at 60°C for 1min, with an increase of 7ºC min^{-1} to 200ºC (hold for 3 min) then again increased by 10°C min^{-1} to 300°C

(hold for 5 min). The total run time of GC-MS system was 39mins. Solvent delay was kept for 8 min. A mass spectrum was observed in Electron Impact positive (EI+) mode at 70eV. A solvent delay of 8 min was there for MS scan. Mass range i.e. m/z range was 50-600 amu (Hema *et al.,* 2010).

3.8.1. Compound identification

The mass spectra of compounds obtained from GC-MS analysis were recognized by relating their peak, peak area, peak height, retention time, molecular weight and mass spectrum patterns to that of known compounds described by National Institute of Standard and Technology (NIST) (2014) library database (Hema *et al.,* 2010).

3.9. FOURIER TRANSFORM INFRARED SPECTROSCOPY (FT-IR) STUDY

Fourier transform infrared spectroscopy (FT-IR) was used to identify the functional groups of compounds in crude powder of the plants. The wavelength of light absorbed features in the chemical bond which can be seen in the annotated spectrum of FT-IR. The infrared absorption spectrum in FT-IR is used to interpret the chemical bonds in the compound. For FT-IR analysis, 10mg dried powder of each sample was encapsulated in 100mg of potassium bromide (KBr) pellet and translucent sample discs were prepared. FT-IR analyses of the samples were performed in THERMO NICOLET IS10 FT-IR Spectrometer (THERMOSCIENTIFIC). The samples were run at infrared region between 400-4000 cm^{-1} and standard DLATGS detector was used at 2.8 mm/sec mirror speed.

3.10. CYTOTOXICITY STUDIES

3.10.1. Cell lines and Culture medium

The cells were procured from the National Centre for Cell Science, Pune, India. 2 human cancer cell lines namely, MCF-7 and HeLa. Stock cells were cultured in MEM supplemented with 10% inactivated Fetal Bovine Serum (FBS), penicillin (100 IU/ml), streptomycin (100µg/ml) and amphotericin B (5µg/ml) in an humidified atmosphere of 5% CO_2 at 37°C until confluent. The cells were dissociated with trypsin solution (0.2% trypsin, 0.02% EDTA, 0.05% glucose in

PBS). The stock cultures were grown in 25cm^2 culture flasks and all experiments were carried out in 96 microtitre plates (Tarsons India Pvt. Ltd., India).

3.10.2. Cell proliferation MTT assay (3-(4, 5-dimethyl thiazol-2 yl)-2, 5-diphenyl tetrazolium bromide) assay

In vitro anticancer activity of the plant extracts was evaluated against MCF-7 and HeLa cell line using MTT assay (3-(4, 5-dimethyl thiazol-2 yl)-2, 5-diphenyl tetrazolium bromide) assay according to the method described by Mosmann, (1983). In MTT assay, cells were placed in the 96 well plates containing 100µl minimum essential medium (MEM) in each microwell. The cells were kept at 37ºC in a CO_2 incubator with an atmosphere of 5% CO_2 in 95% humidified air and were allowed to attach for 24hrs. Different concentrations (5, 10, 25, 50, 75 and 100µg/ml) of the samples were added into the well of the microplates and incubated in the CO_2 incubator. Doxorubicin was used as a standard. After 72hrs of cell plating, 20µl of MTT was added and the microplates were incubated for another 2hrs. The media were removed and the insoluble purple formazan formed was dissolved with DMSO and incubated once again for 4 hrs. Then, the absorbance was taken using a Spectramax M2 Microplate Reader (Molecular Diagnostic, Inc.) at a wavelength of 630nm. The cytotoxicity was calculated:

$$\text{Cytotoxicity (\%)} = \frac{\text{Control} - \text{test sample}}{\text{Control}} \times 100$$

3.10.3. Observation of Morphological Changes

MCF-7 and HeLa cells were seeded in 96-microwell plates at the density of the cell 2×10^4 cells/well and then incubated for 24hr. They were then treated with the extracts and cell morphology was observed by phase contrast inverted microscope (Zeiss, Germany) at 400× magnification after 24hr incubation.

3.10.4. Apoptotic morphological changes by Acridine orange (AO)/Ethidium bromide (Et-Br) (2:1) staining

Acridine orange/ethidium bromide (AO/Et-Br) staining was carried out by the method of Gohel *et al*. (1999) to analyze the morphological alterations induced by the extracts treated in HeLa and MCF-7 cells. Two DNA-binding dyes AO and

Et-Br were used for the morphological detection of apoptotic and necrotic cells. Both the cell lines were seeded separately in 96- well plates for 24hrs at 37^{O}C in 5% CO_2 to allow cell adherence. The cells were treated with the two different concentrations IC_{50} and $2\times IC_{50}$ concentration of the treated group for 24hrs after adherence. Then, the cells ($1x10^5$cells/ml) were washed with ice-cold 1X PBS (Phosphate Buffered Saline) at pH-7.4. Cells were then fixed in 4% formaldehyde in 1X PBS (Phosphate Buffered Saline) at room temperature for 15mins. The dye was incubated with the cells at room temperature in dark for 10minutes. The apoptotic phase of cell death was observed using doxorubicin as a positive control. Inverted fluorescence microscope (Olympus America, Inc., located in Melville, NY, USA) was used for observing the stained cells.

3.10.5. Flow Cytometric Analysis of Apoptosis

Apoptotic cell death was measured using the Annexin V/PI detection kit (BD Biosciences, San Jose, CA). MCF-7 cells (10,000/well) and HeLa cells (30,0000/well) were seeded in 6-well plates for at 24hrs, then treated with 200µg/mL of the plant extracts for 48hrs. Cells were collected after incubation and washed with cold PBS (Phosphate Buffered Saline) and resuspended in 300µl of binding buffer. The pelleted cells were again incubated with 5µl of Annexin V-FITC for 15mins and 5µl of propidium iodide (PI) in the dark at room temperature. After adding 500µL of the buffered isotonic solution, samples were analyzed using the Flow cytometer (FACS Calibure, Becton Dickinson, USA). The total percentage of apoptotic cells was defined as the sum of both early apoptotic (annexin V+/PI-) and late apoptotic (annexin V+/PI+) cells. Necrotic cells were annexin V-/PI+ and viable cells were annexin V-/PI- (Elmore, 2007).