CHAPTER 4

METHODS AND MATERIALS

Present research entitled **"Consumption pattern of *Calamus tenuis* Roxb. shoots of the forest village natives of Dibrugarh, Assam and investigation of its cytotoxicity activity on cancer and normal cells (A549, MCF7 and L132)"** was conducted in five phases.

The study protocol was approved by the Medical Ethical Committee of the Foods and Nutrition department of The Maharaja Sayajirao University of Baroda, Vadodara in compliance with the guidelines issued by Indian Council of Medical Research with the Medical ethics approval number- IECHR/2014/21 [Appendix-1].

First phase of study included, identification and authentication of the plant; sample collection and its primary processing; and investigation of consumption pattern of the shoots, its traditional therapeutic practices and beliefs, storage, sources and background information of the enrolled subjects of the study.

In second phase of study, crude extraction of the plant sample was done using different solvents; MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay of crude extracts on human lung carcinoma cells (A549) and breast carcinoma cells (MCF7); and Qualitative phytoconstituents analysis of extracts was done as per relevant protocols.

In third phase of the study, Fractionation of the crude extract showing highest cytotoxicity was done by Column Chromatography. Fractions (F-2, F-3, F-8) showing majority of phytoconstituents (as per TLC) were tested for cytotoxicity on human

lung carcinoma cells (A549) and breast carcinoma cells (MCF7) by MTT assay. Qualitative analysis of the fractions was done to screen the active phytoconstituents.

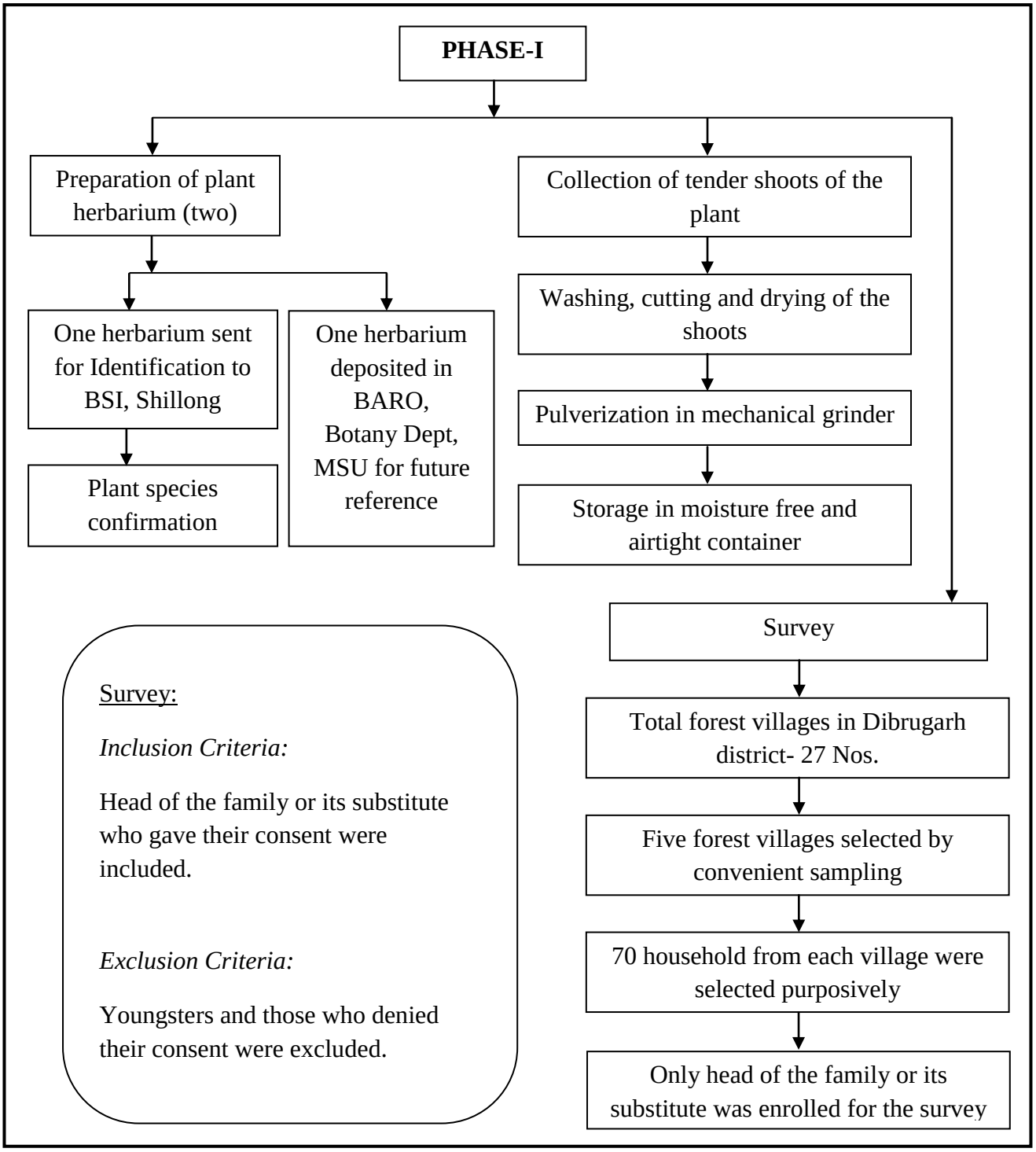
In fourth phase of the study, MTT assay of methanolic precipitate extract (MPCT) and methanolic supernatant extract (MSCT) was done on human lung normal cells (L132). Also, fractions (F-2, F-3, F-8) were tested for cytotoxicity against normal cells (L132).

In fifth phase of the study, Lethal Concentration (LC_{50}) of MPCT, MSCT and fractions (F-2, F-3, F-8) were calculated and compared for cytotoxicity among carcinoma (A549, MCF7) and normal cells (L132).

The detailed experimental design for all the five phases of the study; Phase-I, Phase-II, Phase-III, Phase-IV and Phase-V is presented in figures- 4.1, 4.2, 4.3, 4.4 and 4.5 respectively.

4.1 Phase-I: Plant identification, sample collection and survey

Figure- 4.1: Experimental design for Phase-I



The Phase-I of the study included:

4.1.1 Plant identification

4.1.2 Plant sample collection and its primary processing

4.1.3 Survey of *Calamus tenuis* Roxb. shoots consumption pattern, culinary preparations, source, storage, traditional therapeutic and health issues beliefs and practices

4.1.3.1 Location of the study

4.1.3.2 Determination of sample size for survey

4.1.3.3 Tool used for data collection

4.1.3.4 Statistical analysis of collected data

4.1.1 Plant identification

The plant was identified and authenticated [Ref. No- BSI/ERC/2013/Tech/Plant identification/669)] by Dr. A. A. Mao, Botanical Survey of India, Eastern Regional Centre, Shillong [Appendix-2] and a voucher specimen (Specimen No- MSU/PKT/2013/11, was deposited in BARO herbarium (Plate-4.1.1), Department of Botany, The Maharaja Sayajirao University of Baroda, Vadodara, Gujarat for future references.

Palte-4.1.1: *Calamus tenuis* Roxb. herbarium

Coll. No.	MSU/PKT/2013/11
Family	Aracaceae
Genus & species	*Calamus tenuis* Roxb.
Locality	Jokai, Dibrugarh, Assam
Habitat	Wild
Vern. name	Jati bet

4.1.2 Plant sample collection and its primary processing

Raw 14.09 kg *Calamus tenuis* Roxb. shoots (Plate-4.1.2.1) were collected from Jokai, Dibrugarh, Assam, India in November 2013, which yielded 3.58 kg of fresh edible shoots (Plate- 4.1.2.2). The shoots were cut in pieces (Plate-4.1.2.3), air (Plate-4.1.2.4) and sun (Plates-4.1.2.5) dried, and milled to a coarse powder (275g) (Plate-4.1.2.6) with a mechanical grinder and stored in moisture free air tight container.

Plate-4.1.2.1: Unprocessed *Calamus tenuis* Roxb. shoots

Plate-4.1.2.2: Edible portions of *Calamus tenuis* Roxb. shoots

Plate-4.1.2.3: *Calamus tenuis* Roxb. shoots cutting

Plate-4.1.2.4: *Calamus tenuis* Roxb. shoots air and shade drying

Plate-4.1.2.5: *Calamus tenuis* Roxb. shoots sun drying

Plate-4.1.2.6: *Calamus tenuis* Roxb. shoots milled powder

The moisture content of edible portion of the shoots was calculated as per following:

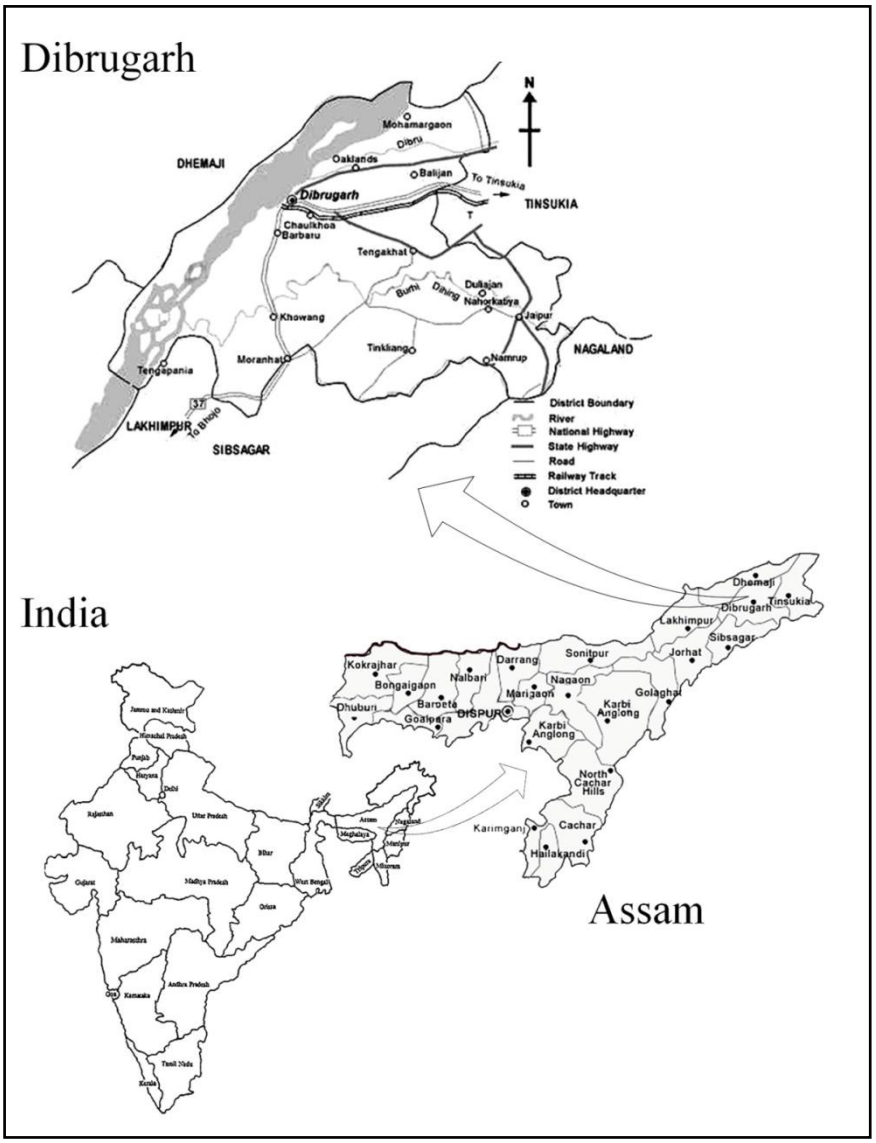
$$\text{Percent moisture content} = \frac{\text{(Fresh edible wt. – dry wt.)}}{\text{Fresh edible wt.}} \times 100$$

4.1.3 Survey of Calamus tenuis Roxb. shoots consumption, culinary preparations, sources, storage, traditional therapeutic and health issues beliefs and practices

4.1.3.1 Location of the study

The study was conducted in Dibrugarh district of Assam. The Dibrugarh district extends from 27^{o} 5' 38" N to 27^{o} 42' 30" N latitude and 94^{o} 33' 46" E to 95^{o} 29'8" E longitude. It is bounded by Dhemaji district on the north, Tinsukia district on the east, Tirap district of Arunachal Pradesh on the south east and Sibsagar district on the north and south west. The area stretches from the north bank of the mighty river Brahmaputra, which flows upto a length of 95 km through the northern margin of the district, to the Patkai foothills on the south [Geography of Dibrugarh district, 2015] (Fig-4.1.3.1). The district occupies an area of 3381 Sq Km [Dibrugarh district, 2015]. The total population recorded is 11,85,072. Among which rural population comprises of 9,56,634 and urban of 2,28,438 number of people [Dibrugarh district census, 2001]. There are 1362 total villages in Dibrugarh district among which 27 are forest villages [Geography of Dibrugarh district, 2015]. From the total forest villages of Dibrugarh district, only five were selected for the survey.

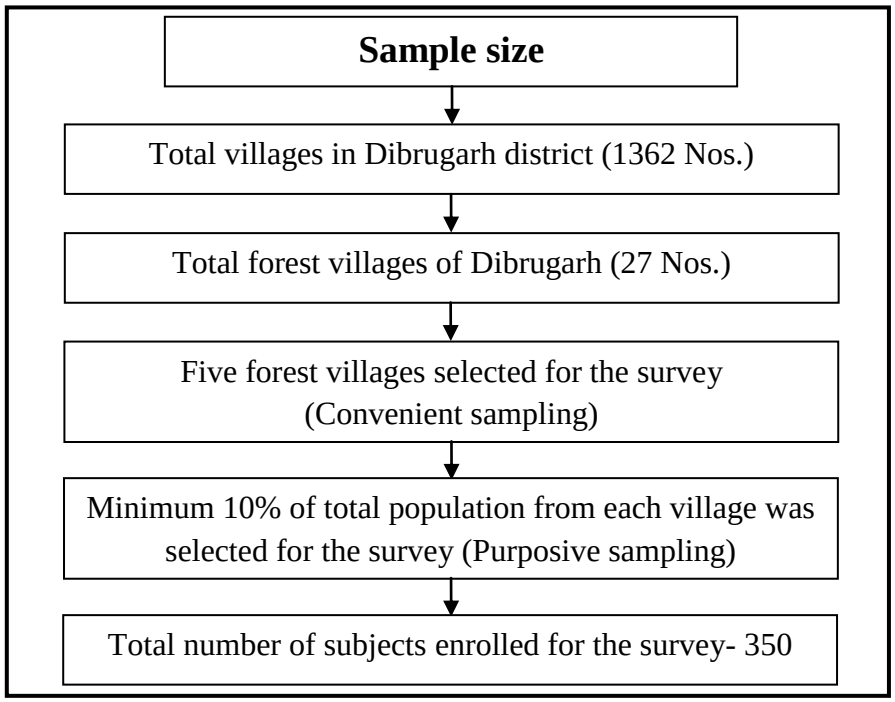
Figure-4.1.3.1: Dibrugarh district of Assam, India



4.1.3.2 Determination of sample size for survey

Five forest villages viz. Tingkhong Village, Hati Gandhori Village, Telpani Village, Modhupur Deori Village and Kaliyoni Village were conveniently selected for the study and minimum 10% of total population (70 households) from each village was purposively selected. Head of the family or its substitute were selected from each household who gave his/her written consent to participate in the survey [Appendix-3 and 4]. A total of 350 adult subjects were enrolled for the study (Fig- 4.1.3.2).

Figure: 4.1.3.2: Determination of sample size for the survey



4.1.3.3 Tool used for data collection

A structured and pretested proforma was used to elucidate the information on various aspects including [Appendix-5]:

- General information and socio-economic status [Patro *et al.*, 2012].
- Use of the shoot in various culinary preparations.
- Frequency and reason of consumption of the shoot.
- Sources of the shoot and its storage practices.
- Beliefs about uses of shoot for healing various diseases.
- Known health issues related to shoot consumption.
- History of various diseases and its association with the consumption of the shoots.

The above points were considered in the tool to get a background of the use of *Calamus tenuis* Roxb. shoots in various culinary preparations, sources, storage practices, traditional therapeutic and health issues beliefs due to consumption. Plates: 4.1.3.3.1 to 4.1.3.3.6 indicates the process of interaction with the subjects during the survey.

Plate-4.1.3.3.1

Plate-4.1.3.3.2

Plate-4.1.3.3.3

Plate-4.1.3.3.4

Plate-4.1.3.3.5

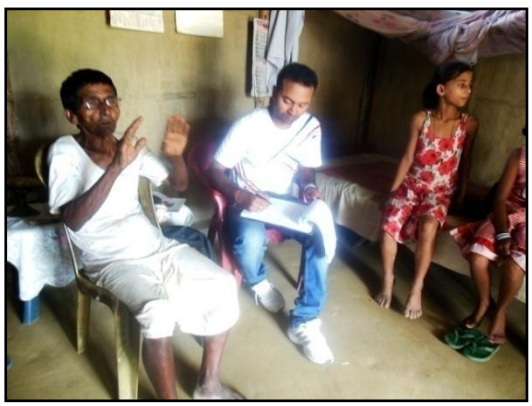


Plate-4.1.3.3.6

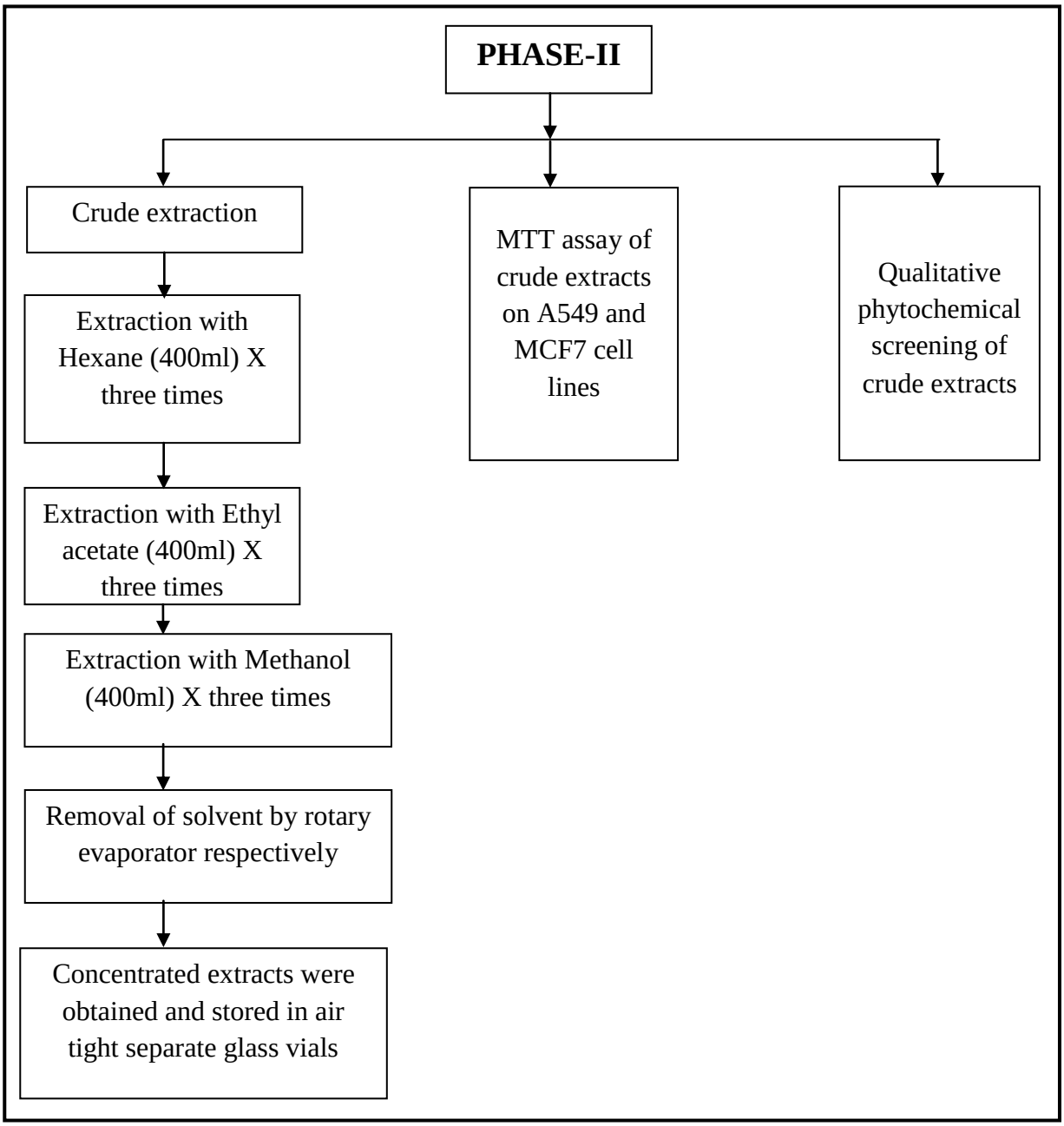
Plates- 4.1.3.3.1 to 4.1.3.3.6: Pictures indicating investigators' interaction with the subjects

4.1.3.4 Statistical analysis of the collected data

The obtained data was entered in a Microsoft excel (2007) spreadsheet. It was cleaned and verified for appropriate statistical analysis. Number and percent calculation was done by using Microsoft excel (2007) and Chi Square was calculated for obtaining association between consumption pattern and other parameters by using Epi Info (7.0).

4.2 Phase-II: Crude extraction of plant sample, cytotoxicity assay on human carcinoma cells, extract fractionation and qualitative phytochemical screening

Figure- 4.2: Experimental design for Phase-II



The Phase-II of the study included:

4.2.1 Chemicals used for extraction, cytotoxicity assay and phytochemical analysis.

4.2.2 Crude extraction of *Calamus tenuis* Roxb. shoots involving successive use of different solvents.

4.2.3 Cytotoxicity assay of crude extracts on human lung carcinoma cells (A549) and breast carcinoma cells (MCF7).

4.2.3.1 Cell culture of human lung carcinoma cells (A549) and breast carcinoma cells (MCF7).

4.2.3.2 MTT assay on human lung carcinoma (A549) and breast carcinoma (MCF7) cells against *Calamus tenuis* Roxb. shoots extracts.

4.2.3.3 Statistical analysis for cytotoxicity

4.2.4 Qualitative phytochemical screening of crude extracts of *Calamus tenuis* Roxb. shoots

4.2.4.1 Frothing test for presence of saponins

4.2.4.2 Hydrochloric acid test for presence of flavonoids

4.2.4.3 Salkowski's test for presence of steroids

4.2.4.4 Ferric chloride test for presence of tannins

4.2.4.5 General glycosides test for its presence

4.2.4.6 Fehling's test for presence of glycosides

4.2.1 Chemicals used for extraction, cytotoxicity assay and qualitative phytochemical analysis

Hexane, Ethyl acetate, Methanol, Chloroform, H_2SO_4, HCl, $FeCl_3$, NaOH, Dimethyl sulphoxide (DMSO) were purchased from Sisco research laboratories Pvt. Ltd. Mumbai, India. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), antibiotic–antimycotic solution, 0.25% Trypsin–EDTA and phosphate buffer saline (PBS) were purchased from Himedia Pvt. Ltd., Mumbai, India.

4.2.2 Extraction of Calamus tenuis Roxb. shoots

Calamus tenuis Roxb. dried shoot powder (275g) was extracted with hexane (3 X 400 ml), ethyl acetate (3 X 400 ml) and methanol (3 X 400 ml). Excess solvent was removed using a rotary vacuum evaporator (Buchi type) which yielded 3.83, 1.38, and 31.63 g of crude extracts of hexane (HECT), ethyl acetate (EACT) and methanol respectively. The methanol extract was syrupy in nature and hence partitioned with 300 ml of distilled water and centrifuged at 10000 rpm for 15 min. The methanolic precipitate (MPCT) and supernatant (MSCT) were lyophilized separately which resulted in 4.64 g of amorphous powder and 15.16 g of sticky paste respectively [Yu *et al.,* 2008].

4.2.3 Cytotoxicity assay of crude extracts on human lung carcinoma cells (A549), and breast carcinoma cells (MCF7)

4.2.3.1 Cell culture of human lung carcinoma cells (A549) and breast carcinoma cells (MCF7)

Human lung carcinoma cells (A549) and breast carcinoma cells (MCF7) obtained from National Centre for Cell Sciences, Pune, India, were seeded (1 X 10^5 cells/25 mm T Flask) and cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% antibiotic/anti-mycotic solution at

37^0C with 5% CO_2 (Thermo scientific, forma II water jacketed CO_2 incubator). Cells were subsequently sub-cultured every third day by trypsinization with trypsin phosphate versus glucose solution (TPVG). All the reagents were sterilized and filtered through 0.22µ filter (Laxbro Bio-Medical Aids Pvt. Ltd.) prior to use for the experiment.

4.2.3.2 MTT assay on human lung carcinoma (A549) and breast carcinoma (MCF7) cells against Calamus tenuis Roxb. shoots extracts

MTT assay was performed as per Thounaojam *et al.* (2011) with minor modification. A549 and MCF7 cells (7 X 10^3 cells/well) were maintain in 96- well culture plates for 24 h in absence and presence of *Calamus tenuis* Roxb. shoot extracts (10–200 µg/ml) and fractions (2-200ug/ml) or vehicle (DMSO) and later, 10µl of 3-(4,5-dimethylthiazol- 2-yl)-2,5-diphenyl-tetrazolium bromide (MTT, 5 mg/ml) was added to the wells. Plates were incubated again at 37˚C for 4 h, culture media was discarded and wells were washed with Phosphate Buffer Saline (Hi-media, India, Pvt. Ltd.). To each well, 150 µl of DMSO was added and incubated for 30 min. Colour intensity was measured at 540 nm in ELX800 Universal Microplate Reader.

4.2.3.3 Statistical analysis for cytotoxicity

Data of cell viability was analyzed for statistical significance (for obtaining cytotoxicity) using one way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test and results were expressed as Mean ± SD using Graph Pad Prism version 5.0 for Windows, Graph Pad Software, San Diego, California, USA.

4.2.4 Phytochemical screening of crude extracts of Calamus tenuis Roxb. shoots

Qualitative phytochemical screening for various types of constituents of *Calamus tenuis* Roxb. shoot extracts was performed as described by Ghani (2005).

4.2.4.1 Saponins (Frothing test)

Water (0.5 ml) was added in the *Calamus tenuis* Roxb. shoot extract and shaken vigorously. Production of persistent froth (1-2 min) even after warming suggested presence of saponins.

4.2.4.2 Flavonoids (HCl acid test)

Few drops of HCl was added to *Calamus tenuis* Roxb. shoot extract and formation of red colour indicated presence of flavonoids.

4.2.4.3 Steroids (Salkowski's test)

1 ml of conc. H_2SO_4 was added along the sides of the test tube in a mixture of *Calamus tenuis* Roxb. shoot extract and chloroform (2 ml). The red colour in the chloroform layer indicated presence of steroids.

4.2.4.4 Tannins (Ferric chloride test)

About 0.5 ml of *Calamus tenuis* Roxb. shoot extract was mixed with 10 ml of water and stirred gently. Formation of blue-black, blue-green, blue or green colour indicated the presence of tannins.

4.2.4.5 Glycosides (General test)

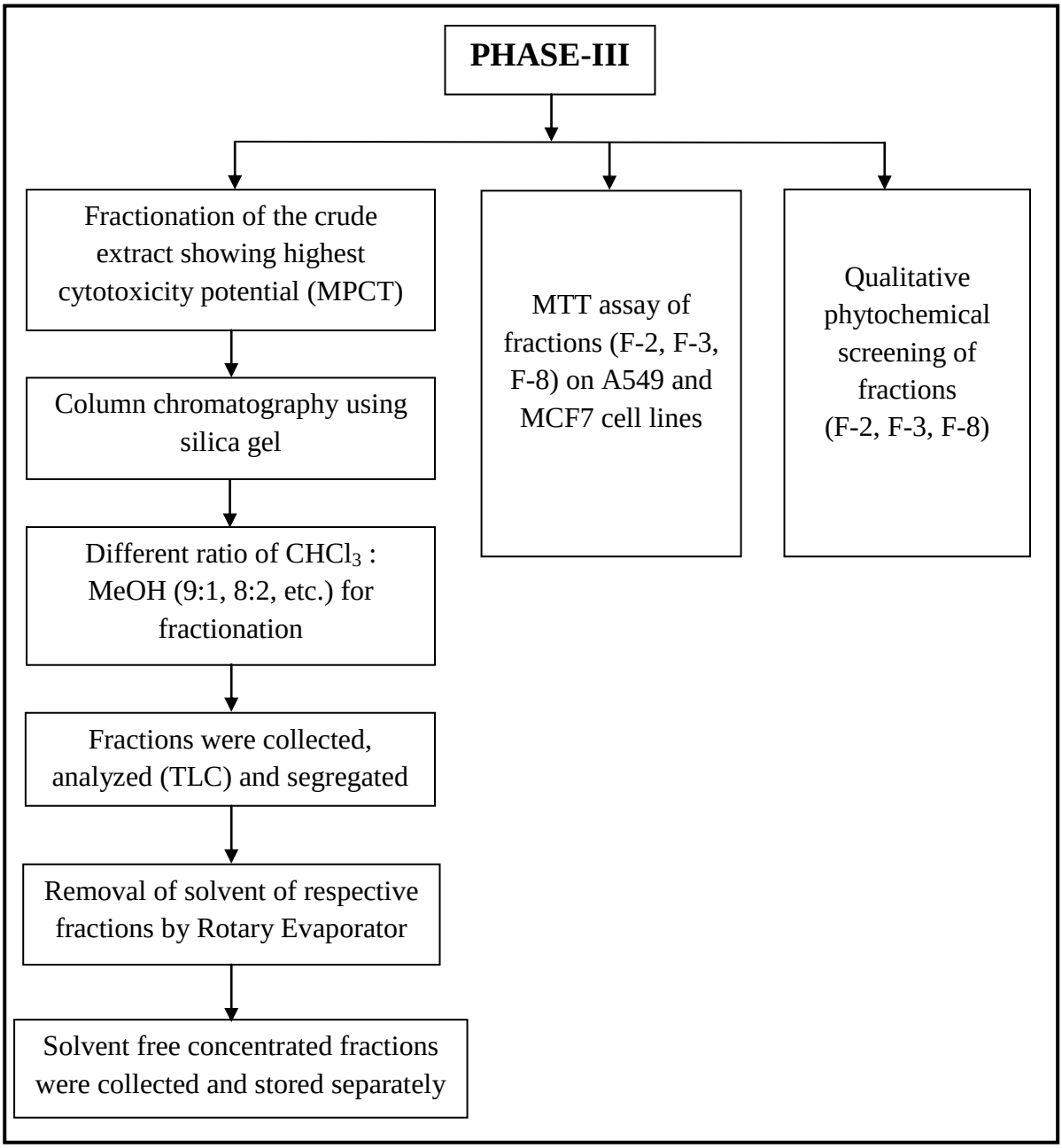
Calamus tenuis Roxb. shoot extract was mixed in small amount of water and a few drops of aqueous NaOH was added leading to a yellow colour indicating presence of glycosides.

4.2.4.6 Glycosides (Fehling's test)

Calamus tenuis Roxb. shoot extract was mixed in small amount of aqueous solution and alcohol and boiled with Fehling's solution wherein brick-red colour was formed. Another portion of extract was dissolved in water and alcohol and boiled with a few drops of dilute H_2SO_4. NaOH solution was added to neutralize the acid and the contents were boiled with Fehling's solution. A brick-red precipitate formation indicated the presence of glycosides.

4.3 Phase-III: Fractionation of Methanol Precipitate extract of *Calamus tenuis* Roxb. shoots (MPCT), cytotoxicity assay on human carcinoma cells, and qualitative phytochemical screening

Figure- 4.3: Experimental design for Phase-III



The Phase-III of the study included:

4.3.1 Fractionation of methanolic precipitate (MPCT) extract of *Calamus tenuis* Roxb. shoots by Column Chromatography.

4.3.2 Cytotoxicity assay of fractions (F-2, F-3, F-8) on human lung carcinoma cells (A549) and breast carcinoma cells (MCF7)

4.3.2.1 MTT assay of fractions (F-2, F-3, F-8) on human lung carcinoma (A549) and breast carcinoma (MCF7) cells

4.3.2.2 Statistical analysis for cytotoxicity

4.3.3 Qualitative phytochemical screening of fractions (F-2, F-3, F-8) of *Calamus tenuis* Roxb. shoots

4.3.1 Fractionation of methanolic precipitate (MPCT) extract of Calamus tenuis Roxb. shoots by Column Chromatography

Fractionation of methanolic precipitate (MPCT) extract was performed by Column Chromatography technique (Plates-4.3.1.1 and 4.3.1.2) using silica gel (60 mesh, 35-70um). Different ratio of $CHCl_3$: MeOH (9:1, 8:2, etc.) was used as solvent system for fractionation. Various fractions of about 25 ml each were collected in separate conical flasks until the solvent system looked clear and no spot were found on TLC plate. Each fraction was analyzed by TLC and observed under UV light at 254 and 366 nm. 10% sulfuric acid solution in methanol was sprayed when it was found insensitive to UV light [Gracia *et al.*, 2008]. Like-fractions were combined and concentrated by removing solvent with the help of vacuum rotary evaporator. TLC was performed for obtained fractions and fractions showing majority of phytoconstituents were selected for cytotoxicity assay.

Plate-4.3.1.1: Column Chromatography

Plate-4.3.1.2: Concentrated fractions

Plates- 4.3.1.1 and 4.3.1.2: Fractionation of methanolic precipitate (MPCT) extract

4.3.2 Cytotoxicity assay of fractions (F-2, F-3, F-8) on human lung carcinoma cells (A549) and breast carcinoma cells (MCF7)

4.3.2.1 MTT assay of fractions (F-2, F-3, F-8) on human lung carcinoma (A549) and breast carcinoma (MCF7) cells

MTT assay of fractions (F-2, F-3, F-8) was done on human lung carcinoma (A549) and breast carcinoma (MCF7) cells as per method described in section 4.2.3.2 [Thounaojam *et al.*, 2011].

4.3.2.2 Statistical analysis for cytotoxicity

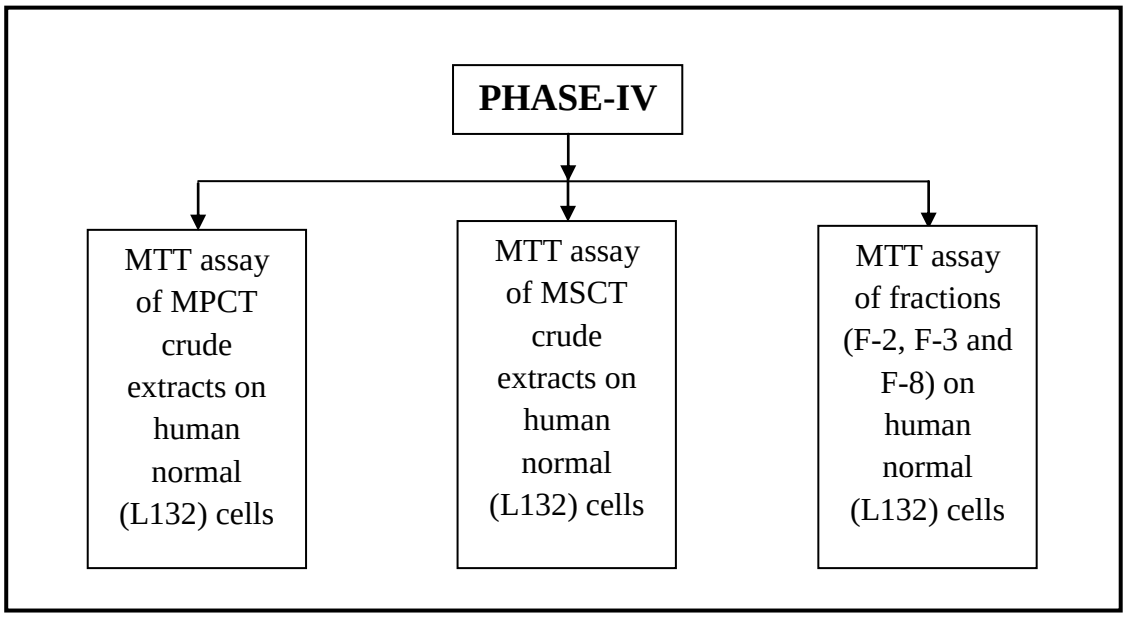
Statistical analysis of cytotoxicity was done as per method described in section 4.2.3.3.

4.3.3 Qualitative phytochemical screening of fractions (F-2, F-3, F-8) of Calamus tenuis Roxb. shoots

Qualitative phytochemical screening of fractions (F-2, F-3, F-8 was done as per methods described in section 4.2.4 [Ghani, 2005].

4.4 Phase-IV: Cell viability assay of MPCT extract, MSCT extract and MPCT fractions (F-2, F-3 and F-8) on human normal cells for evaluation of cytotoxicity activity

Figure- 4.4: Experimental design for Phase-IV



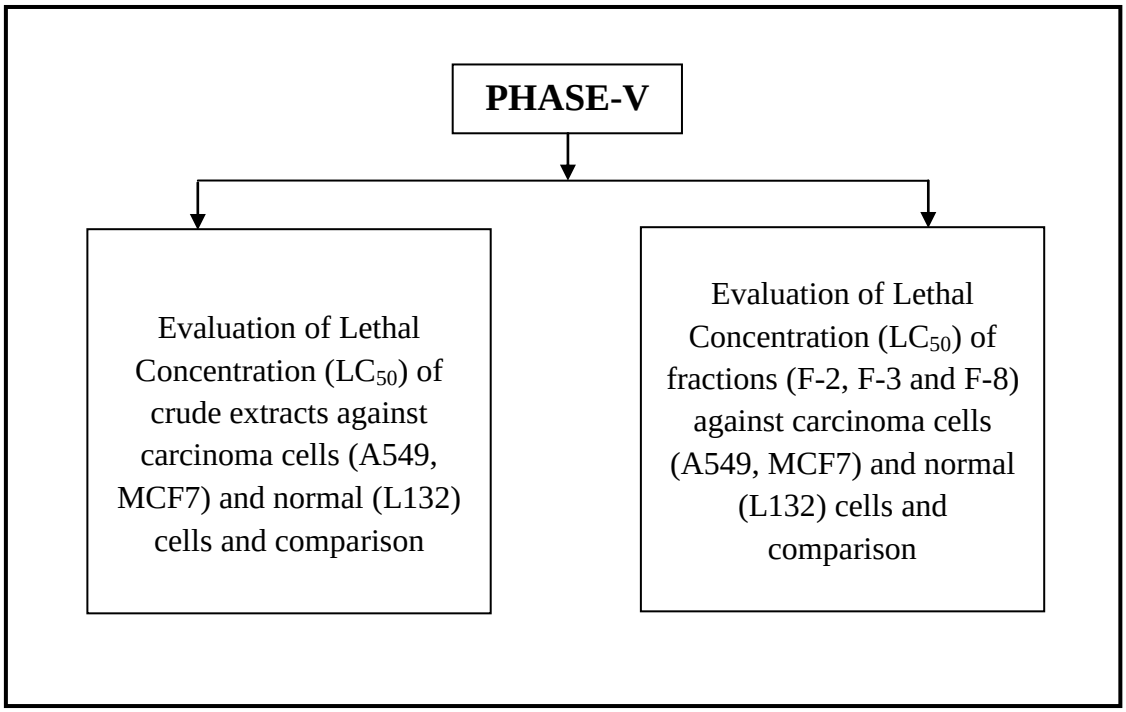
The Phase-IV of the study included:

4.4.1 MTT assay on human lung normal cells (L132) for cell viability and cytotoxicity potential evaluation of Methanol precipitate (MPCT), Methanol supernatant (MSCT) extracts and fractions (F-2, F-3 and F-8) of Calamus tenuis Roxb. shoots

MTT assay of MPCT, MSCT and fractions (F-2, F-3, F-8) was done on human lung normal cells (L132) as per method described in section 4.2.3.2 [Thounaojam *et al.*, 2011].

4.5 Phase-V: Comparison of lethal concentration (LC_{50}) of methanolic precipitate (MPCT) and supernatant (MSCT) extracts; and fractions (F2, F3 and F8) of *Calamus tenuis* Roxb. shoot among human carcinoma and normal cells:

Figure- 4.5: Experimental design for Phase-V



The Phase-V of the study included:

4.5.1 Evaluation and comparison of Lethal Concentration (LC_{50}) of methanolic precipitate (MPCT) and supernatant (MSCT) extracts of *Calamus tenuis* Roxb. shoot against human carcinoma and normal cells.

4.5.2 Evaluation and comparison of Lethal Concentration (LC_{50}) of methanolic precipitate (MPCT) extract fractions (F2, F3 and F8) of *Calamus tenuis* Roxb. shoot extracts against human carcinoma and normal cells.

4.5.1 Evaluation and comparison of Lethal Concentration (LC_{50}) of methanolic precipitate (MPCT) and supernatant (MSCT) extracts of Calamus tenuis Roxb. shoot against human carcinoma and normal cells

The Lethal Concentration (LC_{50}) of methanolic precipitate (MPCT) and supernatant (MSCT) extracts against human carcinoma and normal cells were observed and evaluated based on cytotoxicity caused by the extracts to the respective cell lines. A comparison was done among the extracts towards cytotoxic potential w.r.t. tested cell lines, based on Lethal Concentration (LC_{50}) values obtained.

4.5.2 Evaluation and comparison of Lethal Concentration (LC_{50}) of methanolic precipitate (MPCT) extract fractions (F2, F3 and F8) of Calamus tenuis Roxb. shoot extracts against human carcinoma and normal cells.

The Lethal Concentration (LC_{50}) of methanolic precipitate (MPCT) extract fractions (F2, F3 and F8) against human carcinoma and normal cells were observed and evaluated based on cytotoxicity caused by the fractions to the respective cell lines. A comparison was done among the extracts towards cytotoxic potential w.r.t. tested cell lines, based on Lethal Concentration (LC_{50}) values obtained.