Methods and Materials

CHAPTER NO 3: METHODOLOGY

3.1. SELECTION AND COLLECTION OF PLANT

Based on the open leads from traditional uses and recent laboratory trials, Karphool and Kumarilota plants were selected for the study. These plants are abundantly scattered throughout tropical Asia and India.

Etlingera linguiformis (CAN) of Zingiberaceae family is an important indigenous medicinal and aromatic plant of north east, India, which grows well in warm climates with loamy soil rich in humus. The plant rhizome has medicinal benefits in treating cancer, sore throats, stomachache, rheumatism, and respiratory complaints, while its essential oil is used in perfumery.

Medicinal uses: Pieces of rhizomes are chewed with betel leaf to cure sore throat. A fresh juice extracted from the rhizomes of the plant is taken 5 ml twice a day to cure stomach ache. Pea-sized pills made with the rhizomes of the plant are taken thrice a day, for 15 days, at the same time fomentation (fresh leaves of the plant heated on fire) is applied to swelling to treat rheumatism. Rhizome of the plant is used for the treatment of respiratory complaints. Table 2.1 shows the Plant A identification details and Figure 3.1 provides the location details of selected plant A.

Table 2.1: Plants A Selected for the study

Kingdom	Plantae
Order	Zingiberales
Family	Zingiberaceae
Genus	Etlingera
Species	linguiformis.
Assamese name	Karpoor

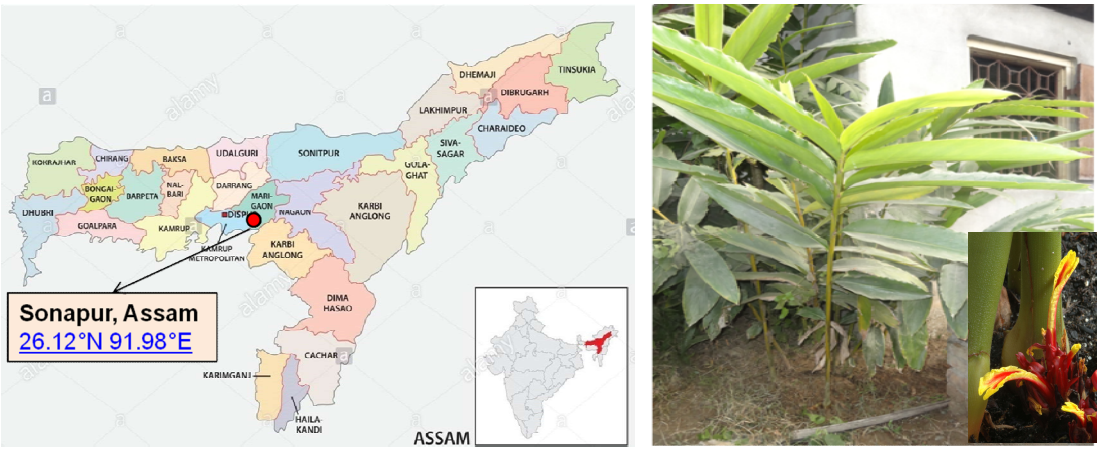

Figure 3.1: Location of selected plant *Etlingera linguiformis*

While the *Smilax ovalifolia* (LEX) of Smilacaceae family is also an important indigenous medicinal of north east, India. Kumari lota (local name) is an armed or unarmed climber.

Medicinal uses: The roots of Kumari lota are used for venereal diseases. Also applied in rheumatic swellings and given in urinary complaints and dysentery.

Both of these plants were selected on the basis of traditional knowledge of the tribal people dwelling in the northeastern states of India. The leaves were collected during November to January. Different facets of plant selection are affected by their action toward cancer. Table 2.2 provides a detailed description of selected plant B and Figure 3.2 provides the location details of selected plant B.

Table 2.2: Plants B Selected for the study

Kingdom	Plantae
Order	Liliales
Family	Smilacaceae
Genus	*Smilax*
Species	*ovalifolia*.
Local name	Kumarilota

Figure 3.2: Location of selected plant *Smilax ovalifolia*.

AUTHENTICATION OF PLANT

Plants were identified by Botanical Survey of India (BSI), Shillong (BSI/ERC/Tech./2019/03 dated 02.04.2019). A voucher specimen number BSI/ERC/Tech./2019/03 dated 02.04.2019 has been preserved for further reference in the laboratory and Botanical Survey of India, Shillong.

3.2. CHEMICALS AND REAGENTS

The human lung adenocarcinoma cell line A549, NCI-H522, NCI-H23 cells were purchased from NCCS, Pune. The following reagents were used in the present study: Ham's F12-K, Dulbecco'"s Modified Eagle Medium (DMEM), Roswell Park Memorial Institute (RPMI)-1640 culture medium and fetal bovine serum (FBS) (both Gibco; Thermo Fisher Scientific, Inc., USA); penicillin (Sigma-Aldrich; Merck, Germany); streptomycin (Sigma-Aldrich; Merck); dimethyl sulfoxide (DMSO) (Sigma-Aldrich; Merck); Trypan blue (Sigma-Aldrich); Annexin V-fluorescein isothiocyanate (FITC)/propidium iodide (PI) double dye (Sigma-Aldrich; Merck); PI and Rhodamine 123 (Rh123) (Sigma-Aldrich; Merck); Dichloro-dihydrofluorescein diacetate (DCFDA) (Sigma-Aldrich; Merck); Acridine orange (Sigma-Aldrich); Ethidium bromide (Sigma-Aldrich) Inverted microscope (Leica); Fluorescent microscope (Leica); Microplate Reader (Eppendorf); and flow cytometer (BD Biosciences, USA).

3.2.1. PLANT EXTRACT PREPARATION

The leaves of Karpoor (CAN) and Kumarilota (LEX) were cleaned, washed and rinsed properly. They were dried in shade and mechanically powdered. All the chemicals used were of analytical grade and were obtained from Merck milipore. The powdered form of plant was extracted using well-established solvent extraction methods [126, 127]. The pH of the concentrated ethanolic extract was adjusted to pH 1.5 with 4 M HCL and re-extracted with ethyle acetate. The maceration was re-extracted with 1 M NaOH and collected supernatant was acidified to pH 1.5 and partitioned into ethyl acetate. The extract was dry by allow pressure rotary flash evaporator and was once kept free of contaminants in a sealed container. Finally, the percentage yields of the dried extract are determined (Figure 3.3).

$$(\%) = \frac{\qquad\qquad\qquad\qquad}{\qquad\qquad\qquad\qquad} \times 100$$

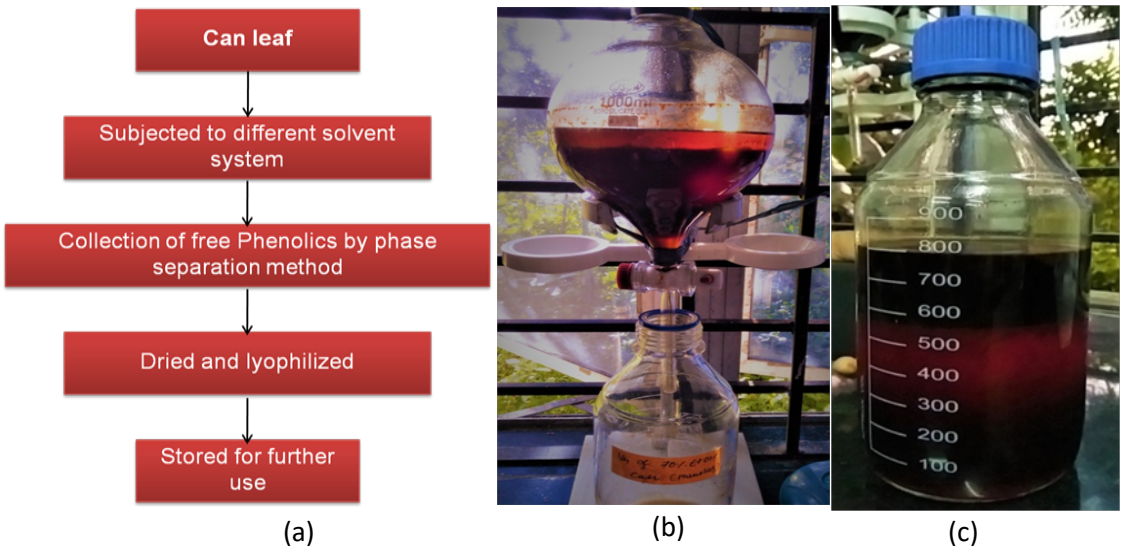

Figure 3.3: Extraction of partially purified free Phenolics fraction from CAN/LEX leaf: (a) extraction process flow chart, (b) extraction process, (c) extracted Phenolics

3.3. SPECTROSCOPIC TECHNIQUES

3.3.1. High Resolution Liquid Chromatography Mass Spectroscopy (Hr LCMS)

Liquid chromatography / Mass Spectroscopy (LC / MS) is a technique which combines high performance liquid chromatography HPLC, a powerful analytical separation technique with mass spectroscopy, a powerful analysis & detection technique. The two common soft ionization techniques used are electrospray ionization (ESI) and atmospheric pressure chemical ionization. This technique enables detection of MW to the nearest 0.001 atomic mass units. The mass resolution of a mass spectrometer is defined mathematically in parts per million (ppm) as given below.

$$= \frac{[\qquad - \qquad h \qquad]}{h} \times 10^{\circ}$$

The ORBITRAP mass filter is a variation of the quadrupole, but instead of forming stable trajectories, ions form a stable orbit between electrodes. The oscillations around these electrodes are a function of the ion's mass. LC-ORBITRAP/MS have a mass resolution of about 2 ppm. Under these conditions, the mass unit error is 0.0006 for a drug with a mass-to-charge ratio of 300 amu. Agilent iFunnel technology combines highly efficient electrospray ion generation and focusing of Agilent Jet Stream technology with a hexabore capillary sampling array and dual-stage ion funnel for increased ion sampling and transmission.

NanoHPLC combined with mass spectrometer can analyse small molecule as well large molecules like proteins. UHPLC separations can be detected by PDA & Mass spectrometer as different detectors.

3.4. IN VITRO CELL CULTURE STUDIES

3.4.1. BASIC CELL CULTURE

Human NSCLC A549 cells were cultured in RPMI-1640 medium, NCI-H522 cells in

DMEM medium and NCI-H23 cells in RPMI-1640 medium supplemented with 10% FBS, 100 U/ml penicillin and 100 mg/l streptomycin in an atmosphere of 5% CO2 at 37˚C. Cells in the exponential growth phase were used in the following experiments Figure 3.4 shows the work flow of study of molecular events in response to the fractionated extract/active molecule using in vitro cell culture system.

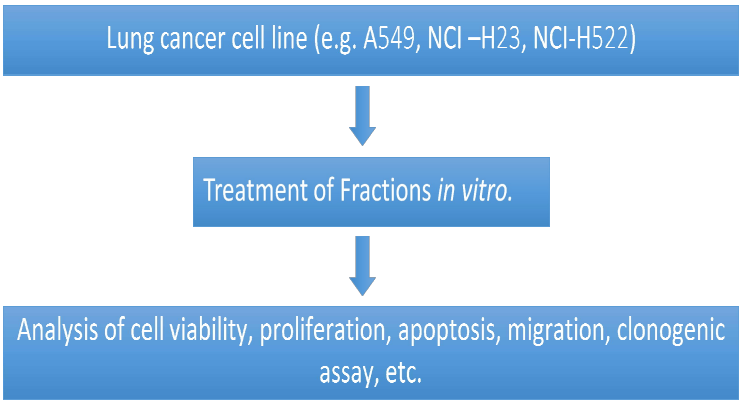

Figure 3.4: Work flow of Study of molecular events in response to the fractionated extract/active molecule using *in vitro* cell culture system.

- **Revival of the cells**

Successfully reviving cells from cryopreservation is one of the critical steps needed to ensure unambiguous experimental results in basic biological research such as cancer research and industrial processes such as vaccine production. Most cell cultures are stored at temperatures below -130°C. To achieve conditions for prolonged storage, the standard procedure for cryopreservation is to freeze cells slowly in a rate controlled freezing container until they reach a temperature below -70°C in medium that includes a cryoprotectant. Vials are transferred to a liquid-nitrogen freezer to maintain them at temperatures below -130°C.

The subsequent recovery of cryopreserved cells is straight forward process: Cells are thawed rapidly in a water bath at 37°C, removed from the freeze-medium by gentle centrifugation and/or diluted with growth medium, and seeded in a culture vessel in complete growth medium. It is important to note that there are numerous factors which affect the viability of recovered cells. Some of the critical parameters for optimization include the composition of the freeze medium,

the growth phase of the culture, the stage of the cell in the cell cycle, and the number and concentration of cells within the freezing solution. Modification of each cell line's procedure can help you attain optimal cell viability upon recovery.

Another point to consider is that, while cells grown in monolayers can be recovered from cryopreservation in multiwell plates, the results are not as consistent as with flasks. Additionally, some cell lines such as hybridoma cultures take several days before they fully recover from cryopreservation 24 hours post-thaw.

- **Splitting of the cells**

  The ceels at 60-70% confluency were trypsinised using 0.25% trypsin-EDTA. After complete detachment from the substratum, the cells were centrifuged at 1500 rpm for 5 mins at room temperature. The pelleted cells were washed with PBS and resuspended with growth medium. The required cell numbers were re-cultured in new flask or used as per the requirement of experiment design.

- **Freezing of the cells**

Cells are cryopreserved to minimize genetic change and avoid loss through contamination. It is best to cryopreserve cells when they are at their optimal rate of growth. Cells were trypsinised using trypsin-EDTA as gently as possible to minimize damage to the cells. Resuspended the detached cells in a complete growth medium and established the viable cell count. Centrifuged at ~200 x g for 5 min. Using a pipette, withdrawn the supernate down to the smallest volume without disturbing the cells. Resuspended the cells in freezing medium to a concentration of 5×10^6 to 1×10^7 cells/mL. Aliquoted into cryogenic storage vials. Placed the vials on wet ice or in a 4°C refrigerator, and started the freezing procedure within 5 min. Cells should be frozen slowly at 1°C/min. This can be achieved using a programmable cooler or by placing vials in an insulated box placed in a –70°C to –90°C freezer, then transferring to liquid nitrogen storage.

3.4.2. CELL VIABILITY ANALYSIS BY MTT

In a 96 well platform, cells (1,000 cells/well) grown in growth medium for 24 hours. Cells were later treated with extracts at 25, 50 and 100 µg/ml concentrations or the vehicle control, 0.1% DMSO. MTT (final 0.5mg/ml concentration) was added to cell culture after incubation. After 2 hours of incubation in CO2 incubator at 37°C, the supernatant culture was eliminated and the matrix cell layer was dissolved in DMSO 100 µl (dimethyl sulfoxide) and optical density is measured at 570 nm using UV VIS spectrophotometer.

3.4.3. CELL COUNTING BY TRYPAN BLUE EXCLUSION METHOD

Trypan blue exclusion method was used to study the effect of polyphenolic extracts in lung cancer cells after 48 hours of treatment. 1 x 10^5 cells/well were seeded in 60 mm plates for overnight and then treated with 25, 50 and 100 µg/ml concentrations or the vehicle control, 0.1% DMSO. Cells were collected by scrapping at the end of the experiment and washed with PBS. Total numbers of live and dead cells are determined using a hemocytometer after trypan blue staining. Photomicrographs of treated and control cells were taken using 60x magnification using inverted microscope (Leica DMi1).

3.4.4. APOPTOSIS ASSAY

Acridine orange and Ethidium Bromide (AO-EtBr) dyes nuclei by intercalating with DNA/RNA. Acridine Orange (AO), being cell-permeable dye may readily enter the normal and initial apoptotic cells with intact membranes and emit green upon attaching to DNA while, EtBr does not permeate through cell membranes but when ruptured, it reaches the nucleus overpowering the effect of AO, stains the nucleus orange to red. This makes it possible to detect fundamental morphological variations in apoptotic cells using the double AO/EtBr fluorescence labeling approach. Since AO/EtBr staining may be used to identify apoptosis qualitatively and quantitatively, it is widely accepted as a reliable approach. 1x10^5 cells are seeded in a 6 well tissue culture plate then treated with CAN/LEX extract 0.1% DMSO as control for 48 hours. At the end of the treatment adherent cells were harvested by scraping and pooled down with dead/floating cells. Thereafter cells were centrifuged

at 3000 rpm for 5 minutes at RT washed with PBS twice and stained with a mixture of AO and EB (100g/ml/ml) for 10 minutes in incubator at a 37^{o}C. The cells were mounted on a glass slide with cover slip and the cells were viewed under fluorescent microscope (LeicaDM300, Germany).

3.4.5. REACTIVE OXYGEN SPECIES (ROS) ANALYSIS

We have used DCFDA test to evaluate the propensity of polyphenolic fraction to produce ROS in the exposed LC cells. DCF-DA is a redox-sensitive dye that can readily cross plasma membranes and get into the matrix, where it is transformed to DCFH by an intracellular esterase. ROS inside the cell subsequently oxidizes this non-fluorescent DCFH to produce fluorescent DCF [128]. Lung cancer cell lines were seeded at a density of 1x10^{5} in 6 well culture plates for 24 hours, followed by treatments with various concentrations of plant extract for 48 hours. Then the cells were trypsinised and washed with PBS. The washed cells were stained with 20 mM DCFDA for 30 minutes at 37^{o}C in the dark. Flow cytometry was used to assess the degree to which DCF had been shifted inside the cells. The Mean Fluorescence Intensity (MFI) represented individual cell levels of ROS (BD FACS LSR III by BD Biosciences, USA).

3.4.6. MITOCHONDRIAL MEMBRANE POTENTIAL (MMP) ANALYSIS

For MMP analysis, the treated cells were staining with Rhodamine-123 and quantified using flowcytometry [129]. The loss of MMP was reflected in the intensity of Rhodamine-123 with respect to the control. The results of this research may be useful in determining the mitochondria's health after being treated with plant extract. The treated cancer cells were incubated with 400 µl of 50 µM Rhodamine 123 at 37^{o}C for 30 minutes with vertical shaking at intervals of 5 min. The cells were washed with PBS for 3 times and placed at vertical shaker for an interval of 5 minutes. The cells were then rewashed with PBS to remove excess extracellular dye. The fluorescence intensity of Rhodamine 123 was measured by flow cytometric analysis (BD FACS LSR III by BD Biosciences, USA) with excitation and emission wavelengths fixed at 488nm and 525-530 nm respectively. The mean fluorescence intensity hence represents the cellular levels of intracellular mitochondrial membrane

potential.

3.4.7. AUTOPHAGY ANALYSIS

The detection and quantification of autophagosome production in lung cancer cells was done using flow cytometry as well as microscopic examination. $1x10^5$ cells/ml cells seeded in 6 well culture plate is then were exposed to polyphenolic fraction (25 µg/ml, 50 µg/ml, 100 µg/ml) and control. Later, media was removed and the cells were stained with Acridine orange (AO) for 15 minutes and washed with PBS and immediately analyzed by the flow cytometer (BD FACS LSR III by BD Biosciences, USA). Lung cancer cells were seeded in 6 well plates containing $3x10^5$ cells in each well and treated with plant extract for 48 hours. At the end of the treatment adherent cells were harvested by trypsinization and pooled with floating cells. Thereafter cells were centrifuged at 3000 rpm for 5 minutes at RT washed with PBS twice followed by staining with 1µg/ml Acridine orange for 15 min at 37^oC. Fluorescent microscope (LeicaDM300, Germany) was used to observe the autophagosomes by 490-nm blue filter.

3.4.8. CELL MIGRATION ASSAY

Cell migration is an essential cellular behavior of cancer cells that plays a crucial role in disease aggressiveness and metastasis. Therefore, reducing the migratory potential of cancer cells is essential for both successful lung cancer eradication and disease prevention. $3x10^5$ lung cancer cells were grown to full confluence in six well plates and wounded by 200µl pipette tips and washed twice with media to remove detached cells. Photomicrographs of initial wounds at 0 hours were taken using digital camera. The cells were exposed to polyphenolic fraction (25 µg/ml, 50 µg/ml, 100 µg/ml) and control, after treated with 0.5 µM mitomycin-C (arrests cell proliferation). The wound sizes with respect to the control were measured using imageJ software.

3.4.9. CLONOGENIC ASSAY

Similar to cell migration, clonogenicity is an essential cellular behavior of cancer cells that plays a crucial role in disease aggressiveness and metastasis. The

clonogenic cell survival assay determines the ability of a cell to proliferate indefinitely, thereby retaining its reproductive ability to form a large colony or a clone. 1 x 10^3 cells were seeded onto 6-well culture plates and incubated for 24 hours in CO2 incubator. After 24 hours, cells were treated with polyphenolic fraction (25 µg/ml, 50 µg/ml, 100 µg/ml) and left to grow undisturbed for next 10-15 days, At the end of treatment, cells were washed twice with ice cold PBS, fixed with 2ml/well of fixing solution for 10 minutes at RT and then stained with 1% crystal violet in methanol for 15 minutes followed by washing with deionized water till background was clear and air dried overnight. Colonies with more than 50 cells were scored and counted under the microscope. Representative photomicrographs were scanned after staining the cells.

3.4.10. CELL CYCLE ANALYSIS

Cell cycle analysis by quantitation of DNA content was one of the earliest applications of flow cytometry. There are Four distinct phases could be recognized in a proliferating cell population: Cells with 2n DNA content denote G0/G1 phase of cell cycle, while G2/M phase cells possess half DNA content (n) to that of G0/G1. Cells in S phase contain and intermediate amount of DNA ranging from (n-2n). Arrest in a particular phase of cell cycle increases the population load in that phase. Cell cycle phases G0/G1, S, G/M, and AP store varying quantities of DNA. The four phases of the cell cycle most generally used to characterise the cell's progress through its life cycle [130]. Many anti-cancer drugs stop a subset of cells from dividing at an appropriate time throughout the cell cycle. We focused on evaluating how polyphenolic fraction affects cell cycle distribution. Treated cells were harvested in the appropriate manner and wash in PBS. Later the cells were pellet down and fixed in ice-cold 70% ethanol by adding drop wise to the pellet while vortexing. This should ensure fixation of all cells and minimize clumping, for 30 min at 4°C. Later the cells were washed 2 times with PBS and centrifused at 850 and cell loss when discarding the supernatant especially after spinning out of ethanol was avoided. Cells were treated with ribonuclease, 50 µl of a 100 µg/ml stock of RNase. This will ensure only DNA, not RNA, is stained. The treated cells were stained with Propidium iodide (200 µl) from 50 µg/ml stock solution and analyzed using flow

cytometer [131]. PI has a maximum emission of 605 nm so can be measured with a suitable bandpass filter. The emitted fluorescent yield determined the relative cellular DNA content hence it enables identification of the distribution and arrest of cells in various stages. (BD FACS LSR III by BD Biosciences, USA). Finally percentage of cells in different phases of cell cycle analysis was carried out using BD FACS Diva software.

3.4.11. OIL RED O STAINING

The histological mechanism of the staining of lipids is invariably a function of the physical properties of the dye being more soluble in the lipid to be demonstrated than in the vehicular solvent. The lipid accumulation in response to the polyphenolic fraction in lung cancer cells was analyzed using Oil Red O staining. Cells treated with CAN free phenolic (25 µg/ml, 50 µg/ml, 100 µg/ml) for 48 hours. Supernatant was removed and washed with PBS and fixed using formalin, briefly wash with running tap water 1-10 mins. After that rinsed with 60% isopropanol followed by staining with freshly prepared Oil Red O working solution 15 mins and observed the a lipid accumulation phenotype using microscope (40X) (LeicaDMi1).

3.5. TOXICITY IN BALB/C MOUSE MODEL

Balb/C mice were grouped into 3 different sets, which included 6 mice each. Mice in control set were not fed with either of the plant extracts (LEX or CAN). The mice in the other two sets were fed with either CAN extract or LEX extract of highest dose 200mg/Kg for 3 months. All the mice were then sacrificed and various toxicity related tests were performed. It was then inferred that the above plant extracts were not toxic to the animals as all the tests were normal. The results of these experiments need to be statistically significant with minimum errors for its scientific as well as biological relevance is taken into account. Survival/mortality rate is to be kept in mind before doing the experiments for statistical significance of the results.

Animals used: Details selected species for experimental study are as follows:

- Species / Common name : ***Balb/c***
- Age/ weight/ size : **5-7 weeks/ 26- 32 gram**
- Gender : **Male**

3.6. PILOT STUDY IN ATHYMIC NUDE MOUSE MODEL

To study the anticancer activity of plant fraction, CF/LF against A459 cell induced carcinoma in Athymic nude mice. Mouse models of cancer have consistently been used to determine the *in vivo* activity of new anti-cancer therapeutics prior to clinical development and testing in humans. The most common models are xenografts of human tumors and cell lines grown subcutaneously in immunodeficient mice such as athymic (nude) or severe combined immune deficient (SCID) mice. These mouse strains exhibit very high take rates for xenografts, making them ideal hosts for *in vivo* propagation of human tumor cells. Xenograft tumors are usually established via subcutaneous inoculation of a predetermined number of tumor cells into the flank of nude mice. Nude mice were grouped into 3 different sets, which included 6 mice each. All the mice were injected with 10^6 A549 cells in the left thigh in order to develop tumors. As soon as the tumors started growing (tumor growth determined visually), mice in control set were not fed with either of the plant extracts (LEX or CAN). The mice in the other two sets were fed with either CAN extract or LEX extract of highest dose 200mg/Kg for 3 month. Mice were sacrificed and tumor size and weight in control mice and those fed with either of the extracts were determined.

Timeline

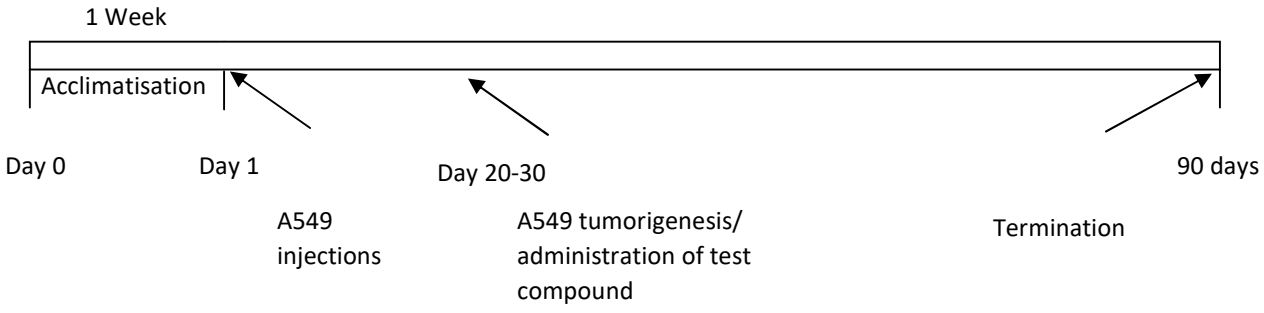

Table 3.1: Treatment given to different groups for study plant fraction CF

Plant Fraction	Experiments	No of mice needed	Duration
CF	Control (Normal diet)	6	3 Months
	Positive Control (A549 cells)	6	
	Dose 1 (25 mg/Kg Body Weight/mice/day) + A549 cells	6	
	Dose 2 (50 mg/Kg Body Weight/mice/day) + A549 cells	6	
	Dose 3 (100 mg/Kg Body Weight/mice/day) + A549 cells	6	

Table 3.2: Treatment given to different groups for study plant fraction LF

Plant Fraction	Experiments	No of mice needed	Duration
LF	Control (Normal diet)	6	3 Months
	Positive Control (A549 cells)	6	
	Dose 1 (25 mg/Kg Body Weight / mice/day) + A549 cells	6	
	Dose 2 (50 mg/Kg Body Weight / mice/day) + A549 cells	6	
	Dose 3 (100 mg/Kg Body Weight / mice/day) + A549 cells	6	

Basic study design

1. The cells to be used for injection will be maintained in aseptic conditions and at exponential growth prior to injection.

2. A549 cells will be prepared by trypsinization and the percentages of viable cells will be determined using trypan blue exclusion method (98% cell viability required). Cell suspensions are adjusted to the appropriate cell density.
3. Each mouse will receive a subcutaneous injection in the hind leg. Each injection contains 100 µl of one million cells with matrigel (A549 suspension).
4. The injection sites will be palpated three times weekly until tumors are established.
5. Animals will be then randomized into the necessary cohorts and administration of the test compound will be performed following the experiment design schedule.
6. The mouse weights will be recorded 3 times weekly.
7. At the end of the study, the mice will be sacrificed and tissues/blood will be collected as defined.
8. All tumors will be excised and weighted; tumors are documented by digital imaging.

All the animal experiments were certified by Centre for DNA Fingerprinting and Diagnostics, Hyderabad- 2035/GO/RBi/S/18/CPCSEA dated 28/09/2018. Protocol No. PCD/CDFD/29. Animals were procured from CPCSEA registered breeders and each animal as housed for 90 days depending on experiment, excluding quarantine and acclimatization period. The experiment was done in Experimental Animal facility, Centre for DNA Fingerprinting and Diagnostics, Opp. Metro Rail Pillar No. NUP 9, Inner Ring Road, Uppal, Hyderabad- 500039.

Furnish details of injections schedule is mentioned in Table 3.3.

Table 3.3: Furnish details of injections schedule

Substances	A549 cells
Doses	1 injection
Sites	Subcutaneous
Volumes	100 µl
Blood Withdrawal	
Volumes	2 ml
Sites	Cardiac puncture after sacrifice

3.7. SELECTION AND HOUSING CONDITIONS OF ANIMALS

A total of 48 nos of 5-7 weeks/25-35gram mice have been, randomly and separately from each other and have been put into a sterile husk propylene enclosure. Relative humidity 50±10 % was maintained and ambiemt temperature at 22±2°C with 12:12 light and dark cycles. Animals were fed regular pellet diet and water ad libitum throughout the experiments.

Figure 3.5: Athymic nude mice

3.8. METHODS OF DISPOSAL POST-EXPERIMENTATION

As soon as the experiments were completed test samples were humanely disposed off through below mentioned techniques.

- **Euthanasia** (Specific method): CO_2 inhalation/ as per CDFD SOP.
- **Method of carcass disposal**: Carcass will be stored in the deep freezer and handed over to the authorized vendor Medicare.
- **Rehabilitation**: Not applicable

Figure 3.6: Housing conditions of animals.

Data are presented as the mean ± standard deviation. Comparisons between multiple groups were performed using one-way analysis of variance. Comparisons between the unpaired two groups were performed using Student's t-test or $\chi 2$ test. Error bars represent standard deviation [a] Significantly different ($p \leq 0.001$) against untreated control. [b] Significantly different ($p \leq 0.01$) against untreated control. [c] Significantly different ($p \leq 0.05$) against untreated control.