CHAPTER 2

MATERIALS AND METHODS

2.1 CHEMICALS AND REAGENTS

$[^3]H$-Thymidine and $[^14]C$ Arachidonic acid were obtained from Amersham Pharmacia Biotech, United Kingdom. Dimethylsulfoxide (DMSO), propidium iodide, Rhodamine 123, phenyl methyl sulfonyl fluoride (PMSF), dithiothreitol (DTT), sodium bicarbonate, NBT (nitroblue tetra zolium), LPS from \textit{E.coli}, serotype 026:B6, EDTA and EGTA were obtained from Sigma Chemicals, Missouri, USA. CytoTox 96 cytotoxicity assay kit was procured from Promega, Madison, WI. Actinomycin D was purchased from Calbiochem, USA. Gentamycin was obtained locally. Primers for RT-PCR (Riverse transcriptase-polymerase chain reaction) were obtained from Integrated DNA Technologies and Ocimum Biosolutions. Fetal Calf serum (FCS), TRIzol reagent for RNA extraction and dNTPs, Taq polymerases for PCR were obtained from GIBCO BRL, USA, and New England Biolabs, UK, respectively. Monoclonal antibodies to IκB, NF-κB p65, caspase 3, caspase 9, Akt, PI3K and cox-2 were obtained from BD Pharmingen. Anti-Cdk 1, 2, 4, 6, anti-cyclin A, B, E, cyclin D1, D3, anti-pRb, anti-E2F, anti-PARP and β-actin antibodies were obtained from Santa Cruz Biotechnology, Santa Cruz, CA and Monoclonal antibody anti-p27 from Calbiochem, USA were used. Calcium chloride, HEPES buffer, sodium chloride and disodium hydrogen phosphate were purchased from Merck, USA. Pre-coated TLC plates (mesh size 60–120) were obtained from E-MERCK India. All other HPLC and analytical grade solvents and silica gel required for column purification were
obtained from SISCO Research Laboratories, India. All reagents were prepared using deionised (Millipore) or glass-distilled water. Leishman and Giemsa stain were obtained from HiMedia, Mumbai, India.

2.2 CELL CULTURE

HL-60 (leukemia cancer cell line), A549 (alveolar epithelial cell carcinoma) and J774 (murine macrophage cells) were procured from ATCC (American Type Culture Collection), cultured in RPMI - 1640 (Roswell Park Memorial Institute) medium for HL-60 and A549, for J774 cell maintenance F-12 Dulbecco's Modified Eagle Medium (DMEM) was used. This is supplemented with 10% heat-inactivated fetal calf serum (FCS) and amphotericin (3 \( \mu g/ml \)), gentamycin (400 \( \mu g/ml \)), streptomycin (250 \( \mu g/ml \)) and penicillin (250 units/ml). Cell cultures were maintained in a carbon dioxide incubator (humidified atmosphere) with 5% \( \text{CO}_2 \) at 37°C.

2.2.1 Preparation of Dulbecco’s Modified Eagle’s Medium (DMEM) and Rosewell Park Memorial Institute Medium (RPMI)

The powdered media (DMEM / RPMI) was dissolved in 900 ml of sterile glass - distilled or Millipore water in an autoclaved glass conical flask under sterile conditions. The antibiotics were added in the concentration as mentioned in section 2.2 and stirred well. 3.7 g of Sodium bicarbonate was added into the flask and stirred until it gets dissolved completely. 10% FBS was added and mixed well. The liquid was slowly poured into the upper portion of a Media sterilisation unit (Corning) and filtered through a 0.2 \( \mu \) filter under negative pressure. The medium was immediately stored at 4°C.

2.2.2 Saline: Trypsin: Versene (STV)

Saline A (10X) was prepared by dissolving 8g NaCl, 0.4g KCl, 1.0g D-Glucose and 0.35 g NaHCO\(_3\) (Tissue Culture Grade) in 100
ml water and stored at 4°C. Versene was prepared with 1g EDTA (Tissue Culture grade) was added to 90 ml distilled water. Then 5N NaOH was added drop wise until it gets dissolved. The solution was filter sterilized and stored at 4°C. 100 ml of STV was prepared by adding 25 mg of trypsin in a mixture of 10 ml of 10X Saline A and 2.5 ml of Versene. Double distilled water was added to make upto 100 ml, sterile filtered, aliquoted, and frozen at –20°C.

2.2.3 Maintaining and Storage of Cell Lines

HL-60 cells, A549 cancer cells showed a steady growth rate with a doubling time of 18 to 24 h. The cells reached confluency in 2 to 3 days and these cells were passaged to get the cells for the experiments and also to store in liquid nitrogen. Passaging was done as follows: The culture medium was removed from the 25ml culture flask by decanting into a clean container inside the laminar airflow chamber, and cells were rinsed with medium without serum, to remove traces of serum, which may inhibit action of trypsin. 2 ml of STV solution was added to the flask containing adherent cells (A549 and J774) and incubated at 37°C for a few minutes. As soon as cells started dislocating from the surface, flask was rinsed with 5ml of serum-containing medium to arrest the trypsinisation. The suspension of cells was collected in a sterile 15 ml centrifuge tube and the cells were pelleted at 1500 rpm for 3 mins. The cell pellet was resuspended in fresh medium with serum and a part of the cells were seeded back into the flask. HL-60 cells being non adherent, the cells were directly collected in a sterile 15 ml centrifuge tube and pelleted as described above. The remaining cells were used for experiment or pelleted as earlier and resuspended in cryopreservative medium (10% glycerol in serum for HL-60 and 10% DMSO in serum for A549) in a cryovial (Nunc) and frozen at -80°C for a day then transferred to liquid nitrogen.
2.3 EXTRACTION OF CENTROSEMA PUBESCENS LEAVES

The *Centrosea pubescens* dried leaves plant material was selected for studying anti-cancer activity. *Centrosea pubescens* Benth was collected from Kolli Hills, Tamil Nadu, India and Taxonomical details of the plant materials was authenticated by the Botanical Survey of India, Southern Circle, TNAU Campus, (Accession No: BSI / SC / 5 / 23 / 07-08 / Tech). Sequential extraction of shade dried leaves of *Centrosea pubescens* (100 g powder) was performed using solvents of varying polarities such as hexane, dichloromethane, ethyl acetate and methanol. Each extraction was carried out by soaking the material overnight and repeated three times with the respective solvent. Extracts were concentrated using rotary evaporator under reduced pressure at the temperature of 45°C to 50°C in order to avoid the evaporation of plant materials and labelled as CPHE, CPDE, CPEE and CPME. The final yields of the extracts were 0.89 %, 3.11 %, 0.37 % and 20 % approximately. Thin Layer Chromatography (TLC) was carried out to identify the similar profile for different batches of the same plant material. The concentrated extracts were reconstituted to obtain concentrations of 100, 50, 25, 10, 5 and 1 µg in dimethylsulphoxide (final concentration not > 0.4 %). For an example 10 mg of the dried extract from each was reconstituted to 400 µl of DMSO. Anti-proliferative studies were done using 1 to 100 µg/ml. Figure 2.1 shows a schematic drawing for the extraction method and the *in vitro* bioassay of the plant extracts.
2.4 \[^{3}H\] THYMIDINE INCORPORATION ASSAY

Thymidine incorporation was performed as reported in an earlier work from our laboratory by Senthil et al (2007) and Giridharan et al (2002). Briefly, different concentrations of (1 to 100 µg / ml) crude extracts and pure compound were checked for anti-proliferative activity on HL-60 and A549 cells in a 24 well plate. Each well contained \(1 \times 10^6\) cells in 1ml of RPMI 1640 medium. DMSO was used as vehicle control and \[^{3}H\]-thymidine (1 µCi / ml) was added to the well 24 h prior to harvesting the cells. Cells were then pelleted and washed sequentially with 10% and 5% TCA and solubilised in 0.1 N NaOH and 0.025% SDS solution. Radioactivity was measured in liquid scintillation counter (Packard Topcount-NXT) and data expressed in terms of percentage inhibition.
2.4.1 Dose Response and Time Course Analysis

To understand the kinetics of the anti-proliferative effect, a dose response and time course analysis of the effect of drug on HL-60 and A549 cells were performed. The amount of $[^3\text{H}]$ thymidine incorporated into cells exposed to various drug concentrations (1 to 100 µg / ml) for 24, 48 and 72 h were analysed as described previously.

2.5 THIN LAYER CHROMATOGRAPHY

Natural plant extracts being tested as potential anti-cancer agents consist of a composite mixture of compounds. In order to identify the active molecule(s) responsible for generating the cytotoxic effect, the optimization of a suitable solvent system is necessary which aids in the downstream purification processes. Such a solvent system must give good resolution of separation. TLC analysis was done with each of the crude extracts to identify and standardize the solvent system which gave for the best separation and resolution for further separation process. TLC plates were visualized under UV light of both short (254 nm) and long (365 nm) wavelength to identify fluorescing compounds. The plates were developed using spray reagents such as ammonium molybdate reagent containing 1 gm of ceric sulphate, 2.5 gm of Ammonium molybdate and 10% Concentrated $\text{H}_2\text{SO}_4$ and 10% concentrated sulphuric acid in methanol. The developed TLC plates were then documented.

2.6 ISOLATION OF PERIPHERAL BLOOD MONONUCLEAR CELLS (PBMC)

Peripheral blood was drawn from healthy volunteers. Mononuclear cells were isolated from the heparinised venous blood by Ficoll Diatrizoate gradient centrifugation using standard procedure (Gayathri et al 2007). Lymphocytes could be separated from the whole blood with the help of Ficoll
Hypaque gradient, which has a density closer to 1.077 g/mL. 20 mL of blood collected aseptically in a syringe was mixed gently with heparin. 20 mL of blood was carefully layered over 10 mL of Ficoll gradient. It was spun at 1800 rpm for 30 mins at room temperature. PBMCs identified as a buffy layer at the interface were collected and washed twice with the RPMI medium without serum and spun at 1500 rpm for 15 mins. The pellet was suspended in RPMI medium with serum and 10 µl of the suspension was mixed with tryphan blue and loaded in the Neubauer’s chamber to check the viability. 0.2 \times 10^6 cells were dispensed in 200 µl of each well of 96-well plate and assessed for viability using MTT kit assay.

2.7 ASSESSMENT OF CYTOTOXICITY USING LACTATE DEHYDROGENASE (LDH) RELEASE ASSAY

To confirm the suppressive effect of crude extracts and pure compound on cell proliferation, the supernatants were also assayed for the enzyme Lactate dehydrogenase (LDH). This stable cytosolic enzyme, which is released upon cell lysis, was measured using the CytoTox 96™ assay kit. The assay was done with 0.2 \times 10^6 cells / 0.2ml / well, seeded in 96 well cell culture plates. Cells on treatment with different concentrations (ranging from 1 µg to 100 µg/ml) of extracts and the pure compound of *Centrosemia pubescens* in HL-60, A549 and Peripheral blood mononuclear cells (PBMC). Triton X-100 was used to induce maximal lysis. The plate was read at 492 nm in a scanning multiwell spectrophotometer. The percentage cytotoxicity was calculated using the following equation.

\[
\text{Cytotoxicity (\%) = \left[ \frac{(A_{492} \text{ of treated cells} - A_{492} \text{ of control cells})}{(A_{492} \text{ of maximal lysis-A_{492} of control cells})} \right] \times 100}
\]
2.8 DETECTION OF APOPTOSIS

2.8.1 DNA Fragmentation Assay by Agarose Gel Electrophorsis Method

Internucleosomal cleavage of DNA was analysed as described previously (Kim et al 2001). Briefly, after treatment with crude extracts and pure molecule for appropriate time periods, cells were washed with ice cold PBS and resuspended in lysis buffer containing 10 mM Tris-HCl [pH 7.6], 2mM EDTA, 10 mM MgCl$_2$ and 10 mM KCl. After centrifugation at 10,000 rpm for 10 min, the pellet was resuspended in lysis buffer with 0.4M NaCl, SDS, and RNAse. It was then incubated at 55°C for 10 min. 125 µl of 5M NaCl was added and mixed well. After centrifuging at 10,000 rpm for 5 min, DNA in the supernatant was precipitated with 2 volumes of ethanol and 0.1 volume of 3M sodium acetate and kept at –80°C for 1 h. DNA was then pelleted by centrifugation at 12,000 rpm for 10 min and washed with 70% ethanol. The final pellet obtained after centrifuging at 12,000 rpm for 3 min was dried at 37°C. DNA was then resuspended in 20 µl of TE buffer and then run on 2% agarose gel stained with ethidium bromide. DNA was then visualized under UV and documented using Alpha Innotech Gel Documentation System (Senthil et al 2007).

2.8.2 DNA Fragmentation Assay by Diphenylamine Method

DNA Fragmentation was assessed by diphenylamine method as described by Francesco et al (2009). After drug treatment A549 cells was resuspended in Tris EDTA buffer (10 mmol / L Tris Hcl- pH 8 and 1 mmol / L EDTA) and lysed for 30 min at 4°C in cold lysis buffer (20mmol / L EDTA, 0.5% Triton X (vol / vol), 5 mmol / L Tris Hcl – pH 8). Intact chromatin (pellet) was then separated from DNA fragments (supernatant) by centrifugation at 13500 rpm for 10 mins at 4°C and the pellets were
resuspended in 500 µl of Tris EDTA buffer and samples were precipitated at 4 °C by adding 10% TCA. After overnight incubation DNA was pelleted by centrifugation at 7500 rpm for 10 mins and the supernatant was discarded and DNA was hydrolysed by boiling for 15 mins in 5 % TCA and then mixed with 150 µl of DPA (88 mmol / L DPA, 98% glacial acetic acid (vol / vol), 1.5% sulphuric acid (vol / vol) and 0.5% (vol / vol) of 1.6 % acetaldehyde). Absorption of the samples was measured at 595 nm and percentage fragmentation was calculated.

2.9 PROPIDIUM IODIDE STAINING

Formation of apoptotic bodies can be qualitatively visualized using this assay. Cell death was assessed using the uptake of the fluorescent dye propidium iodide by the fragmented DNA in dying cells that lack plasma membrane integrity and intact DNA in control untreated cells (Brana et al 2002). 1.0 x 10⁶ cells per well were seeded in 6-well plate. The cells were incubated with or without drug for specific time periods. After incubation, cells were washed with ice cold PBS and centrifuged at 6000 rpm for 10 min at 4 °C. The pellet was resuspended in 70% ethanol in PBS, fixed at –20 °C for 20 min or overnight at 4 °C and centrifuged at 6000 rpm for 10 min at 4 °C. Again the pellet was resuspended in PBS and treated with Rnase A (10 mg ml⁻¹) and incubated at 37 °C for 3 h and then centrifuged at 6000 rpm for 10 min at 4 °C. About 5 µl of propidium iodide (400 µg / ml) was added and incubated overnight. It was then centrifuged at 6000 rpm for 10 min at 4 °C. The pellet was washed with PBS, Fluorescence was excited with an Argon ion laser at 488 nm and visualised under Nikon Fluorescence microscope.

2.10 FLOW CYTOMETRIC ANALYSIS

Apoptosis induced by Centrosema pubescens in HL-60 and A549 cells was determined by DNA content analysis (sub-G₁) by flow cytometry
using propidium iodide (Nicoletti et al 1991). Briefly $2 \times 10^6$ cells/ml seeded in a 6 well plate were treated with crude extracts and pure molecule of *Centrolemma pubescens*. After incubation, cells were lysed and nuclear DNA was stained with 0.2ml of DNA binding buffer (0.3% Triton X-100, 0.2% RNase A, 1mg / ml trisodium citrate and 0.1mg / ml propidium iodide), followed by incubation for 15 min at 37°C and analysis for DNA content. Flow cytometry was performed using a FACScan flow cytometer equipped with Cell Quest software (Becton Dickinson). The fluorescence emitted by Pi-DNA complex, was collected through an FL-2 filter (585 nm).

2.11 PURIFICATION AND ISOLATION OF ACTIVE MOLECULE

2.11.1 Solvent - Solvent Fractionation of Active Crude Extract (CPDE and CPME)

Solvent- solvent fractionation was done for the active extracts CPDE and CPME for easy purification (Figure 2.2). The active extracts (Fraction 1) were dissolved in 70% methanol and filtered. Insoluble extract was labelled as precipitate (Fraction 2) and soluble part (mother liquor) was dissolved in hexane and extracted in separating funnel, the soluble was named as hexane soluble fraction (Fraction 3). The supernatant (mother liquor) was separated using chloroform in separating funnel to get the chloroform soluble fraction (Fraction 4) and hydro-alcoholic fraction. The hydro-alcoholic fraction was further fractionated using butanol to obtain the butanol soluble fraction (Fraction 6). The remaining hydro-alcoholic fraction was considered as fraction 5. (Sundaram R and Mitra SK 2007). All these fractions were subjected to anti-proliferative study by thymidine uptake assay.
2.11.2 Column Chromatography of Chloroform Soluble Fractions of CPDE and CPME

The active chloroform soluble fractions (CSF) of CPDE and CPME were subjected to column fractionation to isolate the molecule that is responsible for the anti-proliferative activity. Column was packed with hexane with silica gel 100-200 µ mesh size as a matrix, sample was loaded as dried slurry of silica gel and the column was eluted with increasing concentration of ethyl acetate (5 %) and methanol to increase polarities for chloroform soluble fraction of CPDE and similar procedure were followed for CPME chloroform soluble fraction, which was subjected to column chromatography with chloroform and followed by increasing concentration of methanol. The ratio of material loaded and silica gel was 1:20. The fractions
were analysed by pre-coated TLC plates and spots were visualised by exposure to UV, ammonium molybdate and 10% sulphuric acid in methanol spray reagents. Fractions with similar TLC profile were pooled together and the pooled fractions were subjected to thymidine uptake screen. The active pooled fraction was subjected to further stages of purification using flash column chromatography followed by 2\textsuperscript{nd} and 3\textsuperscript{rd} stage column with increased silica gel size (200 to 360 µ mesh size) until single active molecule isolation. After confirming the activity of the pure compound by bioassay, structural elucidation was done to analyze the nature of the chemical structure.

2.11.3 Quantification of Molecules Present in the CPDE-CSF using High Performance Thin Layer Chromatography (HPTLC)

High-performance thin layer chromatography is a sophisticated and automated form of TLC. HPTLC is an invaluable quality assessment tool for the evaluation of botanical materials. It allows for the analysis of a broad number of compounds both efficiently and cost effectively. HPTLC analysis was performed to quantify the concentration of the each compounds present in the chloroform soluble fraction of *Centrosema pubescens* dichloromethane extract (CPDE-CSF fraction). HPTLC silica gel, 60 F254 plate (10 cm x10 cm) was dried for 1-2 min at 50°C -60°C before use. The CPDE-CSF to be quantified were applied to the TLC plate and developed in a double trough 10 x 10 cm chamber pre-equilibrated with the mobile phase. The solvent system used for separation of compounds in CAMAG HPTLC is 50% ethyl acetate in hexane. The plates were viewed under UV light (365 nm) and documented.

2.11.4 Structural Characterisation of the Active Compound

2.11.4.1 Nuclear magnetic resonance spectroscopy and mass spectrometry

The structure of the active compound isolated from CPME was determined by $^1$H, $^{13}$C NMR, 2D NMR NOSY, COSY Spectra mapping,
electron spray ionisation mass spectrometry (ESMS), FTIR, UV spectra, LCMS and GCMS. The NMR experiments were done in CDCl₃ solution and ESMS using acetonitrile-water spray condition. The mass spectra confirmed elemental composition of the compounds by mass spectroscopy and gas chromatography mass spectroscopy. Purity of the active molecule was determined by HPLC analysis.

2.12 NF-κB INHIBITION ASSAY IN J774 CELL LINE

J774 is a murine macrophage cell line established from a tumor that arose in a female BALB/c mouse that primarily needs NF-κB for its survival. In this assay, 0.2 million cells were seeded in 200 µl of Dulbecco's Modified Eagle's Medium (DMEM) per well in 96 well plate. Various drug dilutions from Centrosema pubescens were added to the wells, with untreated cells as control and triton X and Bay-11-7082 as positive controls and maintained for 24 h in 5 % CO₂ at 37°C. NF-κB inhibition was measured by cytotoxicity assay as described previously.

2.13 MEASUREMENT OF RHODAMINE 123 UPTAKE BY SPECTROFLUORO METER

The mitochondrial membrane potential was assessed using the Rhodamine 123 uptake assay according to the method illustrated in (Yong Hwan Han et al 2009). Briefly, 0.2 × 10⁶ A549 cells / 0.2 ml / well, seeded in 96 well cell culture plates were treated with CPME (10 µg / ml) and its pure compound and CPDE (25 µg / ml). Actinomycin D (10 µg / ml) treated cells served as positive control. Untreated A549 served as control. The plate was incubated for 6,12,36,24 and 48 h. The cells were washed with PBS and incubated with 10 µg / ml Rhodamine 123 for 30 min at 37°C. Ethanol / water (1:1) was used to extract the dye retained by the cells. Fluorescence was
measured with a fluoriscan spectrofluorometer with an excitation wavelength of 485nm and an emission wavelength of 530 nm.

2.14 NITRIC OXIDE PRODUCTION ASSAY

NO is rapidly converted into the stable end products nitrite and nitrate. Nitrite in culture supernatants was measured by the Griess reaction. A549 cells were treated with 10 \( \mu \text{g} / \text{ml} \) of CPME, lead molecule and 25 \( \mu \text{g} / \text{ml} \) of CPDE for 24 h. 100\( \mu \text{l} \) of culture supernatant were mixed with an equal volume of Griess reaction (0.5% sulfanilamide, 2.5% \( \text{H}_3\text{PO}_4 \) and 0.05% naphthyethylene diamine in \( \text{H}_2\text{O} \)) and incubated for 10 mins at room temperature. Absorbance was measured at 540 nm and compared with a standard curve obtained using sodium nitrite.

2.15 ASSAY OF PHOSPHOLIPASE A\(_2\) ACTIVITY IN A549 CELLS

NSCLC cells have constitutively high expression of cytosolic phospholipase A\(_2\) (PLA\(_2\)) that cleaves AA from membrane phospholipids (Karam El-Bayoumy et al 2006). In order to determine whether our compound inhibits PLA\(_2\) activity, cells are loaded with \( ^{14}\text{C} \) Arachidonic acid. The A23187-stimulated release of incorporated \( ^{14}\text{C} \) Arachidonic acid reflects the PLA\(_2\) activity. Cells are seeded into 24 well plates at a density of 1 \( \times 10^5 \) / well and incubated overnight with \( ^{14}\text{C} \) Arachidonic acid (0.1 \( \mu \text{Ci} \)) along with crude extracts and pure compound at 37\(^\circ\)C. Supernatant is removed to measure the amount of \( ^{14}\text{C} \) Arachidonic acid incorporated into the cells. Cells are resuspended in PBS (pH 7.4) with calcium and exposed to A23817 (5 \( \mu \text{M} \)) for 20 min. Supernatant is collected to count the radioactivity released into the media. The radioactivity released into the medium reflects PLA\(_2\) activity and is measured with liquid scintillation counter. The PLA\(_2\)
activity is expressed as percentage of the total radioactivity incorporated into the cells.

2.16 PREPARATION OF CYTOPLASMIC, NUCLEAR EXTRACTS AND WHOLE CELL PROTEIN EXTRACTS

After drug treatment, A549 cells cytoplasmic and nuclear extracts were prepared according to (Dignam et al 1983). Briefly, the harvested cells were washed with ice-cold PBS and centrifuged at 4000 rpm for 10 mins and the pellet resuspended in 160 µL of buffer A (10 mmol / L HEPES [pH 7.9], 10 mmol / L KCl, 0.1 mmol / L EDTA, 0.1 mmol / L EGTA, 1 mmol / L dithiothreitol [DTT], and 0.5 mmol / L phenylmethylsulfonyl fluoride [PMSF]), and allowed to swell on ice for 15 mins, after which 40 µL of a 2.5% solution of 4-nonylphenolpolyethyleneglycol (Nonidet P-40) was added and the tube was vigorously mixed for 10 sec. The homogenate was centrifuged at 13000 rpm for 5 mins. The supernatant was saved as cytoplasmic extract. The pellet, containing nuclei, was resuspended in 40 µL of buffer B (20 mmol / L HEPES [pH 7.9], 0.4 mol / L NaCl, 1 mmol / L EDTA, 1 mmol / L DTT, and 1 mmol / L PMSF), and the tube was vigorously rocked at 4°C for 20 mins on a shaking platform. The contents, largely nuclear extract, were centrifuged at 13500 rpm for 5 mins at 4°C, and the protein concentrations were measured by Bradford method.

For whole cell extract preparation, 2×10^6 HL-60 and A549 cells/ml were seeded in 6 well plates, treated with appropriate concentrations of drug and after 24 h incubation they were washed with 1X PBS and pelleted at 6000 rpm for 3 mins at 4°C. 40ul of lysis buffer [97.5% TOT-X (20 mM HEPES [pH 7.9], 350mM NaCl, 0.5mM EDTA, 0.5mM EGTA, 0.5mM DTT, 1% NP-40, 25% glycerol, 1mM MgCl2 ), 2% PMSF, 0.5% Aprotinin] was added to the pellet, incubated in ice for 30 and centrifuged at 12000 rpm for 20 mins at 4°C. The supernatant was carefully taken and stored at -80°C.
2.17 WESTERN BLOT

Protein Estimation using Bradford’s method was done to quantify protein concentration. Equal amount of proteins (100 µg) were electrophoresed on SDS-polyacrylamide gel (10%). Then the protein was then transferred onto a nitrocellulose membrane (Hybond C+, Amersham life sciences) by electroblotting at 20 V, 120 mA for 1 h and 30 mins. The membranes were blocked with 5% skim milk overnight at 4°C and then the membrane was washed with PBS, three times for 5 mins each and then incubated with specific primary antibodies to study the protein expression of various cellular targets such as Bcl-2, IκB-α, NFκB p65, Akt, cox-2, PI3K, PARP, CDK-1, 2, 4, 6, Cyclin A, B, E, D1, D3 and p27. Primary antibodies was added with the respective concentration in PBS containing 1% BSA and 0.1% Tween 20 and rocked gently at room temperature for 2 h. The blot was again washed 3 times with PBS for 5 mins each. Secondary antibody (1:5000) in 1% BSA in PBS/TBS and 0.1% Tween 20 was added and allowed to hybridize for 1 h at room temperature. The primary antibody complex was then stained with ALP-conjugated secondary antibodies. The immunocomplexes were developed and the bands were observed using chromogenic substrate NBT-BCIP in alkaline phosphate buffer. The expression levels were normalized using β-actin as internal loading control.

2.18 ELECTROPHORETIC MOBILITY SHIFT ASSAY (EMSA)

Electrophoretic mobility shift assay was carried out using NF-κB oligonucliotides. Double strand oligo synthesis was done using NF-κB Sense (5’ AGC TAT GTG GGT TTT CCC ATG AGC 3’) and anti-Sense (5’ GCT CAT GGG AAA ACC CAC ATA GCT 3’) oligos by denaturing at 95°C for 10 mins, then gradually temperature was reduced to 20°C and snap chilled on ice then the final products were run on 1.5 % agarose gel electrophoresis to
confirm the formation of double strand synthesis. Synthesised double strand oligos were labeled with $^{32}\alpha$ dCTP using RediPrimeII random primer labeling kit (Amersham pharmacia). 50µl of reaction mixture containing 50 µg / ml double stranded poly di:dc, 50µg / ml BSA, binding buffer ( 25 mM HEPES pH 7.5, 0.1 mMol zinc chloride, 1 mMol DTT, 40 mMol KCL, 25mM Mg$^{2+}$, 0.3% brij and 5% glycerol) and 10% glycerol. 15µl of reaction mixture, 0.25ng of labeled probe and nuclear extract containing 15µg of protein with 10µl of loading dye (bromophenol blue) were incubated at room temperature for 1 h. The samples were loaded onto 7 % native polyacrylamide gel (40:1, acrylamide-bisacrylamide), and run with 0.25×TBE at 100 V until the bromophenol blue dye reached approximately $\frac{3}{4}$ down the length of the gel. Gel were then dried on 3 M blotting paper (Whatman) and exposed to X-ray film at -80°C overnight.

2.19 POLYMERASE CHAIN REACTION  RT-PCR

Primer sequences (synthesized by Integrated DNA Technologies and Ocimum Biosolutions) used to analyse different genes (Table 2.1).

After drug treatment total RNA of the cells was extracted using TRIzol reagent and RNA from each group was reverse-transcribed to cDNA as described by Hall et al., 1998. The resulting cDNA was amplified with PCR reaction buffer. For PCR reaction, 1 µl of the cDNA mixture prepared as described earlier was added to a PCR reaction mixture consisting of

i) 1 X PCR buffer

ii) 2.5 pmol dNTPs

iii) 5 pmol of paired primers,

iv) 1.25 units of Taq polymerase (Amersham Pharmacia)

Finally made upto 50 µl with distilled water
Thermal cycler for cyclic reactions as follows:

95°C for 5 mins : Initial denaturation  
95°C for 1 mins : Denaturation  
(X°C) for 1 min : Annealing temperature \{ one cycle \}  
72°C for 1 min : Extension  
72°C for 10 mins : Final Extension  

Total number of cycles = 35

Annealing temperature (X°C) for GAPDH and Bax were 55°C, K Ras was 56°C, Bcl-2 was 63°C, Cytochrome C1 was 60°C, Caspase 3 was 54.6°C, Caspase 8 was 50 °C, TNF-α was 54°C and p53 was 50.5°C.

**Table 2.1** List of primer sequences used in this study

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequence</th>
<th>Product size (bp)</th>
</tr>
</thead>
</table>
| GAPDH      | 5’ TCC CAT CAC CAT CTT CCA 3’  
|            | 5’ CAT CAC GCC ACA GTT TCC 3’ | 597 |
| K-Ras      | 5’ GAC TTC AAG ATC CGA ACC 3’  
|            | 5’ AGC CAC AGA CTC AAA TGC 3’ | 586 |
| Bax        | 5’ ACC AAG AAG CTG AGC 3’  
|            | 5’ ACA AAG ATG GTC ACG 3’ | 364 |
| Bcl-2      | 5’ CGA CTT CGC CGA GAT GTC CAG 3’  
|            | 5’ CTT GTG GCC CAG ATA GGC ACC 3’ | 588 |
| Cytochrome C1 | 5’ TCT CTT CCT TGG ACC ACA CC 3’  
|              | 5’ GTA GAG ACC TTC CCG CAG TC 3’ | 392 |
| Caspase-3  | 5’ TTC TTC AGA GGG CAT CTG TG 3’  
|            | 5’ CGG CCT CCA CTG GTA TTT TA 3’ | 151 |
| Caspase-8  | 5’ AAG CAA ACC TCG GGG ATA CT  
|            | 5’ CGG CCT CCA CTG GTA TTT TA 3’ | 468 |
| TNF-α      | 5’ CAG AGG GAA GAG TTC CCC AG  
|            | 5’ CCT TGG TCT GGT AGG AGA GAC G 3’ | 350 |
| p53        | 5’ GAA GAC CCA GGT CCA GAT GA 3’  
|            | 5’ CTC CGT CAT GTG CTG TGA CT 3’ | 348 |
2.19.1 Agarose Gel Electrophoresis

PCR products were checked by agarose gel electrophoresis. The materials prepared were as follows: Tris-Borate EDTA buffer (TEB) - 89 mM Boric acid, 89 mM Tris-HCl, 2 mM EDTA pH 8.0; loading dye: 50% glycerol in 1X TEB with orange dye, TEB (10X) pH 8.0; staining solution - 2.5 µg Ethidium bromide/ml of distilled water; DNA marker - 100 bp. Depending on PCR product size, 1.5 to 2 % agarose gels were used for DNA separation. Gels were prepared with TEB (0.5X) buffer. 10 µl of PCR products mixed with 3 µl of the Orange G dye was loaded in prestained agarose gel and run at 50-75 mV at room temperature. Then the gel was viewed under UV light and photographed.

2.20 ASSAYS FOR ESTIMATING DIFFERENTIATION

2.20.1 Preparation of Drug Dilutions for Differentiation Assays

The concentrated extracts were weighed and reconstituted to obtain concentrations of 100 µg, 50 µg, 25 µg, 10 µg, 5 µg and 1 µg in

- DMSO (final concentration not > 0.4%) - for anti-proliferation, apoptosis-induction assays and cell cycle analysis
- Methanol (final concentration not > 1.0%) - for differentiation assays to eliminate the effect of DMSO in inducing differentiation. Hence methanol was used for preparation of drug dilutions.

2.20.2 NBT Reduction Assay

The Nitro Blue Tetrazolium (NBT) reduction assay was done as follows. 1 x 10⁶ cells were seeded per ml of medium. 1.25% (v / v) DMSO
was used as positive control. Untreated cells were used as control. Cells were treated with 1 µg / 10 µl concentration of CPDE and CPME were maintained for various time periods viz. 1 day, 2 day, 3 day, 4 day and 5 day. After the appropriate time periods, the different time point samples were collected in 15 ml centrifuge tubes and the cells were pelleted by centrifuging at 1500 rpm for 3 mins.

The cells were counted using a Neubaeur chamber and equal number of cells were suspended in 100 µl of medium and were seeded in flat bottomed 96-well plate in triplicates (atleast 0.5 million cells per well). 50 µl of PMA (a final concentration of 100 ng / ml reaction) dissolved in PBS was added to all the wells. Cells were incubated for half an hour at 37°C. 50 µl of NBT (0.1%) was added to half of the wells and 50 µl of PBS was added to the remaining wells, to make up the reaction volume to 200 µl. The cells were incubated in the dark for 1 h at 37°C. Then the cells were spun at 4000 rpm for 15 mins and the supernatant was discarded. The formazan deposits in the cells were dissolved with 150 µl of DMSO and the absorbance was measured at 595 nm in an ELISA reader.

\[
\text{Superoxide production} = \text{Average OD with NBT} - \text{Average OD without NBT}
\]

This was calculated to eliminate any background hue due to the addition of PMA. The extent of NBT reducing activity of a sample can be correlated with the superoxide production and the latter can be connected directly to the extent of cells differentiated.

2.20.3 Thymidine Uptake Assay of Differentiated Cells

The cells were prepared as described for NBT reduction assay. 1 million cells per ml of medium were seeded and 1 µCi of [³H] thymidine
was added per well. They were incubated for 24 h, harvested and radioactivity was counted as mentioned above.

2.20.4 Superoxide Dismutase Assay

The samples were loaded onto the non-denaturing polyacrylamide gel and the gel was run for 7 h. For the initial 3 h, the gel was run using running gel buffer (RGB) without SDS at a pH of 8.8. For the next 4 h, the gel was run using RGB without SDS at a pH of 8.3. After electrophoresis, the gel was incubated in NBT solution (2.5 mM) for 30 min in the dark with gentle shaking, followed by 30 min incubation with a solution containing 10 mg / ml of riboflavin and 4 µl of TEMED. SOD activity was detected as the achromatic band on the violet-coloured gel, obtained after light exposure.

2.20.5 Wright Giemsa Staining

A clean glass slide was taken and the cells were smeared on the glass slide using another glass slide held at 45°. The cells were then stained with giemsa stain uniformly and kept for 2-3 mins. The excess stain was then removed by washing with PBS and the slide was allowed to air dry. After drying, the slide was kept under a fluorescence microscope to study the morphology of the cells.

2.21 ANIMAL STUDY

Male Swiss Albino Mice 5-7 weeks old weighing approximately 20 to 25g were obtained from Kings Institute of Preventive Medicine, Chennai, India. Animals were housed in polypropylene cages, in rooms with controlled temperature (24 ± 4°C), relative humidity (60 ± 5%) and proper lighting one week prior to start the experiment supplied with standard pellet diet (Hindustan lever limited, Bangalore, India) and water ad libitum. The animals
were used for the study on approval by Institutional animal Ethical Committee (Registration no or IAEC no 550/02/a/CPCSEA dated 22.01.2002 Meeting held on 6th October 2008 in the centre for biotechnology at 10.30 A.M)

2.21.1 Toxicological Studies

The toxicological studies were done with dosages selected based on the in vitro studies for a period of 30 days (alternative days for 15 doses) in experimental mouse model to check its toxicity towards normal cells by recommended dosage in the treatment of cancer. Toxicity if any was determined by assay of by histological observation.

Group I – control (Corn oil alone), Group II – Centrosema pubescens dichloromethane extract (CPDE) 25 mg/kg b.w, Group III, IV, V was similar as that of group II with 50, 75 and 100 mg/kg b.w concentrations. Group VI, VII, VIII and IX - Centrosema pubescens Methanol extract (CPME) 25, 50, 75 and 100 mg/kg b.w respectively.

We found at 50 mg/kg b.w of CPDE and CPME showed maximum efficacy. The dosage chosen for the study (50 mg/kg b.w for CPDE and CPME respectively) maintained normal architecture of hepatocytes of the liver and no significant alterations were seen in columnar epithelial lining of bronchioles of the lung as that of control mice in histological studies, so these dosage was chosen because of its non toxicity.

2.21.2 Experimental Design

Animal Grouping (n=6) for in vivo studies. Group I Normal (untreated mice), Group II B(a)P Induced control, Group III Post-treated CPDE (50mg/kg b.w) administered 15 days continuously after B(a)P
administered at post initiation stage (tumour state), Group IV was same as that of Group III with CPME (50mg / kg b.w).

2.21.3 Induction of Lung Carcinoma

Development of lung carcinoma in Swiss albino mice involved the oral administration of Benzo(a)pyrene (50mg/kg body weight) in corn oil for four weeks (twice weekly) (Speir et al 1978).

2.21.4 Histology

The liver and lung tissues were dissected out and washed in ice-cold PBS immediately. A portion of the tissues was fixed in 10% buffered neutral formalin solution for histological studies. After fixation, tissues were embedded on paraffin and solid sections were cut at 5µM. All section were stained with haemotoxylin and eosin. The sections were examined under light microscope and photomicrographs were taken.

2.21.5 Immuno Histochemistry

Animal tissue sections of 4.5µm size were taken from paraffin embedded tissue blocks and placed on pre coated slides with poly-L-lysine or egg albumin and then incubated at 56°C for overnight. Section were deparaffinized in xylene, using three changes of fresh xylene, 10 min each and further rehydrated gradually through descending grades of alcohol (95, 90, 70, 50, 30 %), 5min each. Finally, the sections were hydrated in distilled water. In order to quench endogenous peroxidase activity, sections were incubated in 0.3% \( \text{H}_2\text{O}_2 \) in methanol for 30 min and washed in distilled water for 5 min. Selected sections were rinsed with 1x PBS and the sections were incubated with 3% BSA in PBS, for 30 min in a humid chamber, to suppress non-specific binding of antibody. Then, sections were washed with
1x PBS thrice and incubated with primary antibody at appropriate dilution of 1:100 in 1% BSA overnight at 4°C in a humid chamber and again rinsed with three changes of 1X PBS. Sections were dried and incubated with secondary antibody at 1:200 dilutions in 1X PBS, for 45 min at room temperature in humid chamber and rinsed again with 1X PBS thrice. The sections were then incubated with substrate solution (0.7 mg/dL diaminobenzidine and 3% hydrogen peroxide in 5ml of distilled water) for 10 min in humid chamber. The sections were then rinsed in distilled water 1 or 2 drops ammonia solution for 2 min for better color development. Then dehydrated gradually in ascending grades of alcohol (70, 75, 100 %), 5 min each. Then the sections were mounted in DPX with cover slips and allowed to air dry. The sections were analysed for the immunoreactivity under light microscopy and photographed.

2.21.6 Tissue Homogenization

After sacrifice, 100 mg of the lung tissue were cut in to small pieces and homogenised using Potter-Elvehem homogenizer in 1ml of Tris HCl homogenate buffer, pH 7.4 to give a 10% homogenate. This homogenate was estimated for its protein content by bardfoed’s method and Western blotting analysis were done similar as that of section 2.17 in materials and methods to study in detail about the markers involved in cell cycle regulation.

2.22 DENSITOMETRIC ANALYSIS

Densitometric scanning was performed for the necessary experiments and the integral density values (IDV) of untreated controls were assigned an arbitrary value of 1. The arbitrary values of treated samples were represented in comparison to the untreated control values.
2.23 DATA OR STATISTICAL ANALYSIS

All experiments were repeated three times in triplicates and the values expressed as mean± SEM. The statistical significance between means of the independent groups was analyzed using one way ANOVA and p < 0.05 was considered to be statistically significant.