CHAPTER 3
RESULTS AND DISCUSSION

3.1 ASSESSMENT OF THE ANTI-PROLIFERATIVE ACTIVITY OF CENTROSEMA PUBESCENS BENTH USING HL-60 AND A549 AS AN IN VITRO MODEL

3.1.1 Selection and Extraction of Medicinal Plants for Anti-Proliferative Screening

Numerous reports in folklore on herbal plants have attributed various medicinal benefits for the treatment of diseases. But, only a few of them claim to have some scientific validity. From early days, plants have been a good source for several compounds, which are being widely used to treat diseases like cancer (Stefania Nobili et al 2009). These molecules represent the outcome of random screens, and one such example is taxol (Wall and Wani 1996). Interplay between modern technology and traditional knowledge would aid in the isolation of effective anti-cancer drugs. Based on literature and folklore studies, the plants Enicostemma auxilleria, Execaria agallocha, Dodonea viscosa, Leucas aspera, Tridax procumbens, Centrosemab pubescens and Gauzima ulmifolia were screened for anti-proliferative activity in HL-60 and A549 cancer cell lines. The dried plant powder were sequentially extracted using solvents of varying polarities from non-polar to polar range such as Hexane, dichloromethane, ethyl acetate and methanol. The concentrated extracts were weighed and reconstituted to obtain concentrations of 1, 5, 10, 25, 50 and 100µg/mL in DMSO (final concentration not > 0.4%). Dose response analysis were performed at concentrations ranging from 1 to 100µg/mL at 24 h and assayed for [³H]-
thymidine incorporation to study the anti-proliferative activity. All the plants except *Centrosema pubescens* showed less <50% anti-proliferative activity whereas *Centrosema pubescens* showed maximum activity of 99.44%.

### 3.1.1.1 Yield of *Centrosema pubescens* extracts in different organic solvents

100 g dried, powdered leaves of *Centrosema pubescens* yielded approximately 0.89% of hexane extract (CPHE), 3.11% of Dichloromethane extract (CPDE), 0.37% of Ethyl acetate extract (CPEE) and 20% of Methanol extract (CPME) respectively.

### 3.1.2 Anti-proliferative Effect of *Centrosema pubescens* Extracts on Leukemic Cancer Cells using $[^{3}\text{H}]$ – Thymidine Incorporation Assay

Thymidine incorporation assay was done to select the best extract of *centrosema pubescens* based on its anti-proliferative activity (Giridharan et al 2002). Incorporation of radioactive thymidine is an index of the extent of proliferation exhibited by a proliferating cell. Growing and dividing cells take up thymidine for incorporation into DNA during active DNA synthesis. HL-60 is derived from a patient with acute promyelocytic leukemia and has been comprehensively used as an *in vitro* model for studying various events involved in the proliferation and differentiation of normal and leukemic cells of the granulocyte/monocyte/macrophage lineage (Li-Yun Luo et al 2006). Anti proliferative screening and dose response assay exhibited that dichloromethane (CPDE) and methanol (CPME) extracts of *Centrosema pubescens* showed high anti-proliferative activity of 99.44 % and 87.22 % respectively at 100µg/mL in HL-60 cells at the 24h time point using $[^{3}\text{H}]$ - thymidine incorporation assay. CPHE and CPEE had very minimal influence on the proliferating leukemic cancer cells (13.88 % and 62.45 % at100µg/mL respectively) (Figure 3.1).
Figure 3.1 Screening for anti-proliferative effect of Centrosema pubescens extracts on HL-60 cells

Dose response analysis from 1 to 100µg/mL of the different solvent extracts of Centrosema pubescens on HL-60 at 24 h. Results are expressed as % of inhibition with respect to untreated control. DMSO is the solvent control and showed no effect on the cells. Hex - Hexane, DCM - Dichloromethane, EtOAC - Ethyl acetate, MeOH - Methanolic extract

3.1.3 Anti-Proliferative Effect of Centrosema Pubescens Extracts on Lung Cancer Cells using [³H] – Thymidine Incorporation Assay

A549 is human alveolar basal epithelial lung carcinoma adherent cell line derived from a 58-year-old caucasian male. Constitutive activation of NF-κB in this cell line has been established by Linxiang et al (2007). Analysis of the dose response by the extracts showed that CPHE, CPDE, CPEE and CPME at 100µg/mL exhibited maximum anti-proliferative activity. Among these CPDE and CPME showed marked inhibition of proliferation at 84.85 % and 94.96 % respectively (Figure 3.2). CPHE and CPEE showed minimal activity (35.23% and 66.37% respectively).
Figure 3.2 Screening for anti-proliferative effect of *Centrosema Pubescens* extracts on A549 cells

[^3]H-Thymidine incorporation assay with 1 to 100µg/mL of different solvent extracts on A549 cells at 24 h. Results expressed as % of inhibition with respect to untreated control. Solvent control (DMSO) had no effect on cells.

3.1.4 Time Course and Dose Response Analysis of CPDE and CPME in HL-60 Model

Dose response analysis (1, 5, 10, 25, 50, 100µg/mL) suggested that optimum dose for 50% inhibition (IC$_{50}$) in the HL-60 cells was found to be 5µg/mL for dichloromethane extract and 10µg/mL for methanol extract of *Centrosema pubescens*. CPDE and CPME treatment inhibited the uptake of radio-labelled thymidine in HL-60 cells in a dose and time dependent manner (24, 48 and 72 h) (Figure 3.3a and b respectively). CPDE and CPME treatment exhibited similar effect of anti-proliferative activity at the higher doses tested and very slight differences at the lower doses.
Figure 3.3a  Time course and dose response analysis of CPDE in HL-60 cells
Anti-proliferative effect of CPDE in HL-60 cells by $[^3]H$-Thymidine incorporation assay. Different concentrations (from 1μg/mL to 100μg/mL) of extract was treated for 24, 48, and 72 h. % of inhibition is expressed with respect to untreated control. Solvent control (DMSO) had no effect on cells.

Figure 3.3b  Time course and dose response analysis of CPME in HL-60 cells
Anti-proliferative effect of CPME in HL-60 cells by $[^3]H$-Thymidine incorporation assay. Different concentrations (from 1μg/mL to 100μg/mL) of extract was treated for 24, 48, and 72 h. % of inhibition is expressed with respect to untreated control. Solvent control (DMSO) had no effect on cells.
3.1.5 Time Course and Dose Response Analysis of CPDE and CPME in A549 Model

Dose response analysis of dichloromethane and methanol crude extract was carried out using thymidine uptake assay to identify the minimal dose required for maximum inhibition of proliferation in A549 cells. The cells were incubated with various doses (1, 5, 10, 25, 50 and 100 µg/mL) of crude extracts for 24, 48 and 72 h time duration. The studies reveal that 25 µg/mL of CPDE and 10 µg/mL of CPME was sufficient for maximum inhibition of proliferative response in *C. pubescens* as shown in Figure 3.4a and b respectively.

![Figure 3.4a](image)

**Figure 3.4a Time course and dose response analysis of CPDE in A549 cells**

Anti-proliferative effect of CPDE in A549 cells by [³H]-Thymidine incorporation assay. Different concentrations (from 1 µg/mL to 100 µg/mL) of extract was treated for 24, 48, and 72 h. % of inhibition is expressed with respect to untreated control. Solvent control (DMSO) had no effect on cells.
Figure 3.4b Time course and dose response analysis of CPME in A549 cells

Anti-proliferative effect of CPME in A549 cells by [$^3$H]-Thymidine incorporation assay. Different concentrations (from $1\mu$g/mL to $100\mu$g/mL) of extract was treated for 24, 48, and 72 h. % of inhibition is expressed with respect to untreated control. Solvent control (DMSO) had no effect on cells.

3.1.6 Cytotoxicity Effect of CPDE and CPME on PBMC, HL-60 and A549

The assessment of apoptosis versus necrosis is an essential parameter to distinguish between toxicity and efficacy of the treatment (Zamai et al 2001). To confirm that the anti-proliferative potential of CPDE and CPME on HL-60 cells was due to apoptosis and not because of necrosis, LDH release assay was performed in HL-60, A549 cells and compared with human peripheral blood mononuclear cells (PBMC) isolated freshly from healthy volunteers. LDH release was measured quantitatively in HL-60, A549 and PBMCs at 24 h using a cytotox 96 assay kit and the results are expressed as % cytotoxicity with respect to control (untreated cells) and Triton X 100 as positive control. Treatment of PBMC, HL-60 and A549 cells with various concentrations of CPDE and CPME exhibited minimal LDH release and cytotoxicity in the range of 0.83 % to 20 % at 24 h (Figure 3.5a, b and c).
CPDE and CPME exhibited cytotoxicity less than 20% at the highest tested concentration. The results show that even a high dose of the extract (100µg/mL) did not evoke any toxicity in PBMCs, HL-60 and A549 cells.

Figure 3.5 (Continued)
Figure 3.5  Effect of cytotoxicity on (a) PBMC, (b) HL-60 and (c) A549 treated with CPDE and CPME was studied by LDH release assay at a different concentrations ranging from 1µg/mL to 100µg/mL at 24 h.

The results are expressed as % cytotoxicity with respect to control and Triton X 100 as a positive control.

In conclusion, our studies confirmed the potential anti-proliferative effect of _Centroesea pubescens_ dichloromethane (CPDE) and methanolic (CPME) extracts on lung and leukemic cancer model without causing adverse effect to normal cells. Thus _C. pubescens_ was taken for further purification process for the isolation of molecules with potential anti cancer activity.
3.2 TO ISOLATE AND CHARACTERIZE ANTI-CANCER COMPOUNDS FROM THE ACTIVE EXTRACTS OF C. PUBESCENS USING IN VITRO BIOASSAY-BASED SCREENING AND COLUMN CHROMATOGRAPHY

3.2.1 Isolation of Anti-cancer Compound from CPDE using Fractionation, Column Chromatography and Bioactivity Based Screening

Since CPDE showed significant anti-proliferative properties in both leukemia and lung cancer models, it was taken for further purification in order to identify the bio-active molecule(s) responsible for its activity (Figure 3.6a). Solvent–solvent fractionation of CPDE yielded four different fractions such as precipitate or insoluble fraction, hexane fraction, chloroform fraction and hydro-alcohol soluble fractions (Figure 3.6b). [³H] thymidine uptake assay revealed that the chloroform soluble fraction (CSF) showed maximum anti-proliferative activity greater than 80% even at 1μg/mL concentration whereas the other three fractions did not show much activity (Figure 3.6c). CPDE CSF (chloroform soluble fraction of CPDE) was subjected to further purification using conventional column chromatography.

![Figure 3.6a Thin Layer Chromatography (TLC) profiling of compounds in present C. Pubescens extracts](image)

(A) Hexane extract (Solvent system: 40% ethyl acetate in hexane), (B) Dichloromethane extract (Solvent system: 50% hexane in ethyl acetate), (C) Ethyl acetate extract (Solvent system: 40% isopropyl alcohol in dichloromethane) and (D) Methanolic extract (Solvent system: 30% ethyl acetate in chloroform).
Figure 3.6b TLC profiling of compounds present in solvent-solvent fractions of CPDE. 50% Hexane in ethyl acetate solvent system were used to resolve the fractions
Lane 1: CPDE, Lane 2: Precipitate, Lane 3: Hexane Soluble Fraction, Lane 4: Chloroform Soluble Fraction, Lane 5: Hydro-alcoholic Fraction.

Figure 3.6c Anti-proliferative activity for the solvent-solvent fractions of CPDE in HL-60
The % inhibition of the different solvent-solvent fractions (precipitate, hexane soluble fraction, chloroform soluble fraction and hydro alcoholic fraction) of CPDE. Fraction 3 (Chloroform soluble fraction) showed maximum inhibition compared to other fractions at 24 h.
3.2.2 Purification of CPDE CSF Using Chromatography Techniques

CPDE CSF was subjected to silica gel column chromatography that yielded several column fractions. These column fractions were pooled based on the similarity in TLC finger printing and 13 pooled fractions were obtained (Figure 3.7). These 13 fractions were subjected to bioassay to assess its inhibitory activity on the proliferation of leukemic cancer cells (HL-60) (Figure 3.8). Fractions 3 to 10 showed significant activity in thymidine incorporation assay whereas fraction 11 showed moderate inhibition of proliferation. But fractions 12 and the last fraction 13 showed very negligible percentage of proliferation. From the above results it is seen that the column chromatography fractions of CPDE-CSF showed a significant inhibition of proliferation similar to that of CPDE.

![TLC of the first stage column fractions of CPDE CSF to resolve the compound(s) using the solvent system (50% hexane in ethyl acetate).](image_url)

Profile of TLC was detected using (A) UV at 365 nm, (B) UV at 254 nm, (C) ammonium molybdate spray reagent. First Lane in the TLC was spotted with CPDE CSF as reference
Figure 3.8  Anti-proliferative study of CPDE CSF fractions (Fr 1 to 13) in HL-60 leukemia cancer cells.

The graph shows the % inhibition of different column fractions (Fr1 to Fr 13) of CPDE CSF fractions. Fraction 3 to 10 showed maximum inhibition compared to other fractions at 50 and 100µg/ml concentrations.

Fractionation of CPDE CSF and TLC finger printing analysis indicates that the 6\textsuperscript{th} fraction was enriched with a molecule, visible to a lesser extent in the CPDE CSF. The fraction 6 showed maximum activity (97.99 %) similar to fraction 5 (96.88 %), and which was greater than the activity of fractions 3 (91.17 %) and 4 (91.35 %), at 100µg/mL concentration. Even though fractions 5 and 6 showed more or less equal activity, fraction 5 did not show the prominent compound which was seen in fraction 6. Hence fraction 6 was taken through further purification to isolate the expected compound (Figure 3.9a). At the end of this process, the expected molecule was successfully isolated and named as CP1 the TLC profile as shown in Figure 3.9b. However, the CP1 in bioassay was found to be inactive when compare with its mother fraction 6.
Figure 3.9a  TLC profile of 2nd stage column purification of fraction 6 from CPDE CSF

TLC analysis of the second stage column of fraction 6 of CPDE CSF using 50% hexane in ethyl acetate. Profile of TLC was detected using (A) UV at 365 nm, (B) UV at 254 nm, (C) ammonium molybdate spray reagent. First Lane in the TLC was spotted with Fraction number 6 as reference.

Figure 3.9b Isolated single pure molecule (CP1) from Fraction 6 of CPDE CSF

TLC Profile was documented using (1) UV at 365 nm (2) UV at 254 nm (3) Ammonium molybdate spray reagent.

During the TLC profiling of fractions 9, 10 and 11 another major molecule was observed prominently in the TLC. In the bio assay, the activity
of fraction 9 was found to be similar to that of CPDE CSF, unlike fractions 10 and 11. So fraction 9 was taken to column chromatography for purification. The molecule was isolated and named as CP2 (Figure 3.10a and b). Interestingly CP2 also exhibited decrease in activity after isolation.

**Figure 3.10a** Expected marker molecules from Fractions 6 and 9 of CPDE CSF
1. CPDE, 2. Chloroform soluble fraction (CPDE CSF), 3. Fraction 6
4. Fraction 9. Profile of TLC was documented using (A) UV at 365 nm and (B) ammonium molybdate spray reagent. 50% hexane in ethyl acetate solvent system was used to resolve the molecules.

**Figure 3.10b** Isolated single pure molecule (CP2) from Fraction 9 of CPDE CSF
Profile of TLC was detected using (1) UV at 365 nm, (2) UV at 254 nm, (3) ammonium molybdate spray reagent.
The other minor compounds present in CPDE CSF, visible under UV at 365nm (Figure 3.11a), were isolated and named as CP3, CP4 and CP5. All the compounds exhibited less than 30% anti-proliferative activity.

In order to understand this loss in activity of individual isolates, synergistic study was undertaken. The 5 isolated molecules from CPDE-CSF were pooled back in their relative ratios, based on the HPTLC profile of CPDE-CSF (Figure 3.11a and b). However, the pooling did not yield the expected synergy (Figure 3.12).

Figure 3.11a Densitometry Scanning of TLC profile of CPDE CSF to find out the relative ratios of the molecules present

TLC resolved using 50% hexane in ethyl acetate and detected using UV at 365 nm.
Figure 3.11b HPTLC profile of CPDE CSF to find out the exact relative ratios of the molecules present

Figure 3.12 Anti-proliferative effect of compounds isolated from CPDE and pooled compounds

Compounds compared with CPDE in HL-60 leukemia cancer cells and A549 lung cancer cells using thymidine incorporation assay at 24 h.

Simultaneously all the 5 isolated compounds were tested in lung carcinoma cell line A549 to check its anti-proliferative activity, CPDE exhibited 90.94% of inhibition of proliferation whereas isolates from CPDE showed less than 25% activity. Again activity loss was observed in lung cancer carcinoma cell line (Figure 3.12).

The above observations lead to a conclusion that the whole extract showed better activity (CPDE) and isolating the compounds or pooling them together leads to a loss of activity in *Centrosea pubecens*. This led us to
investigate the methanolic extract of *Centrosema pubescens* for isolation of bioactive molecule.

3.2.3 Isolation of Anti-cancer Compound from CPME using Fractionation, Column Chromatography and Bioactivity based Screening

The active methanolic leaf extract (CPME) exhibited 94.96 % and 87.22 % in A549 and HL-60 respectively at a concentration of 100µg/mL. Since CPME exhibited better anti-proliferative activity in A549 cell line it was selected for further work. Solvent–solvent fractionation of CPME yielded five different fractions namely, precipitate or insoluble (fraction1), hexane (fraction2), chloroform (fraction3), butanol (fraction4), hydro-alcohol soluble fractions (fraction5) (Figure 3.13). The fractions 1 to 5 were assayed for activity in which chloroform soluble fraction (CSF) showed maximum anti-proliferative activity of 94.96 % in A549 cells. The other four fractions showed less activity (Figure 3.14). CPME CSF (chloroform soluble fraction of CPME) was subjected to further purification using conventional column chromatography.

![Figure 3.13](image)

**Figure 3.13** TLC profiling CPME solvent-solvent fractions

A) CPME and B) Precipitate were resolved using Ethyl acetate: Acetic acid: Formic acid: Water(10:1:1:2.5) (C) Hexane soluble fraction was resolved using 20% Ethyl acetate in Hexane (D1) Chloroform soluble fraction compounds separated using 0.5% Methanol in Chloroform and (D2) Ethyl acetate: Acetic acid: Formic acid: Water (10:1:1:2.5) and (E) Hydro-alcoholic fraction: 40% Methanol in Isopropanol.
TLC Profile was detected using (1) UV at 365 nm, (2) UV at 254 nm, (3) 10% sulphuric acid in methanol and (4) ammonium molybdate spray reagent.

Figure 3.14 Anti-proliferative activity of solvent-solvent fractions of CPME in A549 cells

The graph shows the % inhibition of different solvent-solvent fractions (Precipitate, Hexane soluble fraction, Chloroform soluble fraction, Butanol soluble fraction and Hydro alcoholic fraction) of CPME.

3.2.3.1 Purification of CPME CSF using chromatography techniques

CPME CSF was subjected to silica gel column chromatography. Based on the TLC profile, the collected elutes were pooled together to obtain 7 different fractions. All these fractions were assayed for $[^3]$H-thymidine incorporation, wherein fraction 2 showed maximum anti-proliferative activity similar to that of CPME in A549 cells (Figure 3.15). The active fraction was further purified by flash column chromatography (bio assay guided purification) and isolated an active molecule (Figure 3.16) which is comparable with activity similar to CPME in A549. The active isolate was subjected to structural characterization.
Figure 3.15  Anti proliferative effect of CPME CSF column fractions (Fr 1-7) on lung cancer cells A549 at 100µg/mL concentrations
The graph shows the %inhibition of different column fractions (Fr1 to Fr 7) of CPME CSF.

Figure 3.16  TLC profile of semi-pure fractions of CPME CSF and single active molecule Lupenone
Lane 1-4 semi pure fractions, D: Lupenone. The profile was detected using (A) UV at 365 nm, (B) UV at 254 nm and (C and D) ammonium molybdate spray reagent.
Time course and dose response analysis of CPME in A549 cells (1 to 100µg/mL for 24, 48 and 72 h) revealed the IC$_{50}$ concentration for CPME and its active isolate at 24 h in A549 cells to be 10µg/mL.

3.2.4 Structure Elucidation

The purified active molecule from CPME was characterized based on spectroscopic studies using NMR, Mass spectroscopy, etc and identified to be Lupenone (Figure 3.17).

3.2.4.1 Proton NMR

$^1$H NMR (CDCl$_3$, 400 MHz) $\delta$: 0.73, 0.87, 0.96, 1.01, 1.05, 1.70 (each 3H, s, Me x7), 4.69 (1H, s, H-29b), 4.57 (1H, s, H-29a).

3.2.4.2 $^{13}$C NMR

$^{13}$H NMR (CDCl$_3$, 100 MHz) $\delta$: 214.0 (C-3), 151.1 (C-20), 109.6 (C-29), 59.7 (C-5), 58.4 (C-9), 53.3 (C-18), 43.0 (C-19), 42.4 (C-17), 41.8 (C-4), 41.4 (C-14,8), 40.2 (C-22), 39.5 (C-1), 36.2 (C-10, 16), 35.2 (C-13), 35.0 (C-2), 33.1 (C-7), 32.3 (C-23), 32.0 (C-24), 30.2 (C-15), 30.0 (C-21), 29.6 (C-12), 22.5 (C-11), 21.2 (C-30), 20.5 (C-28), 18.9 (C-25), 18.5 (C-6), 18.2 (C-26), 14.9 (C-27).

Mass data EIMS m/z: 424 [M +], Characterization studies elucidated the compound to be Lupenone, which was verified with existing report (Supaluk Prachayasittikul et al 2010). The molecular formula of the compound is C$_{30}$H$_{48}$O. Melting point is 168-170°C. IR $V_{\text{max}}$ cm$^{-1}$ 3434, 1715, 1459, 918. (Figure 3.18).
Figure 3.17 Structural elucidation of *Centrosema pubescens* (CPME) pure molecule
Left top Proton NMR, right top Carbon NMR, left bottom IR and right bottom Mass spectrum.

Figure 3.18 Chemical structure of pure compound from *Centrosema pubescens* methanolic extract (CPME) was determined as Lupenone (C$_{30}$H$_{48}$O)
3.2.5 Nature and Pharmacological Activity of Triterpenoids

The isolated active molecule from CPME was identified to be Lupenone (C\textsubscript{30}H\textsubscript{48}O), chemically referred to as 18-Lupen-3-one or lup-18-en-3-one, which has been reported for anti-cancer activity (Triterpene) but reported from \textit{Centrosema pubescens} for the first time. It is a pentacyclic triterpenoid present in a variety of plant species. Triterpenoids are highly multifunctional and the antitumor activity of these compounds is measured by their ability to block nuclear factor-κB activation, induce apoptosis, inhibit signal transducer, and activate transcription and angiogenesis. (Petronelli Alessia et al 2009). Lupenone was reported to exhibit a variety of biological activities such as anti-malarial, anti-inflammatory, anti-tumor, anti-fungal, anti-viral, anti-oxidant, parasitic lysis activity along with inhibitory effect on acetylcholinesterase and alpha glucosidase (Supaluk Prachayasittikul 2010; Ankita Wal et al 2010; Petronelli Alessia et al 2009; Madureira 2003; Flekhter 2000; Roman Paduch 2007; Satish Kumar et al 2008).

3.2.6 Comparative Dose Response Study of CPME and Lupenone Using Thymidine Incorporation Assay

On comparison of the effect of CPME and Lupenone on thymidine uptake, CPME showed a dose dependant increase in thymidine uptake which was found to be optimum at 10\textmu g/mL, after which a dose dependent increase in the activity was noticed. Similarly lupenone showed a similar dose response profile with moderate increase in activity on par with CPME and the optimum effect was observed at 24\textmu M (Figure 3.19).
Figure 3.19 Comparative dose response analysis of CPME and Lupenone on A549 using \(^3\text{H}\)-thymidine uptake assay

Different concentrations (from 1\(\mu\)g/mL to 100\(\mu\)g/mL) of CPME and Lupenone were treated for 24 h. % inhibition is expressed with respect to untreated control. Solvent control (DMSO) had no effect on cells. All the experiments were performed twice in triplicates and expressed as mean ± S.E.

3.2.7 Evaluation of CPME and TRI Terpinoids (Lupenone) on Cytotoxicity

To test the effect of CPME and Lupenone on cytotoxicity, MTT assay was performed in PBMC and A549 cells. The release of lactate dehydrogenase was measured at 492 nm (Figure 3.20a and b). Both CPME and Lupenone showed no toxicity (<20%) even at the highest concentration (100\(\mu\)g/mL) tested proving its non toxic nature and ensuring its compatibility over the in vitro system of study. The observed effects were compared with Triton X 100, a detergent which showed maximum lactate dehydrogenase release.
Figure 3.20  Cytotoxicity effect OF CPME and Lupenone
(a) PBMC and (b) A549 at different concentrations ranging from 1µg/mL to 100µg/mL at 24 h. The results are expressed as % cytotoxicity with respect to control.
3.3 AN IN VITRO INVESTIGATION TO ASSESS THE EFFICACY OF THE ACTIVE EXTRACTS AND THE BIOACTIVE COMPOUND IN ARRESTING CELL CYCLE AND TO EVALUATE THE FATE OF THE ARRESTED CELLS USING APOPTOTIC MARKERS IN A549 CELLS

In recent years, cancer therapy has been targeted by novel approaches using anti-cancer drugs that can mediate the cell death through the induction of apoptosis (Hengartner 2000). We investigated the apoptotic activity of *Centrosema pubescens* and tried to elucidate the possible mechanism of action.

3.3.1 Dose Response and Time Course Analysis of CPDE, CPME and Lupenone on A549

Since Lupenone isolated from CPME exhibited better anti-proliferative effect on lung cancer model (A549), the study was extended to elucidate the molecular mechanism behind the action of both the extracts (CPDE and CPME) and Lupenone on A549.

CPDE, CPME and Lupenone showed maximum anti-proliferative effect at 100µg/mL concentration of 84.85 % and 94.96 % and 90.40% respectively (Figure 3.21). Time course and dose response analysis was performed to find the IC\textsubscript{50} concentration. (Figure 3.22). The concentration required to inhibit A549 cell growth by 50 % (IC\textsubscript{50}) at 24 h was found to be 25µg/mL for CPDE and 10µg/mL for CPME and 24µM for Lupenone. Slight increase in inhibition of proliferation was seen at 48 and 72 h time point with increasing dose concentration.
Figure 3.21 Anti-proliferative effect of CPDE, CPME and Lupenone (100μg/mL) on A549 lung carcinoma cell line at 24 h
Results expressed as % of inhibition with respect to untreated control. Solvent control (DMSO) had no effect on cells. CPDE - Centrosema Pubescens dichloromethane extract, CPME - Centrosema Pubescens Methanolic extract and Lupenone isolated from CPME.

Figure 3.22 Dose response and time course analysis of CPDE, CPME and Lupenone on A549 lung carcinoma cell line
Different concentrations (from 1μg/mL to 100μg/mL) of extracts and pure compound were treated for 24, 48, and 72 h. % of inhibition is expressed with respect to untreated control. Solvent control (DMSO) had no effect on cells.
The lactate dehydrogenase release assay in human PBMCs and A549 cells confirmed the anti-proliferative effect of CPDE, CPME and Lupenone is due to apoptosis and not because of necrosis. The cytotoxicity of CPDE, CPME and Lupenone was found to be less than 20 % at the highest tested concentrations (Figure 3.23a and b) (Renz et al 2001).

**Figure 3.23** The cytotoxicity effect of CPDE, CPME and Lupenone on

PBMC

(a) and A549 cells (b) studied by LDH release assay at a different concentrations ranging from 1µg/mL to 100µg/mL at 24 h.
### 3.3.2 Assessment of Apoptosis using DNA Fragmentation in A549 Cells

Several opportunity studies have reported the mechanism of action of chemotherapeutic drugs to induce cancer cell death through the activation of apoptotic pathways (Lu et al. 2003). Cells undergoing apoptosis are characterized by morphological changes including cell shrinkage, membrane blebbing, chromatin condensation and DNA fragmentation (Kerr et al. 1972; Thompson 1995). DNA fragmentation is considered to be the biochemical hallmark and signature of apoptosis, where in the nuclear DNA is cleaved into nucleosome sized fragments (Compton 1992). To elucidate whether the observed anti-proliferative activity of CPDE, CPME and Lupenone is associated with the induction of apoptosis in A549 lung cancer cells, the mode of cell death by DNA fragmentation was assessed using diphenylamine (DPA) method and propidium iodide staining at a time point of 24 h. A dose dependant DNA fragmentation was observed in A549 cells treated with the crude extracts and Lupenone. The dose response studies for DNA fragmentation as shown in Figure 3.24a, revealed that the IC₅₀ concentration of 10µg/mL of crude extracts and Lupenone was optimum enough to induce fragmentation in A549 cells. Further nuclear fragmentation was confirmed by staining untreated and treated A549 cells with propidium iodide stain. The clear fragmented nuclei were observed in the cells treated with 10µg/mL of CPME and 24µM of Lupenone and 25µg/mL of CPDE whereas the untreated control cells did not show any apoptotic characteristics like nuclear fragmentation, cytoplasmic membrane blebbing etc. These findings suggest that the CPDE, CPME and Lupenone inhibited proliferation of A549 cells through the induction of apoptosis. Microscopic examination also revealed the morphological changes of apoptosis in A549 treated cells when compared to the untreated cells (Figure 3.24b). Our studies indicate that CPDE, CPME and Lupenone have significant anti-proliferative activity. The percentage of fragmented DNA was found to be nearly 75.43 % at 100µg/mL of Lupenone which is higher than that of the positive control used in the study indicating the novelty of the effect of Lupenone on the induction of apoptosis in A549 cells.
Figure 3.24a DNA fragmentation assay in A549 cells using DPA method. A549 cells treated with CPDE, CPME and Lupenone of *Centrosema Pubescens* showed significant % fragmentation after 24h treatment.

Figure 3.24b Microscopic examination of CPDE, CPME and Lupenone treated A549 cells for morphological assessment of apoptosis in A549 treated cells
1. Untreated control A549 cells, 2. CPDE treated A549 cells, 3. CPME treated A549 cells and 4. Lupenone treated A549 cells at 24h.
3.3.3 Flow Cytometry Analysis

The activation of apoptosis by CPDE, CPME and Lupenone was further confirmed quantitatively using the flow cytometric analysis, which helps to analyze the changes in DNA content. To quantify the percentage of apoptotic cells upon incubation with CPDE, CPME and Lupenone, the A549 cells were treated with the extracts and the compound for 24 h and the cells were stained with the fluorescent dye propidium iodide and subjected to FACS analysis. Cells in the M1 region are apoptotic and are sub-diploid (<2n), Cells falling under M2 region are considered as G0/G1 phases of the cell cycle and diploid in nature (2n). S-phase cells i.e., M3 region have a DNA content greater than 2n and less than 4n whereas Cells within the G2/M phases (M4) have a DNA content of 4n. Propidium Iodide staining clearly distinguishes nuclei with normal diploid DNA in control cells from the nuclei with hypo diploid DNA found in treated cells. Based on this the apoptotic cells and each phase of the cell cycle were quantified. Figure 3.25 shows the DNA content histograms obtained after PI staining of permeabilised cells treated with extracts and lupenone compound. The proportion of cells in the sub-G1 region increased from 2.34% in control untreated cells to 25.02%, 23.09 % and 25.58 % in CPDE, CPME and Lupenone treated cells at a time point of 24 h. Taken together, these results indicate that CPDE, CPME and Lupenone were capable of inducing apoptosis in A549 cells.

![Figure 3.25 Flow cytometry analysis of A549 cells](image)

Figure 3.25 Flow cytometry analysis of A549 cells

Treatment with the crude extracts (CPDE and CPME) and pure compound (Lupenone) at 24 h using propidium iodide in A549 cells. A. Untreated control cells, B. CPDE treated, C. CPME treated and D. Lupenone treated A549 cells.
The activation of apoptosis was further confirmed quantitatively using the flow cytometric analysis. It was found that 25.02 %, 23.02 % and 25.58 % were triggered by the treatment of CPDE, CPME and Lupenone to undergo apoptosis as indicated by distinct Sub G1 phase.

3.3.4 Effect of CPDE, CPME and Lupenone on Apoptotic Markers

A well-orchestrated apoptotic programme was observed after treatment with CPDE, CPME and lupenone as manifested by a decrease in the gene expression levels of anti-apoptotic Bcl-2 and oncogene k-Ras, an increase of tumor suppressor gene p53, pro-apoptotic Bax and cytochrome C mRNA levels and the activation of caspase-3 (Figure 3.26a and b).

![Figure 3.26a RT-PCR analysis of effect of the CPME, Lupenone and CPDE of Centrosema pubescens on p53, Bax, Bcl-2, Cytochrome-C, Caspase-3, k-Ras, TNF-α, Caspase-8 and GAPDH in A549 cells. M. 100 bp size marker.](image)


The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3. PARP-1 is specifically proteolysed by
caspases into DNA-binding domain (DBD) and to catalytic fragment during the execution of the apoptotic program (Kaufmann et al 2001; Damien et al 2001). PARP was confirmed by immunoblot analysis. Cleavage of PARP by the extracts (CPME and CPDE) and Lupenone was observed (Figure 35.13). GAPDH was used as internal control. No significant alteration in the gene expression levels of Caspase-8 and TNF-α were observed suggesting that the apoptosis induced by CPDE, CPME and Lupenone follows mitochondrial pathway of apoptosis.

Figure 3.26b Integral densitometry values of the above RT-PCR analysis for p53, Bax, Bcl-2, Cytochrome-C, Caspase-3, k-Ras, TNF-α, Caspase-8 and GAPDH mRNA in A549 cells.

3.3.5 Assessment of Mitochondrial Membrane Potential using Rhodamin 123 Uptake Assay

Mitochondrial fluorescence has been shown to decrease quantitatively in response to alteration in the mitochondrial membrane potential. The uptake of Rh123 associated with mitochondria is proportional to the mitochondrial membrane potential (Rahn et al 1991). The uptake of Rh123 was measured quantitatively in A549 cells on treatment with CPDE, CPME and Lupenone (25, 10µg/mL and 24µM concentration respectively) at
24 h. Rh123 uptake was decreased by 36.42 % in A549 treated with CPME, 27.24 % decrease was observed in cells treated with Lupenone whereas a decrease of 21.44 % was observed for cells treated with CPDE. Time course analysis of cells treated with (IC₅₀ concentration in µg/mL) CPME, Lupenone and CPDE for 6, 12, 24, 36 and 48 showed a significant decrease in Rh123 uptake compared to untreated control cells. The positive control Actinomycin D treated A549 cells showed a decreased Rh123 uptake up of 30.67 % at 24 h (Figure 3.27). The disturbed mitochondrial membrane potential indicates the release of cytochrome C and confirms the activation of apoptosis through mitochondrial pathway.

![Figure 3.27 Determination and Time course analysis of mitochondrial membrane potential in A549 cells by Rhodamine 123 uptake](image)

3.3.6 Assessment of NF-κB Inhibition in J774 Murine Macrophage Cell Line

NF-κB is known to participate in apoptosis modulation by regulating the expression of important apoptosis-related genes including Bcl-2 family and IAP family (Shishodia and Aggarwal 2002). Primary assay for NF-κB inhibition was performed in J774 cell line. MTT assay was performed
to find out the percentage inhibition of NF-κB and was calculated for various concentrations of CPDE, CPME and Lupenone using cytotoxicity assay with Bay-11-7082, a commercial inhibitor of IκBα phosphorylation, as positive control. A dose-dependent inhibition was observed. At 24 h, 25μg/mL of CPDE, 10μg/mL of CPME and 24μM of Lupenone showed 50 % inhibition (Figure 3.28).

![Figure 3.28](image)

**Figure 3.28** Dose response analysis of NF-κB inhibition in J774 murine macrophage cell line. Cells treated with CPDE, CPME and Lupenone in J774 cells for 24 h. Bay-11-7082 (25 μM) was used as positive control.

### 3.3.6.1 Western blot analysis of NF-κB and IκBα

The kinetics of CPDE, CPME and Lupenone on NF-κB inactivation was further assessed using antibodies that specifically recognize IκBα protein and NF-κB p65 subunit in the cytoplasmic fraction and nuclear fraction respectively prepared from A549 cells. The inactive form of NF-κB (IκBα) was nearly undetectable in control cells while the active p65 subunit was clearly seen. Upon treatment with CPDE, CPME and Lupenone, marked inhibition in degradation of IκBα protein was observed in the cytoplasmic
extract and considerable inactivation of NFκB p65 was seen in the nuclear fraction (Figure 3.29). Thus it can be concluded that *Centrosema pubescens* suppresses NF-κB activation by inhibiting IκBα degradation.

![Image](image_url)

**Figure 3.29** Analysis of *Centrosema pubescens* crude extracts (CPDE and CPME) and pure compound (Lupenone) on IκBα degradation and NF-κB p65 activation in A549 cell line

Inhibitory effect of CPME, Lupenone and CPDE on IκBα degradation in cytoplasmic extracts and NF-κB p65 translocation in nuclear extracts were analysed by western blotting using monoclonal antibody to IκBα and NF-κB p65. Lanes represents 1. Control (untreated A549 cells), 2. Actinomycin D (positive control), 3. CPME, 4. Lupenone and 5. CPDE treated A549 cells at 24 h.

### 3.3.6.2 Assessment of NF-κB DNA-binding activity

In order to investigate NF-κB response in CPDE, CPME and Lupenone treated A549 cells, nuclear protein extracts of A549 cells were exposed to IC₅₀ concentrations of CPDE, CPME and Lupenone for 24 h and
tested for specific NF-κB DNA-binding activity by electrophoretic mobility shift assay (EMSA). The results show that the NF-κB DNA binding activity in untreated A549 cells was moderately high, indicative of constitutive activation of NF-κB in this type of cancer cells whereas the treated cells showed significant inhibition (Figure 3.30). The confirmatory assay (EMSA) for NF-κB proved the role of Centrosema pubescens on inhibition of transcription factor.

This coincides well with our earlier result on the decrease in the expression of the anti-apoptotic gene Bcl-2 and inhibition of TNF-α gene, suggesting that NF-κB may participate in the down-regulation of the expression of these genes. Therefore it can be inferred that NF-κB may contribute to CPDE, CPME, Lupenone induced A549 cells apoptosis, at least in part.

![Electro Mobility Shift Assay (EMSA) binding study in A549 cells](image)

**Figure 3.30** Electro Mobility Shift Assay (EMSA) binding study in A549 cells

3.3.7 Immunoblot analysis of AkT and COX-2

Among various activities of NF-κB, it is involved in regulating the production of prostaglandins via the pro-inflammatory gene COX-2, which plays an important role in both inflammation and carcinogenesis (Acquisto et al 2002). Cyclooxygenase/PGHS-2, catalyses the first step in the biosynthesis of prostaglandins, which are implicated in various physiological events including progression of inflammation, immunomodulation and transmission of pain (Baeuerle and Henkel 1994). Immunoblot analysis revealed that the expression of COX-2 was inhibited significantly by CPME whereas a moderate inhibition was observed for CPDE and Lupenone (Figure 3.31). Since an over expression of COX-2 and a constitutive activation of NF-κB is known to be associated with poor survival in lung cancer patients, the blockade of COX-2 and NF-κB activation by Lupenone may have important clinical implications.

Human Caspase-9, a member of the protease family intimately associated with the initiation of apoptosis, is thought to be phosphorylated and inhibited by Akt, a serine-threonine kinase (Cardone et al 1998). In addition to the inhibition of pro-apoptotic factors, AKT can also activate the transcription of anti-apoptotic genes through the activation of the transcription factor NF-κB (Kane et al 1999). Immunoblot analysis for phospho-Akt revealed down regulation in its expression upon treatment with CPDE, CPME and Lupenone, suggesting its role in NF-κB inactivation (Figure 3.31).
Figure 3.31 Western blot analysis of the CPDE, CPME and Lupenone on COX-2 and AKT in A549 lung carcinoma cells

1. Untreated A549 cells (Control), 2. Positive control (Actinomycin D), 3. CPME (10µg/mL), 4. Lupenone (24µM) and 5. CPDE (25µg/mL).

3.3.8 Analysis of Nitric Oxide (NO) Production in A549 Cells

Release of NO is known to result in the activation of caspases thereby inducing apoptosis. The result of nitric oxide production assay revealed that A549 cells produced 2.5, 2 and 2.4 µM of nitric oxide after 24 h of treatment with 10µg/mL of CPME, 24µM of Lupenone and 25µg/mL of CPDE respectively on par with untreated A549 cells (Figure 3.32). This result corroborates our earlier findings of C. pubescens induced apoptosis.
Figure 3.32  Analysis of *Centrosema pubescens* crude extracts (CPDE and CPME) and Lupenone on NO release in A549 cell line at 24 h and expressed in uM concentrations of nitrite release.

3.3.9 Effect on PLA\(_2\) Inhibition by \(^{14}\)C\] Arachidonic Acid Incorporation in A549 Cells

PLA\(_2\) cleaves arachidonic acid from the membrane phospholipids, thus providing the substrate for COX-2, the cell survival enzyme. Non small cell lung carcinoma cells have constitutively high expression of cytosolic phospholipase A\(_2\) (PLA\(_2\)). The effect of CPDE, CPME and Lupenone on the prostaglandin bio-synthetic enzyme PLA\(_2\) activity was assessed by *in vitro* activity assay. The commercial cPLA\(_2\) inhibitor and curcumin were used as the positive controls for the PLA2 activity assay, showing a 61.69 % and 79.45% inhibition of PLA\(_2\) activity respectively; while IC\(_{50}\) concentrations of CPDE, CPME and Lupenone exhibited a 53.04 %, 69%, 60.09% inhibition of cPLA\(_2\) activity (Figure 3.33).
Collectively, these results elucidate that the possible mechanism of apoptosis induced by CPDE, CPME and Lupenone in A549 cells is by inhibiting AKT, PLA$_2$ and COX-2. This is followed by inhibition of degradation of I$_{kB}$ and suppression of NF-$\kappa$B and a decrease in the NF-$\kappa$B DNA-binding activity which consequently down-regulates the expression of anti-apoptotic gene Bcl-2 and up-regulates pro-apoptotic genes. Simultaneously increased NO production was observed which in turn led to the activation of caspases. Finally mitochondrial mediated apoptosis occurs via the intrinsic pathway in CPDE, CPME, and Lupenone treated A549 cells.

### 3.3.10 Effect of CPDE, CPME and Lupenone on Cell Cycle Regulation in A549

The cell cycle is regulated through a complex network of cell cycle associated proteins such as Cyclin Dependent Kinases (CDKs), cyclins and CKIs. These multiple members of CDK family are associated with specific
cyclin and are the driving force for progression of the cell cycle through the different restriction points (Cooper and Hausman 2007). A major hallmark of cancer is hyperproliferation due to loss of cell cycle regulatory mechanisms (John Clarke et al 2008). Thus to clarify the molecular mechanism of the CPME, Lupenone and CPDE induced inhibition of A549 cell proliferation, we assessed their effect on the expression of these cell cycle regulatory proteins. The cells were treated with 10µg/mL of CPME and 24µM of Lupenone and 25µg/mL of CPDE for 6, 12, 24 and 48 h and the expression levels of related proteins were examined by western blot analysis (Hsu et al 2006).

To examine whether the anti proliferative effect of CPME, Lupenone and CPDE on A549 cells was mediated by induction of cell cycle arrest or apoptotic cell death (Sun-Jack Kim et al 2008), A549 cells were treated with 10µg/mL of CPME and 24µM of Lupenone and 25µg/mL of CPDE for 12h and 24h and the distribution of cells in each phase of cell cycle was examined by flow cytometric analysis. Figure 3.34a illustrates that 49.87% (G1 phase) cells in the control increased to 73.83%, 78.18% and 82.28% in the CPDE, CPME and Lupenone treated A549 cells respectively. Similarly results in Figure 3.34b show an accumulation of cells in the sub G1 phase (apoptotic) at 24h as there was an increase in the number of cells from 2.34% in the control to 25.03%, 23.09% and 25.58% in CPDE, CPME and Lupenone treated cells respectively. Also the percentage of cells in the G1 Phase was found to be 52.87% of cells in control and 55.14%, 52.16%, and 50.38% in CPDE, CPME and Lupenone treated cells respectively. As cells in the G1 phase remained unaltered when compared to the control it can be inferred that cells that entered apoptosis were from G2/M phase. Thus the extracts and lupenone triggers apoptosis at 24h and causes cell cycle arrest at G1 phase. The apoptosis data was confirmed by a previous result which shows the induction of apoptosis through intrinsic pathway at 24h. The results obtained in flow cytometry was further confirmed by performing a western
blot analysis for the regulatory proteins involved in the various phases of the cell cycle.

Figure 3.34a Flow cytometric analysis of CPDE, CPME and Lupenone on cell cycle progression in A549 cells at 12h
A- control cells (untreated A549 cells), B- cells treated with CPDE, C- cells treated with CPME, D- cells treated with Lupenone. This data is a representative of three independent experiments with similar results.

Figure 3.34b Flow cytometric analysis of CPDE, CPME and Lupenone on cell cycle progression in A549 cells at 24h
A - Control cells (untreated A549 cells), B- cells treated with CPDE, C- cells treated with CPME, D- cells treated with Lupenone. This data is a representative of three independent experiments with similar results.
3.3.10.1 Effect of CPME, Lupenone and CPDE on CDKS and cyclins

CDK 4/6 belong to the early G1 phase of the cell cycle and play a key role in the transition of the cells through the G1 check point. CDK4/6 are activated upon forming complexes with cyclin D3 providing them with kinase activity (Alicia Russo et al 1998). These proteins then phosphorylate the retinoblastoma protein (Rb) with subsequent release of the transcriptional factor E2F, which in turn activates the synthesis of necessary proteins for the S phase (Sherr 1996). When checkpoint regulation fails, cells enter an uncontrolled proliferative cycle being the cause of different cancers. Cyclin D3 is also considered as the key regulator of G1 phase and is synthesized in the G0 phase due to mitogenic stimulation of the cells and is the first cyclin produced in the cell cycle (Ann Bode and Zigang Dong 2007). However when mitogens are removed the level of cyclin D3 rapidly decreases and the cells get arrested in the G1 phase (Zhilin Qu et al 2002). The complex of CDK 4 with cyclin D3 plays a critical role in the progression of the cell through the check point in G1 (Ekholm and Reed 2000). Thus, when the complex formation between CDK4 and cyclin D3 is hindered, cells get arrested in G1 phase.

3.3.10.2 Expression level of CDK4

A slight decrease in the expression of CDK4 at 12h in CPME, Lupenone and CPDE treated cells was observed when compared with untreated A549 cells (Figure 3.35a) whereas a marked down regulation was observed at 24h. Treatment of cells for 6h and 48h did not cause any sizeable difference between the control and treated cells.
Figure 3.35a Western blot analysis of CPME, Lupenone and CPDE on the expression of CDK4 in A549 cells

1. Control (untreated A549 cells), 2. 10μm of ActinomycinD (positive), 3. CPME, 4. Lupenone, 5. CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of CDK4 expression. This data is a representative of three independent experiments with similar results.

3.3.10.3 Expression level of CDK6

A549 cells treated with CPME, Lupenone and CPDE showed similar expression of CDK6 to that of untreated cells at 6h. The level of CDK6 had a mild decline in expression at 12h in CPME, Lupenone and CPDE treated cells as compared to control cells (Figure 3.35b). A considerable decrease was observed in CDK6 on treating the cells for 24h. The extracts and lupenone were observed to exhibit similar expression of CDK6 when compared to control cells. At 48h only the CPDE and Lupenone treated cells showed a decrease in the expression of CDK6 compared to control cells.
Figure 3.35b Western blot analysis of CPME, Lupenone and CPDE on the expression of CDK6 in A549 cells

1. Control (untreated A549 cells), 2. 10µm of ActinomycinD (positive), 3. CPME, 4. Lupenone, 5. CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of CDK6 expression. This data is a representative of three independent experiments with similar results.

3.3.10.4 Expression levels of cyclin D3

There was a decrease in the level of cyclin D3 in cells treated with CPME, Lupenone and CPDE for 24h as compared to control cells (Figure 3.35c). Lupenone showed an improved decrease in the level of cyclin D3 at 24h as against CPME and CPDE. The decrease in cyclin D3 expression started at 12h wherein it was observed to be faintly lower than the control cells. The low expression of cyclin D3 and CDK4/6 at 12h confirms the accumulation of cells at the G1 phase.
Figure 3.35c Western blot analysis of CPME, Lupenone and CPDE on the expression of cyclin D3 in A549 cells

1. Control (untreated A549 cells), 2. 10 µm of ActinomycinD (positive), 3. CPME, 4. Lupenone, 5. CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of cyclin D3 expression. This data is a representative of three independent experiments with similar results.

Once the cell enters the late G1 phase it has to pass through a point between the G1 and S phase called the restriction point which is a critical point late in the G1 phase. Here the mammalian cell has to enter the S phase completing the cell cycle, even in the absence of growth factors (Planas Silva and Weinberg 1997). Progression through the restriction point and entry into the S phase is mediated by the activation of CDK2/cyclin E complexes. An increased activity of CDK 2 is required for the G1 – S transition which is regulated by the transcriptional mechanism of cyclin E (Bartek and Lukas 2001). The release of E2F upon phosphorylation of Rb by CDK 4/cyclin D, stimulates the synthesis of cyclin E (Dyson 1998). The binding and activation of cyclin E and CDK 2 is stimulated by the dephosphorylation of cdc25A (Sexl et al 1999). During cell cycle arrest at the DNA damage check points, cdc25A gets phosphorylated inhibiting the activity of CDK 2. In the absence of CDK 2 activity, it does not form complex with cyclin E and progression into S phase is blocked and cells remain blocked in G1 phase.
3.3.10.5 Expression levels of CDK2

In Figure 3.35d, the expression of CDK2 was observed to be reduced due to the treatment of CPME, Lupenone and CPDE with A549 cells beginning at 12h and maximum at 24h when compared to the control cells with Lupenone showed a much better decrease in comparison. The expression level of CDK2 in A549 cells at 6h, and 48h did not have any noticeable difference between the treated and untreated cells.

**Figure 3.35d Western blot analysis of CPME, Lupenone and CPDE on the expression of CDK2 in A549 cells**

1. Control (untreated A549 cells), 2.10µm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of CDK2 expression. This data is a representative of three independent experiments with similar results.

3.3.10.6 Expression levels of cyclin E

Treatment with Lupenone and CPDE for 24h showed a decrease in cyclin E expression as compared with CPME treatment (Figure 3.35e). Treatment of cells for 12h showed a marked decrease in the levels of cyclin E as compared to control cells. There was a mild decrease in the levels of cyclin
E on Lupenone treatment of A549 cells for 48h when compared to control, CPME and CPDE. As the activation of CDK2/ cyclin E complex is crucial for the cell cycle progression through G1 check point and entry into S phase, the reduced expression of both CDK2 and cyclin E by CPME, Lupenone and CPDE suggest that the CPDE, CPME and Lupenone partially block the entry of the cell into S phase.

Figure 3.35e Western blot analysis of CPME, Lupenone and CPDE on the expression of cyclin E in A549 cells

1. Control (untreated A549 cells), 2. 10µm of ActinomycinD (positive), 3. CPME, 4. Lupenone, 5. CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of cyclin E expression. This data is a representative of three independent experiments with similar results.

3.3.10.7 Expression levels of cyclin A

Cyclin A and B are the only kinases involving in the regulation of G2/M transition, they accumulate and reach a maximal level before mitosis at which point they are degraded. Cyclin A binds with both CDK1 and CDK2, giving two distinct cyclin A kinase activities, one appearing in S phase, the other in G2 (Michele Pagano et al 1992) and cyclin B binds with CDK1 (regulates G2/M phase). The CDK1/cyclin B complex rise through the cell
cycle until mitosis and is also called as maturation promoting factor, involved in phosphorylation of pRb and release E2F thus playing a crucial role in the progression of cells through the G2/M phase of the cell cycle (Katrien Vermeulen et al 2003). The results in Figure 3.35f showed a significant decrease in the expression levels of cyclin A in CPME, Lupenone and CPDE treated A549 cells for 48h when compared to control cells. CPME showed a comparatively lower expression than CPDE and Lupenone treated cells. At 24h the expression level of cyclin A had reduced in A549 cells treated with CPME, Lupenone and CPDE.

![Figure 3.35f](image)

**Figure 3.35f  Western blot analysis of CPME, Lupenone and CPDE on the expression of cyclin A in A549 cells**

1. Control (untreated A549 cells), 2.10µm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of cyclin A expression. This data is a representative of three independent experiments with similar results.

### 3.3.10.8 Expression levels of cyclin B

The treatment of A549 cells with CPME, Lupenone and CPDE illustrated a clear reduction in expression level of cyclin B at 48h (Figure 3.35g). At 24h, A549 cells treated with CPME showed a significant
decrease in cyclin B levels than CPDE and Lupenone treatment. Cyclin B showed reduced expression in Lupenone and CPDE treated lanes at 6h and 12h.

Figure 3.35g Western blot analysis of CPME, Lupenone and CPDE on the expression of cyclin B in A549 cells
1. Control (untreated A549 cells), 2. 10µm of Actinomycin D (positive), 3. CPME, 4. Lupenone, 5. CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of cyclin B expression. This data is a representative of three independent experiments with similar results.

3.3.10.9 Expression levels of CDK1

There was a mild reduction in the expression of CDK1 at 12h in CPME, Lupenone and CPDE treated cells when compared with untreated A549 cells (Figure 3.35h). The level of CDK1 was markedly down regulated at both 24h and 48h in CPME, Lupenone and CPDE cells when compared to untreated A549 cells. Treating cells for 6h did not make any sizeable difference between the control and treated cells. These results thereby demonstrate that, with low levels of cyclin A, B and CDK 1, the cell got arrested at the G2/M of the cell cycle at 48h.
Figure 3.35h Western blot analysis of CPME, Lupenone and CPDE on the expression of CDK 1 in A549 cells

1. Control (untreated A549 cells), 2.10μm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of CDK1 expression. This data is a representative of three independent experiments with similar results.

3.3.10.10 Effect of CPME, Lupenone and CPDE on CKI

The activities of CDK and cyclin are interrupted by the inhibitory protein called the CDK inhibitors or the CKIs thereby down regulating cell cycle progression. There are two major families of CKI (a) INK4 family is specific for CDK4 and CDK6, having four members: p16, p15, p18 and p19 and (b) Kip family inhibiting all the G1 and S phase CDK enzymes, having three members: p21, p27 and p5 (Haeyoung Lima et al 2006). Thus the expression of one member from each family (p16 and p27) was checked in CPME, Lupenone and CPDE treated A549 cells. p16 INK4a is a tumor suppressor protein which binds to CDK4/ cyclin D3 or CDK2/ cyclin E complexes, thus blocking their kinase activity and inhibiting progression of cells into S phase of the cell cycle (Milde-Langosch et al 2001). It acts by hampering the phosphorylating activity of the functional CDK4/cyclin D
complex, thus abrogating the E2F-induced activation of genes important for driving the cell cycle into S-phase progression (Kommoss et al 2007). p27 inhibits the activity of CDK2/ cyclin E complex in G0 or early G1 phase leading to cell cycle arrest at G1. It is known that by binding p27, cyclin D inhibits p27 from binding to CDK2/ cyclin E complex (Cooper and Hausman 2007).

### 3.3.10.11 Expression levels of p16

The results shown in Figure 3.35i shows that treatment of CPME, Lupenone and CPDE results in significant induction of p16 in A549 cells at 24h. CPME and Lupenone treated lanes showed a greater increase in the p16 level when compared to CPDE treated lane. There was a mild increase in the expression levels in cells treated for 48h when compared to untreated cells.

![Figure 3.35i](image)

**Figure 3.35i Western blot analysis of CPME, Lupenone and CPDE on the expression of cell cycle inhibitory protein p16 in A549 cells**

1. Control (untreated A549 cells), 2.10µm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of p16 expression. This data is a representative of three independent experiments with similar results.
3.3.10.12 Expression levels of p27

The intensity of CDK inhibitor p27 was observed to be increased in CPME, Lupenone and CPDE treated cells at 12h and 24 h when compared to the control cells (Figure 3.35j), whereas in 48h there was moderate increase in expression level of p27. However Protein levels of both the CKIs (p16 and p27) were significantly detectable only at 24h.

![Western blot analysis of CPME, Lupenone and CPDE on the expression of cell cycle inhibitory protein p27 in A549 cells](image)

**Figure 3.35j** Western blot analysis of CPME, Lupenone and CPDE on the expression of cell cycle inhibitory protein p27 in A549 cells

1.Control (untreated A549 cells), 2. ActinomycinD 10µm concentration (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of p27 expression. This data is a representative of three independent experiments with similar results.

3.3.10.13 Effect of CPME, Lupenone and CPDE on pRb and E2F

Rb is a tumor suppressor gene that plays an important role in tumor development. In its phosphorylated form it is the most important regulator of G1/S transition in cell cycle and the phosphorylation status of this protein was tested in CPME, Lupenone and CPDE treated A549 cells. In its unphosphorylated form Rb binds to a family of transcription factor E2F that regulates the expression of several genes encoding for proteins involved in cell cycle. One of the important gene’s expressions being regulated by E2F is the one encoding for cyclin E (James Degregori et al 1995). So the effect of
CPME and its active isolate on E2F was investigated. When cells pass through the restriction point in G1, the CDK4/cyclin D complex in particular phosphorylates Rb. Rb represses the binding of E2F to its target sequences thus suppressing the transcription of the gene encoding for cyclin E. The phosphorylation by the CDK/ cyclin complex, dissociates pRb from E2F thus liberating the proteins essential for the S phase entry. E2F activates the transcription of several genes responsible for DNA synthesis and also stimulates the synthesis of cyclin E which in turn forms complexes with CDK2 and promotes transition of the cell from G1 to S phase of the cell cycle (Cooper and Hausman 2007).

3.3.10.14 Expression levels of pRb

It was observed that in control cells, pRb was hyperphosphorylated whereas treatment with CPME, Lupenone and CPDE for 24h caused a considerable decrease in the phosphorylation of pRb (Figure 3.35k). The decrease was very significant with CPME treatment than with Lupenone and CPDE treatment.

![Figure 3.35k](image)

**Figure 3.35k Western blot analysis of CPME, Lupenone and CPDE on the expression of pRb in A549 cells**

1. Control (untreated A549 cells), 2.10µm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of pRb expression. This data is a representative of three independent experiments with similar results.
3.3.10.15 Expression levels of E2F

When compared to control cells the levels of E2F markedly decreased on treatment of A549 cells with CPME, Lupenone and CPDE for 24h (Figure 3.35l). The decrease was significantly observed in Lupenone and CPDE treated lanes than in the CPME treated lane. There was no major difference seen in cells treated for 6h, 12h and 48h when compared to control cells. Thus the results state that the hyperphosphorylation of pRb was not efficient, which repressed the binding of E2F to cyclin E consequently not leading the transition of the cell into S phase.

![Western blot analysis of CPME, Lupenone and CPDE on the expression of E2F in A549 cells](image)

**Figure 3.35l Western blot analysis of CPME, Lupenone and CPDE on the expression of E2F in A549 cells**

1. Control (untreated A549 cells), 2.10µm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of E2F expression. This data is a representative of three independent experiments with similar results.

3.3.10.16 Expression levels of PARP

The enzyme poly (ADP-ribose) polymerase, or PARP, is one of the first proteins identified as a substrate for caspases. PARP is involved in the
repair of DNA damage and functions by catalyzing the synthesis of poly (ADP-ribose) by binding to DNA strand breaks and modifying nuclear proteins. The ability of PARP to repair DNA damage is prevented following cleavage of PARP by caspase-3. PARP-1 is specifically proteolysed by caspases into DNA-binding domain (DBD) and to catalytic fragment during the execution of the apoptotic program (Kaufmann et al 2001; Damien Amours et al 2001). The sequence at which caspase 3 cleaves PARP (DEVD) is conserved in the PARP protein from very distant species, indicating the potential importance of PARP cleavage in apoptosis (Hamid boulares et al 1999). A significant increase in PARP expression was observed at 24h in treated A549 cells when compared with control (Figure. 3.35m). An increased cleavage of PARP confirms the activation of apoptosis correlating with the gene expression analysis.

**Figure 3.35m Western blot analysis of CPME, Lupenone and CPDE on the expression of PARP in A549 cells**

1. Control (untreated A549 cells), 2.10µm of ActinomycinD (positive), 3.CPME, 4.Lupenone, 5.CPDE. Bars represent the mean ± S.E. n=3 of densitometry representation of PARP expression. This data is a representative of three independent experiments with similar results.
3.3.11 SUMMARY

In conclusion, the growth inhibitory effect of CPDE, CPME and Lupenone of *Centrosema pubescens* was observed in A549 cells. Purification of CPME led to the isolation of a pure compound structurally characterised using NMR and Mass spectroscopy to be Lupenone. The compound Lupenone exhibited an activity comparable to that of CPME while the other compounds on fractionation and purification did not show any marked anti-proliferative effect. Hence this compound can be considered as a marker molecule contributing in greater part to the activity of the CPME. Similar activity profile has been demonstrated in other plant extracts (Giridharan et al 2002). Further it was observed that the anti-proliferative activity of the CPDE, CPME and the Lupenone is associated with the induction of apoptosis in A549 lung cancer cells. Collectively, these results may elucidate the possible mechanism of apoptosis induced by CPDE, CPME, Lupenone in A549 cells acting via the inhibition of AKT, PLA$_2$ and COX-2 followed by inhibition of degradation of I$\kappa$B and suppression of NF-$\kappa$B was observed. The decreased NF-$\kappa$B DNA-binding activity consequently down-regulates the expression of anti-apoptotic gene and an up-regulation of the pro-apoptotic genes was observed.

An activation of caspases was observed leading to increased NO production, suggesting that CPDE, CPME, Lupenone acts via mitochondrial mediated apoptosis in treated A549 cells. The anti proliferative effect exhibited by CPME, CPDE and Lupenone in A549 cells could be either because of cell cycle arrest or the induction of apoptosis. Since, the induction of apoptosis was confirmed at 24h upon treatment with CPME. The other possibility of anti proliferation in A549 cells by cell cycle arrest was studied by examining various cell cycle markers. Flow cytometric analysis of A549 cells demonstrated the marked accumulation of cells in G1 phase at 12h
which might be due to the arrest in the cell cycle at G1 phase (Jordan and Wilson 2004). At 24h, CPME, CPDE and Lupenone showed significant increase of peak in the apoptotic region (sub G1) as well as the amount of cells from G2/M phase were reduced in control cells when compared with treated cells and the treated cells in the G1 phase were similar to the control cells, which indicates that the cells from G2/M phase entered apoptosis.

Since cells in the G1 phase remained unaltered it signifies a G1 phase arrest at 24h. This indicates CPME, CPDE and Lupenone are capable of inducing apoptosis and arresting cell cycle at 24h (Sun-Jack Kim et al 2008). As Cell cycle distribution analysis showed increased percentage of cells in G1 phase at 12h and 24h, these results were further confirmed with western blot analysis in order to examine the molecular mechanism.

Western blot analysis of the effect of CPME, CPDE and Lupenone on various cell cycle regulatory proteins revealed the decreased expression of CDK4,6/cyclinD3, CDK2/cyclin E, pRb and E2F and increased expression of CKIs p16 and p27 on treatment of A549 cells with CPME, CPDE and Lupenone for 12h and 24h. The efficacy of the C. pubescens extracts and its active molecule on cell cycle arrest started at 12h and was very prominent at 24h. 6h treatment did not have any impact on the cell cycle regulation as this time point might not have been sufficient enough to induce cell cycle arrest. These proteins are involved in the regulation of G1/S phase transition and these results corroborated with previous flow cytometry data (Haeyoung Lima et al 2006). These results thereby demonstrate that with low levels of cyclin D3, the hyperphosphorylation of pRb was not efficient, which indeed did not regulate the activation of CDK2/cyclin E complex consequently not leading to the transition of the cell into S phase (R). Thus it can be said that cells were blocked at G1 phase. Also cell cycle progression through G1 check point and entry into S phase is intervened by the activation of CDK2/ cyclin E complex,
the reduced expression of both CDK2 and cyclin E by CPME, Lupenone and CPDE, suggest that they partially block the entry of the cell into S phase. Our results illustrate the down regulation of cyclin D and E, thereby revealing the fact that p16 had been efficiently bound to CDK4/ cyclin D3 complex and p27 had been bound to CDK2/cyclin E complex blocking the kinase activity involved in the transition of the cells into S phase thus arresting cell cycle at G1 phase. Therefore, by the inhibition of CDK/cyclin activity and induction of CKIs, CPME, Lupenone and CPDE hampers cell cycle progression of A549 cells inducing G1 arrest at 24h. This was also checked by the analysis of the expression of PARP wherein an up regulation was observed in treated cells at 24h further confirming the occurrence of apoptosis following cell cycle arrest.

The *Centrosema pubescens* extracts and the pure compound also induced G2/M arrest at a later time point (48h) which was observed by the down regulation of CDK1/cyclin A, B complexes. The cyclin A/ CDK1 complex helps in the regulation of G2 phase and the cyclin B/ CDK1 complex is involved in the transition of cells from G2 to M phase. In summary, CPME, Lupenone and CPDE could be competent drugs to treat lung carcinoma by targeting cell cycle arrest at G1/S and G2/M phases by inducing apoptosis (Rajesh et al 2007).

### 3.4 *IN VIVO VALIDATION OF THE EFFECT OF THE ACTIVE EXTRACTS ON CELL CYCLE ARREST USING SWISS ALBINO MICE MODEL*

*In vitro* results confirmed the anti-cancer effect of *Centrosema pubescens* in lung cancer cell line (A549). To further proceed with this work, *Centrosema pubescens* was taken to the next stage of drug discovery i.e., *In vivo* studies. To confirm the effect of *Centrosema pubescens* on lung cancer, Swiss albino mice were chosen as *in vivo* model for the study.
3.4.1 Toxicological Studies
3.4.1.1 Histology of liver and lung

Histology reveals the collective architecture of any tissue type and the comparison of normal and pathological tissues helps in understanding the finer aspects of the pathological conditions like infections, toxicity, carcinoma etc.

The liver plays a central role in the transformation and clearing of chemicals and is susceptible to toxicity from these agents. Chemical agents, such as those used in laboratories and industries, natural chemicals and herbal remedies can also induce hepatotoxicity. More than 900 drugs have been implicated in causing liver injury (Friedman Scott et al 2003), and is the most common reason for a drug to be withdrawn from the market. Drug induced liver injury is responsible for 5% of all hospital admissions and 50% of all acute liver failures (McNally Peter 2006; Ostapowicz et al 2002). Hence histopathology was done to check the effect if CPDE and CPME on Liver toxicity.

In the present study, the protective effect of CPDE and CPME on Lung cancer mice models was investigated initially. Cytotoxicity levels of CPDE and CPME was studied using histology in lung and liver tissues of normal mice. In normal mice, hepatocytes maintain normal architecture. Sinusoid spaces are normal and Kupffer cells are very few and do not showed enlargement. CPDE and CPME administered mice also showed normal architecture of hepatocytes. Sinusoid spaces are normal but Kupffer cells are mildly increased.

Histology was performed to study the effect of CPDE and CPME on normal Swiss albino mice. Cytotoxicity levels of the CPDE and CPME were observed in liver and lung tissues using histological studies, which
showed normal architecture (haematoxylin and eosin 20 X) in treated animals (Group III and VII- 50 mg/kg b.w for CPDE and CPME ) as that of untreated control mice (Group I- corn oil). There was no mortality and no prominent changes in the histology of the liver (Figure 3.36a) and lung (Figure 3.36b).

**Figure 3.36a** Histological investigations of the liver for toxicological evaluation in experimental animals

A (Group I): Control animals showing a normal architecture of liver (20X Magnification)

B (Group II): CPDE (50 mg/kg body weight) administered mice showing normal liver architecture with no prominent damages and no irregular cells (20X)

C (Group III): CPME (50 mg/kg body weight) administered mice showing normal liver architecture with no histological abnormalities (20X)

**Figure 3.36b** Histological investigations of the Lung for toxicological evaluation in experimental animals

A (Group I): Control animals showing a normal architecture of lung (20X Magnification)

B (Group II): CPDE (50 mg/kg body weight) administered mice showing normal lung architecture with no prominent damages and no irregular cells (20 X)

C (Group III): CPME (50 mg/kg body weight) administered mice showing normal lung architecture with no histological abnormalities (20 X)
3.4.2 Confirmatory Experiments for the Induction of Lung Cancer using Benzo(A)Pyrene Induced Animals and the Effect of CPDE and CPME Post-Treated Animals Using Histological Studies and Western Blotting

Histological analysis of control and experimental groups were evaluated. The lung section from control animals revealed a normal architecture and no appreciable changes in histological abnormalities (haematoxylin and eosin 20 X) was observed in group I mice. In lung cancer bearing mice (Group II), the lung section showed alveolar damages and more number of pyknotic nuclei. Further the alveolar damage was accompanied by increase in the number of hyperchromatic irregular nuclei in the cells of the alveolar walls (haematoxylin and eosin 20X). The lung histology of group IV illustrated almost normal histology where a marked difference was characterised by reduced number of hyperchromatic irregular cells in the alveolar wall and reduced alveolar damage when compared with group II mice lung. These findings showed much more pronounced effect in the CPDE and CPME post-treatment animals (Group III and IV) (Figure 3.37a). The results obtained from histological studies confirmed the induction of lung cancer and the effective role of the CPDE and CPME in controlling the cancer progression.

Similarity in the histopathology and tumour progression stages between mice and human lung adenocarcinoma thus lung tumour model has been used extensively to evaluate the efficacy of putative lung cancer chemopreventive agents (Ana Pavla Diniz Gurgel 2009; Herzog et al 1997). In induced lung cancer bearing animals the lung showed alveolar damage which was accompanied by enhanced number of hyperchromatic irregular nuclei in the cells of alveolar walls and increase number of pyknotic nuclei. These conditions were reverted back to normal in the CPDE and CPME treated groups. This confirms the protective nature of both the extracts of Centrosema pubescens in the treatment of cancer. Chemoprevention of lung
cancer can be envisioned by retardation in blockage of steps leading to genetic damage, or stimulation of protective processes, which would delay or stop the appearance of lung cancer (Cohen and Khuri 2006). DNA repair ability, apoptosis, varying effects on genes involved in signal transduction pathways and regulation of the cell cycle are the major contributing factor for reduced tumour cells, thereby reduced tumour progression seen in post-treated groups (III and IV) mice lung was further confirmed with immunohistochemistry and western blotting analysis.

**Figure 3.37a Histological investigations of the Lung of control and experimental animals**

A (Group I) – Control animals showing normal architecture (20X)
B (Group II) – Lung cancer bearing mice (Induced control) showing damage alveolar region with more pyknotic nuclei and glandular from of NSCLC (20X)
C (Group III) – Lung section of mice post-treated with CPDE showing reduced number of hyperchromatic irregular cells in alveolar wall (20X)
D (Group IV) – Lung section of mice post-treated with CPME showing reduced alveolar damage and reduced hyperchromatic irregular cells (20X)
3.4.3 Tumor Markers

Tumor markers are not only of significance to the researchers in understanding tumor biology, but also to clinicians in treating patients with cancer (Pamies et al 1996). In oncology practice, the use of tumor markers may be helpful in the diagnosis and pathologic classification of tumors. Tumor targets are molecular markers existing either in blood or tissues and are associated with cancer. Their identification is useful in patients during diagnosis for clinical management. Tumor markers can be used for one of four purposes: (1) screening a healthy population or a high risk population for the presence of cancer, (2) making a diagnosis of cancer or of a specific type of cancer, (3) determining the prognosis in a patient; (4) monitoring the course in a patient remission or while receiving surgery, radiation, or chemotherapy.

Tumor markers include many substances that are not readily systematically organised. Carcinoembryonic antigen (CEA), an important tumor marker is a protein found in many types of cells but associated with tumor and the developing foetus. CEA was one of the first oncofetal antigens to be described and exploited clinically. It is a complex glycoprotein associated with plasma membrane of tumor cells, from which it may be released into the blood. Although CEA was identified in colon cancer, abnormal CEA considerations are particularly high in adenocarcinoma and large cell lung cancer. CEA may be helpful in the differential diagnosis of non-small cell lung cancer (Ebert et al 1994; Schalhorn et al 2001; Molina et al 2003; Bates et al 1997; Vivian Barak et al 2004). CEA provides prognostic information in NSCLC, particularly in adenocarcinoma of the lung (Molina et al 2003; Barlesi et al 2004; Vivian Barak et al 2004; Tomita et al 2004). Usually the CEA returns to normal upon treatment. In cancer of the breast, lung, pancreas, stomach and ovary the CEA may be elevated and can be used to monitor the progress of disease or response to treatment.
CA-125 is an antigen present in lung carcinomas which has shown tremendous usefulness as a serum tumor marker in various solid tumors (Salgia et al 2001). They are abnormally elevated in patients with lung cancer and is a good indicator of the disease an useful clinical therapeutic marker, and may have important prognostic value (Salgia et al 2001). The CA-125 is elevated in other cancers including endometrial, pancreatic, breast, colon cancer and in menstruation, pregnancy, endometriosis, and other gynaecologic and non gynaecologic conditions.

Given the importance of these markers in cancer diagnosis the expression levels of CEA and CA-125 by western blotting analysis was studied. In B(a)P induced animals both the tumor markers showed an elevated expression of CEA and CA-125 with prominent band when compared with normal untreated animals (Figure 3.37b). The results indicate that the Benzo(A)Pyrene (B(a)P) administered mice were bearing lung cancer. This data helps to proceed further by administering the drug in B(a)P induced animals to analyse the effect of CPDE and CPME post-treatment in lung cancer bearing mice models.

![Western blotting showed the expression of tumor markers (CEA and CA-125) in untreated and treated mice for the confirmation of lung cancer](image)

Lane 1 represents normal mice (Group I) 2. B(a)p induced mice (Group II).

Abnormalities in mucin-type glycoprotein expression have been documented in a variety of cancers. Epithelial mucin proteins are synthesized by cells lining the ducts and lumens of various epithelial surfaces and
contribute to the protective and lubricating functions of mammalian mucus (Barbara Driscoll 2002). Several different mucins are expressed in normal and neoplastic human respiratory epithelium. MUC1 has also been shown to be highly and frequently expressed in non-small cell lung cancers (NSCLC). Human tumor antigen MUC1 which is over expressed in B(a)P induced cancer was studied whereas the mice treated with CPDE and CPME showed decreased levels of MUC1 (Figure 3.37c). This indicates the induction of lung cancer as well as the reduction in prognosis of cancer in animals treated with CPDE and CPME. MUC1 has a role in monitoring therapy in advanced stages and detecting recurrent disease of non-small cell adenocarcinoma. Usually the tumor markers returns to normal upon treatment thus MUC1, CEA and CA-125 may be elevated and can be used to monitor the progress of disease or response to treatment.

![MUC1 protein expression in treated and untreated experimental mice](image)

**Figure 3.37c** MUC1 protein expression in treated and untreated experimental mice to cross check the occurrence of lung cancer and the effect of CPDE and CPME

Lane represents 1. Control mice, 2. B(a)P induced mice, 3. CPDE treated mice (post treated) and 4. CPME treated mice (post treated).

### 3.4.4 Immunohistochemistry

Immunohistochemical or IHC staining is widely used in the diagnosis of abnormal cells such as those found in cancerous tumors. Immunohistochemistry was performed to analyse the anti-cancer potential of CPDE and CPME in lung cancer models. The IHC refers to the localizing antigens or proteins (eg. CDKs, cyclins, etc.) in the cells of a tissue section.
Specific antibodies to the cell cycle regulators were examined in fixed tissue samples obtained from the lung cancer and control mice groups using immunohistochemistry. Post treatment with CPDE and CPME showed significant modification in the architecture of the lung sections with respect to cell cycle proteins responsible for G1 arrest (Figure 3.38). These results proved the effective role of *C. pubescens* on cell cycle regulation thereby controlling cancer progression.

(a) CDK 2

(b) CDK 4

Figure 3.38 (Continued)
(c) Cyclin D1

(d) Cyclin D3

(e) Cyclin E

Figure 3.38 (Continued)
Figure 3.38 (Continued)
Figure 3.38 Localisation of (a) CDK 2, (b) CDK 4, (c) cyclin D1, (d) cyclin D3, (e) cyclin E, (f) E2F, (g) pRb, (h) p16, (i) p27, and (j) PARP expression in mice lung tissue by histochemical analysis (20X Magnification).

Picture represents A. Control (Normal albino mice), B. Induced B(a)P induced lung cancer mice), C.CPDE post-treated mice and D. CPME post-treated mice.
Figure 3.39 Western blotting analysis to study the effect of CPDE and CPME on Cell cycle regulatory markers such as CDKs and cyclins to assess its effect on cell cycle arrest.

(a) Protein expression of CDK 1, 2, 4, 6, Cyclin A, B, D3 and E proteins were observed in untreated and treated mice models. Lane represents 1. Control mice, 2. B(a)P induced mice, 3. CPDE treated mice (post treated) and 4. CPME treated mice (post treated).

(b) Densitometric representation of CDK 1, 2, 4, 6, Cyclin A, B, D3 and E expression in treated and untreated swiss albino mice model
Figure 3.40 Western blotting to study the effect of CPDE and CPME on Cell cycle regulatory proteins (E2F-1 and pRb), Cell cycle inhibitors (p16 and p27) and Apoptotic protein (PARP) to assess its effect on cell cycle arrest.

(a) Expression of E2F-1, pRb, p16, p27 and PARP proteins were observed in untreated and treated mice models. Lane represents 1. Control mice, 2. B(a)P induced mice, 3. CPDE treated mice (post treated) and 4. CPME treated mice (post treated).

(b) Densitometric representation of E2F, pRb, p16, p27, PARP and β-actin expression in treated and untreated swiss albino mice model.
3.4.5 Immunoblot Analysis of Cell Cycle Proteins

To further confirm the results obtained from immunohistochemistry in the control and treated mice models, Immunoblot analysis was done to check the protein level expression of CDK 1, 2, 4 and 6, Cyclin A, B, D3 and E, cell cycle inhibitors p16 and p27, elongation factor E2F, pRb and the apoptosis marker PARP.

3.4.5.1 CDK 1 Expression by western blot analysis

Cell cycle progression is governed by cyclin dependent kinases (CDKs) that are activated by cyclin binding and inhibited by CDK inhibitors. CDKs regulate checkpoints that integrate mitogenic and growth inhibitory signals, coordinating cell cycle transitions (Silvia Lapenna and Antonio Giordano 2009). cyclin D/CDK4, cyclin D/CDK6 and cyclin E/CDK2 – regulates transition from G1 to S phase, which is essential for the control of the cell cycle at the G1/S transition. In S phase cyclin A/CDK2 complex were found to be active whereas cyclin B/CDK1 regulates progression from G2 to M phase and is essential for the control of the cell cycle at the mitosis phase. G2/M cyclins accumulate steadily during G2 and are abruptly destroyed as cells exit from mitosis at the end of the M-phase.

CDK 1 is the only important kinase that plays a vital role in the control of G2/M phase transition (Castedo et al 2002). The effect of CPDE and CPME on CDK1 expression was verified in B(a)P induced lung carcinoma mice model. There was no difference in expression of the CDK 1 as seen in western blot by CPDE and CPME (Figure 3.39). The treatment with CPDE and CPME showed similar expression in post treatment mice (Group III and IV) as that of control (Group I). Slight increase in expression was observed in B(a)P induced (Group II) mice. The result concludes CPDE
and CPME do not showed any effect on regulating the cell cycle by arresting at G2/M phase.

### 3.4.5.2 CDK 2 expression by western blot analysis and immunohistochemistry

CDK2 is considered as one the essential marker and has been observed to play an important role in G1 to S phase transition of the cell cycle. CDK2 is regulated by the regulatory subunits of the complex including cyclin E or A (Jeremy Myers et al 2007). Cyclin E binds G1 phase CDK2, which is required for the transition from G1 to S phase while binding with cyclin A is required to progress through the S phase.

The treated and untreated mice were investigated for the expression levels of CDK2 in Swiss albino mice model. A decrease in the expression levels of the CDK 2 was seen in immunohistochemistry (Figure 3.38a) which was confirmed by western blot analysis (Figure 3.39) by CPDE and CPME. The treatment with CPDE showed significant decrease in the expression of CDK 2 in post treated (Group III) mice as compared to the control group (Group I). CPME also showed similar expression (Group IV) but CPDE was found to be more effective than CPME. Increased expression of CDK 2 was observed in B(a)P induced (Group II) mice which indicates the severity of the lung cancer. Outcome of this analysis conveys the potential effect of the extracts on controlling the transition from G1 to S or arresting G1/S phase of the cell cycle.

### 3.4.5.3 CDK 4 expression by western blot analysis and immunohistochemistry

CDK4 is an enzyme activated only with D-type cyclins and CDK inhibitor p16(INK4a), whereas cyclins A, B1, and E are not (Kato et al 1993).
This kinase was shown to be responsible for the phosphorylation of retinoblastoma gene product (Rb). Mutations in this gene as well as in its related proteins including D-type cyclins, p16 (INK4a) and Rb were all found to be associated with tumorigenesis of a variety of cancers. Analysis of CDK4 expression after treatment with the extracts in experimental lung cancer mice models, there was a higher expression level of CDK 4 as seen in immunohistochemistry (Figure 3.38b) confirmed by western blot (Figure 3.39) in B(a)P induced (Group II) mice. Upon treatment with CPDE there was a reduced expression in post-treated (Group III) mice similar to control (Group I) with CPME (Group IV) showing significant decrease as compared to CPDE. These studies prove the efficacy of CPDE and CPME in cell cycle G1/S progression arrest.

3.4.5.4 CDK 6 expression by western blot analysis

The activity of CDK6 first appears in mid-G1 phase, which is controlled by the regulatory subunits including D-type cyclins and members of INK4 family of CDK inhibitors. CDK6 is important for cell cycle G1 phase progression and G1/S transition (Lin et al 2001). This CDK6 as well as CDK4, has been shown to get phosphorylated thereby regulate the activity of tumor suppressor protein Rb. Since CDK4 expression was observed to be reduced by CPDE and CPME, CDK6 was taken for analysis to confirm its effect on G1/S phase arrest. There was a decrease in expression of the CDK 6 as seen in western blot analysis due to the extracts (Figure 3.39). The treatment with CPDE and CPME showed significant decrease in expression of CDK 6 in post treated (Group III and IV) mice when compared with induced control (Group II). Increased expression of CDK 6 was observed in B(a)P induced mice when compared with control. This experiment signifies that both CDK4, 6 were inhibited by the potential effect of CPDE and CPME leading to cell cycle arrest at G1/S phase.
3.4.5.5 Expression of cyclin A and B by western blot analysis

Cyclin A and B are the only kinases involving in the regulation of G2/M transition also called mitotic cyclins. Cyclin A binds with CDK1/2 (regulates S and G2/M phase) and cyclin B binds with CDK1 (regulates G2/M phase) complexes. Also called as maturation promoting factor, cyclin B is involved in phosphorylation of pRb and release of E2F for further progression of cell cycle. In the current study, cylin A and B showed no difference in their expression levels as seen in the western blot analysis due to CPDE and CPME (Figure 3.39). The treatment with CPDE and CPME showed no effect in controlling the expression of cyclin A and B in post treated (Group III and IV) mice which was similar as that of control and induced control (Group I and II). This data finally concludes that CPDE and CPME are not capable of inducing G2/M phase arrest in lung cancer mice.

3.4.5.6 Cyclin D1 expression by immunohistochemistry

The cyclin D1 proto-oncogene is an important regulator of G1 to S phase progression in many different cell types. Together with its binding partners cyclin dependent kinase 4 and 6 (CDK4 and CDK6), cyclin D1 will form active complexes that promote cell cycle progression by phosphorylating and inactivating the retinoblastoma protein (Rb) (Clarke et al 1992). Cyclin D1 is important for the development and progression of several cancers including those of the breast, oesophagus, bladder and lung.

The formation of all possible complexes between the D-type cyclins (D1, D2 and D3) and CDK4/6 is promoted by the proteins, p21 (CIP1/WAF1) and p27 (KIP1) (John Alao 2007). The cyclin-dependent kinases are then activated due to phosphorylation by CAK. The cyclin-dependent kinases phosphorylate the Rb protein leading to release of the E2F
transcription factors, which then results in proper G1/S transition (Yew et al 2001).

There was a higher expression of the cyclin D1 as seen in immunohistochemistry (Figure 3.38c) in B(a)P induced (Group II) mice, which upon treatment with CPDE and CPME had reduced expression in post-treated (Group III and IV) mice similar to control (Group I).

The downregulation in the level of cyclin D1 indicates that the formation of cyclin D1 with CDK4/CDK6 complex is restricted. Thus it leads to inhibition of the phosphorylation process of Rb-E2F complex followed by inhibition of E2F release. Ultimately the cell will get arrested in G1 phase.

3.4.5.7 Cyclin D3 expression by western blot analysis and immunohistochemistry

Cyclin D3 forms a complex with CDK4 or CDK6 and functions in G1/S transition of the cell cycle. This active complex then gets involved in phosphorylation of tumor suppressor protein Rb (Mendelsohn et al 2002). The CDK4 activity associated with this cyclin was reported to be necessary for cell cycle progression through G2 phase into mitosis. Whose activity was studied with treatment of CPDE and CPME using specific cyclin D3 antibody. There was a decrease in expression of the cyclin D3 as seen in immunohistochemistry (Figure 3.38d) and also seen in western blot (Figure 3.39) by CPDE and CPME. The treatment with CPDE and CPME showed significant decrease in expression of cyclin D3 in post treated (Group III and IV) mice when compared with induced control (Group II). Expression pattern of CPDE and CPME was similar as that of control (Group I) which indicates that severity of the cancer was reverting back to normal. In the previous data CDK 4 and 6 were seen to be down regulated this partially conveys the effect of CPDE and CPME in the treatment of lung cancer by arresting the cell cycle
at G1/S phase. The reduction in the expression of cyclin D3 in this analysis further confirmed the arrest at G1/S phase in lung cancer bearing mice.

3.4.5.8 Cyclin E expression by western blot analysis and immunochemistry

Cyclin E is essential for progression through the G1-phase of the cell cycle and initiation of DNA replication by interacting with and activating its catalytic partner, the cyclin dependent kinase 2 (CDK 2) which was originally designated as cdc2. Rb is a target involved in the phosphorylation of cyclin E/CDK 2. There are a number of putative binding sites for E2F in the cyclin E promoter region, suggesting an E2F-dependent regulation (Qin et al 2004). The tight regulation of cyclin E expression, both at the transcriptional level and by ubiquitin-mediated proteolysis, indicates that it has a major role in the control of the G1- and S-phase transitions (Mazumder 2004 et al). Over expression of cyclin E has been observed in several malignancies and is associated with high proliferation, aberrant expression of other cell cycle regulators and chromosomal instability in vitro (Thomas Lindahl et al 2004).

There was decrease in expression of the cyclin E as seen in immunohistochemistry (Figure 3.38e) and also in western blot (Figure 3.39) by CPDE and CPME. The treatment with CPDE and CPME showed significant decrease in expression of cyclin E in post treated (Group III and IV) mice when compared with induced control (Group II). Expression pattern of CPDE and CPME was similar as that of control (Group I) which indicates that severity of the cancer was reverting back to normal.

The down regulation in the expression level of cyclin E indicates that the formation of cyclin E-CDK2 might have been restricted, which might not allow the phosphorylation pRb and thus inhibiting the release of E2F. If
E2F is not available for the transition of G1/S phase, the cell will get arrested in G1 phase.

3.4.5.9 E2F-1 and pRb expression by western blot analysis and immunohistochemistry

The retinoblastoma protein (Rb) is a nuclear phosphoprotein that regulates proliferation, differentiation, and apoptosis. Tumor suppressor Rb inhibits proliferation by repressing E2F1 mediated transcription when hypophosphorylated (Weinberg 1995). Hyperphosphorylation of Rb relieves E2F1 repression and allows cell cycle progression to occur. The importance of Rb is underscored by the fact that Rb function is disrupted in virtually all human cancers (Taya 1997). Paradoxically and inconsistent with its role as a tumor suppressor, hyperphosphorylated wild-type Rb inhibits apoptosis in both cell culture and animal models (Onikepe Adegbola and Gary Pasternack 2005).

During the latter stages of G1, pRb is extensively modified by phosphorylation, generating hyper phosphorylated forms that persist until exit from mitosis (Buchkovich et al 1989; DeCaprio et al 1989). The correlation between cell proliferation and pRb phosphorylation suggests that the ability of pRb to constrain cell cycle progression is inhibited by phosphorylation. A model emerges wherein pRb regulates a cell cycle transition late in G1 that must be traversed to continue with cell division. Appropriate signals lead to activation of regulatory kinases, phosphorylation of pRb, and passage through G1 (Weinberg 1995). G1 cyclin-dependent kinases (CDKs), particularly cyclin D-type/CDK4 and cyclin E/CDK2, are maximally active near the time of pRb phosphorylation, and these kinases can phosphorylate pRb (Weinberg 1995). Phosphorylation by cyclin E/CDK2 affects the ability of pRb to bind and inhibit the transcription factor E2F, a major target of pRb function (Lisa Connell-Crowley 1997).
The E2F transcription factor family plays a crucial and well-established role in cell cycle progression (Irena Royzman et al 2002). This family comprises six different polypeptides (E2F1–E2F6) that pair with a heterodimeric partner (DP1 or DP2). E2F is thought to function by activating a panel of genes involved in progression through the G1 phase as well as DNA replication. An important function of E2F is the recruitment of the retinoblastoma (pRb) tumor suppressor family of proteins. E2F-mediated recruitment of pocket proteins in quiescent cells and the early G1 phase of the cell cycle results in repression of genes that are subsequently activated at the G1/S phase transition. Over expression of E2F induces entry into S phase and DNA synthesis. Over expression of E2F-1 also leads to apoptosis by p53-dependent mechanisms (Yinyin Huang et al 1997).

There was decrease in expression of the E2F-1 and pRb as seen in immunohistochemistry (Figure 3.38f and 3.38g) and also in western blot (Figure 3.40) by CPDE and CPME. The treatment with CPDE and CPME showed significant decrease in expression of E2F-1 and pRb in post treated (Group III and IV) mice when compared with induced control (Group II). Expression pattern of CPDE and CPME were moderately lower than that of control (Group I).

The down regulation of phosphorylated retinoblastoma tumour suppressor protein and the down regulation of E2F in the results indicate the dephosphorylation of Rb. The results conclude the retinoblastoma protein binds to an E2F–DP complex in G0/G1 cells and this leads to repression of E2F-responsive genes. pRb inhibits E2F from activating transcription by binding to its transactivation domain and preventing its interaction with the core transcriptional machinery.
3.4.5.10 Cell cycle inhibitors p16 and p27 expression by western blot analysis and immunohistochemistry

CKIs have the ability to stop cell cycle progression by interacting directly with several cell cycle regulators and also are considered to be potential tumour suppressor genes (Morgan 1995). At present, 2 different groups of CKIs can be separated on the basis of sequence homology criteria. (a) INK4 family is specific for CDK4 and CDK6, and has four members: p16, p15, p18 and p19 (b) Kip family inhibit all G1 and S phase CDK enzymes, and have three members: p21, p27 and p5.

The INK4a gene is located at the chromosome locus 9 p21, a region that undergoes frequent hemi- and homozygous deletion in human cancers. The p16 gene encodes a 16 kD protein first identified in transformed cell lines. The detection of significant levels of p16 in late G1 and S phase only confirms its role in the inhibition of the cell cycle machine. In addition, p16 is believed to block the activation of cyclin dependent 4 and 6 by competing for D cyclin binding (Angela Groeger et al1999).

p27KIP1 belongs to the family of cell cycle regulators called cyclin-dependent kinase inhibitors (CDKI), which bind to cyclin-CDK complexes and cause cell cycle arrest in the G1 phase (see figure). p27 KIP1 is often known as a universal CDKI, since it interacts with all subtypes of "cyclin-CDK" complexes to inhibit cell cycle progression. In proliferating cells p27 is prevalently bound to cyclin D/CDKs, whereas in G1-arrested cells p27 is found in complexes with cyclin E/CDK2 (Nahum et al 2001). Therefore, the competition for p27 between cyclin D/CDKs and cyclin E/CDK2 complexes seems to be crucial for cell cycle progression, because cyclin D/CDKs can sequester p27 from the cyclin E/CDK2 complex and favour progression into S phase (Roberto Chiarle et al 2002).

In addition, p27KIP1 has been postulated to promote apoptosis, play a role in terminal differentiation of some tissues, and mediate
chemosensitivity in solid tumors. p27KIP1 status is also an independent prognostic factor, and loss of p27KIP1 expression is associated with tumour progression, lymph node metastasis, early relapse, and reduced overall survival. These findings have been validated on a host of cancers occurring at various anatomic sites such as breast, colon, esophagus, stomach, lung, and prostate, and virtually all studies were performed using immunohistochemistry for determining p27KIP1 expression. Assessment of p27KIP1 status in solid tumors is thus an useful adjunct to more conventional markers for predicting prognosis (Charles Catzavelos et al 1997; Vincenzo Esposito et al 1997; Ricardo Lloyd et al1999).

There was increase in expression of the p16 and p27 as seen in immunohistochemistry (Figure 3.38h and 3.38i) and also in western blot (Figure 3.40) by CPDE and CPME. The treatment with CPDE and CPME showed significant increase in expression of p16 and p27 in post treated (Group III and IV) mice when compared with induced control (Group II). Expression pattern of CPDE and CPME were moderately higher than that of control (Group I).

Results thus indicate that the increased expression of p16 inhibits the formation of CDK4/6 cyclin D complex by acting as a competitive inhibitor for cyclin D binding site and inhibits the transition of G1 to S phase. Same way increased expression of p27 might inhibit the cyclin E/ CDK2 complex formation and not allow the phosphorylation of Rb thus inhibiting E2F release and ultimately arresting the cell in G1 phase.

3.4.5.11 PARP and β-Actin expression by western blot analysis and immunohistochemistry

PARP is known to repair mutated or damaged DNA through modification of nuclear proteins. Caspase 3 is activated when there is an
increased unrepaired DNA, which leads to the cleavage of PARP thereby activating apoptosis.

There was increase in expression of the PARP as seen in immunohistochemistry (Figure 3.38j) and also in western blot (Figure 3.40) by CPDE and CPME. The treatment with CPDE and CPME showed significant increase in expression of PARP in post treated (Group III and IV) mice when compared with induced control (Group II). Expression pattern of CPDE and CPME was similar as that of control (Group I). Data represents the over expression of PARP which indicates the induction of caspase 3 by cleaving PARP, that is involved in DNA degradation which will ultimately result in apoptosis of the cell.

β-actin was used as internal control to normalize the protein concentrations, which showed equal expression (Figure 3.40) in control (Group I), Induced (Group II), CPDE (Group III) and CPME (Group IV) treated mice. The concentrations of protein were also quantified using Bradford’s method.

3.4.6 Summary

The results reveal the capability of CPDE and CPME to induce G1 phase arrest in Swiss albino mice model (Figure 3.40). These in vivo results corroborate with the data obtained in the in vitro lung cancer model used in the study.

G1 phase-related cell cycle-regulators such as CKIs, CDKs and cyclins were investigated. The G1 phase arrest in the treated mice models were associated with a marked up-regulation of the p27KIP1 and p16 INK 4 along with a significant reduction in cyclin D1, D3, E, CDK 2, 4, 6, E2F and pRb. Over expression of PARP indicates the occurrence of apoptosis in CPDE and CPME treated mice models. Reduced kinase activities of CDK2, 4 and 6
with its cyclins and CKIs were accompanied by the under-phosphorylation of the Rb protein, which is known to sequester the transcription factor, E2F, thereby preventing the cell cycle-progression by blocking the cells in G1 phase and restricting its entry into the S-phase. This study demonstrates that CPDE and CPME potently causes the cell cycle arrest in G1 phase of the cell cycle in Swiss albino mice model leading to the induction of apoptosis. Whereas, there was no effect on the G2/M phase markers such as cyclin A, B and CDK 1. The therapeutic efficacy of CPDE and CPME as seen in (Group III and IV) mice, when compared with induced (group II) mice confirms its anti-neoplastic therapy. CPDE and CPME thus minimize the damage caused by lung carcinogenesis, by suppressing the progression of malignancy via induction of cell cycle arrest. Finally, these results suggest that CPDE and CPME may be useful as one of the investigational drugs for treating lung cancer patients.

3.5 TO STUDY THE ROLE OF C. PUBESCENS EXTRACTS IN DIFFERENTIATION THERAPY USING HL-60 AS AN IN VITRO MODEL AND ASSESSING THE FATE OF DIFFERENTIATED CELLS USING APOPTOTIC MARKERS

3.5.1 Selection of CPDE and CPME Dose Concentration for Differentiation Therapy in HL-60 Cells

Sequential organic solvent extraction from non-polar to polar yielded four different extracts namely Centrosema pubescens hexane extract (CPHE), Centrosema pubescens dichloromethane extract (CPDE), Centrosema pubescens ethyl acetate extract (CPEE) and Centrosema pubescens methanol extract (CPME). Amongst the four extracts of C.pubescens, both CPDE and CPME were found to possess good anti-proliferative activities at their IC<sub>50</sub> concentrations (5 and 10µg/mL respectively); but at 1µg/mL concentration, very minimal anti-proliferative activities was seen with both CPDE and CPME (Figure 3.41a and b),
indicating that the dose is insufficient to induce cell death. Hence, both extracts (1µg/mL concentration) were selected for the present study.

Figure 3.41 Anti proliferative activity of *Centrosema pubescens*
(a) Dichloromethane [CPDE] and (b) Methanol extract [CPME] HL-60 cells were incubated with [³H] thymidine and different concentrations of DMSO-dissolved extract for 24, 48 and 72 h and assayed for thymidine uptake. Values (% inhibition over DMSO-control) are mean ± SE from triplicate experiments.
3.5.2 High Concentration of CPDE and CPME Induces Apoptosis Mode of Cell Death but Low Concentration Does Not

Internucleosomal DNA fragmentation is a key event in apoptosis. Most anti-tumor agents have been shown to induce apoptosis (Georg Seifert et al 2008). DNA was extracted by high salt method and run on a 2% agarose gel. The results have been shown in Figure 3.42a. At high doses, anti-cancer agents commonly cause necrosis instead of apoptosis. Results from the DNA fragmentation assay, showed a fragmented, ladder-like appearance of the genomic DNA, in HL-60 cells treated with 100µg/mL concentrations of CPDE and CPME extracts; similar results were obtained with the actinomycin D treated cells. In contrast, untreated cells had intact DNA. This proved that both the extracts induce the apoptosis mode of cell death and not necrosis, even at higher concentrations. In order to understand the result from the anti-proliferative studies performed at lower doses, cells treated with 1µg/mL concentration of both CPDE and CPME were subjected to DNA fragmentation assay, 24 h post treatment. Results from the above experiment did not show an induction of apoptosis (Figure 3.42b). This explains the low % inhibition values (3-4%) obtained via proliferation studies at 1µg/mL. So this concentration was used for preparation of samples for NBT reduction assay. MTT assay results already confirmed its negligible cytotoxicity levels in normal cells (PBMC), indicating that they do not induce cell death through necrosis. Results from DNA fragmentation studies, demonstrated the mode of cytotoxicity observed at higher concentrations. Differentiation therapy forces malignant cells to undergo terminal differentiation instead of killing them through cytotoxicity, making it less toxic than conventional cancer treatments (Figure 3.43). Hence, 1µg/mL concentration of both CPDE and CPME dissolved in methanol, were used for studies on HL-60 differentiation.
Figure 3.42 Agarose gel electrophoresis of DNA fragment of HL-60 cells
(a) (1) 100 bp ladder (2) control (3) CPDE 100µg/mL, (4) CPME 100µg/mL at 24 h showed prominent DNA fragmentation when compared with control, which showed intact DNA. (b) (1) 100 bp ladder (2) control (3) CPDE 1µg/mL, (4) CPME 1µg/mL at 24 h showed no fragmentation as that of control.

Figure 3.43 Schematic diagram of differentiation
3.5.3 NBT Reduction Assay Reveals Induction of Differentiation by CPDE and CPME

The mature and differentiated HL-60 cells respond to chemotaxins, phagocytose then develop complement receptors and produce superoxide. Upon activation, superoxide is produced, by a membrane oxidase. Tetrazolium salts are reduced to formazan by superoxide \textit{in vitro}. Reduction of NBT is dependent on an intact respiratory burst with production of hydrogen peroxide ($\text{H}_2\text{O}_2$) and superoxide ($\text{O}_2^-$) (Peter Newburger et al 1979). Thus, macrophages and granulocytes have the potential to reduce NBT, but their less mature progenitors do not. NBT reduction assay was done to more accurately quantify differentiation and to eliminate observer subjectivity associated with morphological assessment alone. A plot of absorbance versus the number of days of incubation of the sample Figure. 3.44a and b shows a progressive increase and a maximum NBT reducing activity were seen in the 3\textsuperscript{rd} day and 4\textsuperscript{th} day samples of CPME and CPDE treated cells, respectively. When the incubation time increased, the levels of NBT reduction also increased, reached its maximum and started declining by the 4\textsuperscript{th} and 5\textsuperscript{th} days of incubation, (CPME and CPDE respectively). DMSO is a proven inducer of differentiation in HL-60 cells (Peter Newburger et al 1979), which requires a latent period of at least 3 days between the induction by DMSO and the appearance of the earliest functions of mature cells (Corrado Tarella et al 1982). The lesser amounts of NBT reduction seen in the 1\textsuperscript{st} and 2\textsuperscript{nd} day samples compared to the 3\textsuperscript{rd} day samples could be due to the incomplete induction of differentiation. The differentiation appeared to be complete by the 3\textsuperscript{rd} day of CPME treatment. Similarly, CPDE required 4 days to achieve complete differentiation induction.

\textit{In vitro}, HL-60 cells differentiate into granulocytes and die via apoptosis like their normal counterparts in blood (Ruta Navakaukiene et al 2004). This justifies the descending trend in superoxide production after 4 and
5 days of CPME incubation and 5 days of CPDE incubation, as dying cell membranes cannot be stimulated by PMA. Majority of the HL-60 cell population are promyelocytic in morphology and histochemistry, but 4-15% of them show morphological characters of more mature myeloid cells (Steven Collins et al 1987). This explains the occurrence of basal levels of superoxide production in the control population.

Figure 3.44 Effect of CPME (a) and CPDE (b) on the activity of NBT reduction assay in HL-60 cells for 1µg/mL at indicated time point was performed. This showed 3, 4 day of respective differentiation induction.
3.5.3.1 Thymidine uptake assay confirms the induction of differentiation by CPDE and CPME

Maximum % inhibition was observed in the 3rd and 4th day incubated samples; declining values were noted on either side of the peak (Figure 3.45). The results are in line with the observations from NBT reduction assay (third and fourth day peak). Differentiated cells permanently lose their proliferative potential (Yoav Sharoni et al 2002) and therefore showed low proliferation in the assay. In other words, an inverse relationship was found between the proportion of mature cells in a population and their DNA-synthesizing capacity.

Figure 3.45 Effect of CPME and CPDE on $[^3]$H thymidine incorporation assay in HL-60 cells

Results shows 3 and 4 day maximum inhibition of proliferation by CPME and CPDE induction respectively at 1µg/mL concentration taken under mean ± SD of three separate experiments.

3.5.4 Elevation of SOD Activity of CPDE and CPME

During differentiation, there is an increase in the activity of SOD and hence measuring the levels of SOD activity can be a useful indicator of
the extent of differentiation of acute promyelocytic leukemia cells. The effect of 1µg/mL concentration of CPME and CPDE at 3 and 4-day time points respectively, showed significant inhibition of proliferation in HL-60 cells. Third and fourth days were chosen as the optimum time points for differentiation, since significant reduction in NBT was observed at these time points. Hence using the superoxide dismutase assay, the SOD activity was assessed in HL-60 cells at the 3rd and 4th day time points. Results showed that there is an increase in the size of the zone of clearance corresponding to the position of Cu/Zn SOD, in cells treated with 1µg/mL concentration of CPME and CPDE, in comparison to control cells (Figure 3.46). However, the zones of clearance for Mn SOD did not change. These results showed that the Cu/Zn SOD activity increases in the 1µg/mL concentration of CPME and CPDE treated cells, whereas the activity of Mn SOD remains unchanged. The increase in SOD activity in 1µg/mL concentration of CPME and CPDE treated cells reflects the fact that these cells have undergone differentiation (Yu-Ling Zhoua et al 2006). Thus, the results of SOD assay results correspond with that of NBT reduction assay.

**Figure 3.46  Expression of SODs in HL-60 cells**

In case of Cu/Zn SOD lanes represent (1) Untreated HL-60 cells showed no zone of clearance. (2) DMSO induced HL-60 cells showing prominent zone of clearance. (3) 1µg/mL concentration of CPDE (4 days) treated cells showed increase zone of clearance (4) 1µg/mL concentration of CPME (3 days) treated cells showed increase zone of clearance. The zones of clearance for Mn SOD haven’t changed much for both 1µg/mL and IC₅₀ concentrations of CPME and CPDE.
3.5.5 Morphological Changes of HL-60 Cells Induced by CPME and CPDE

Mostly, HL-60 leukemic cells remain blocked at the promyelocytic stage. The inducer used determines the differentiation of HL-60 cells into neutrophils, monocytes, eosinophils or basophiles (Li-Yun Luo et al 2006). This study made use of the HL-60 differentiating properties of DMSO. Morphology of untreated or treated (with or without 1µg/mL concentration of CPME and CPDE) HL-60 cells were observed under phase contrast microscopy. The untreated cells were ovoid in shape with large round nucleus. DMSO treated positive control cells showed neutrophil like morphology. Whereas CPME and CPDE treated cells showed a kidney or bean shaped nucleus, thus providing a morphological indication of the differentiation of HL-60 cells into monocytes, shown in Figure 3.47 (Defang Li 2009).

![Morphological changes induced by CPDE and CPME in HL-60 cells](image)

**Figure 3.47** Morphological changes induced by CPDE and CPME in HL-60 cells were stained with Wright-giemsa stain and observed by microscopy

The lanes represent (1) Untreated HL-60 control cells (Undifferentiated nucleus) (2) positive control cells treated with 1.25% DMSO (Neutrophil like morphology), (3) Cells were treated with CPDE for 4 days & (4) Cells were treated with CPME for 3 days in 1µg/mL concentration (CPDE and CPME showed monocyte like morphology).
3.5.6 FACS Analysis for Control Cells and 1µg/mL CPME and CPDE Treated Cells

Flow cytometric analysis was performed to determine if the observed differentiation was mediated by an underlying cell cycle arrest. Control cells were mostly present in the G1 phase, whereas 1µg/mL CPME treated cells at 3 days showed a higher percentage of cells in the S (28.83%) and G2-M (30.43%) phases. There was a reduction in the percentage of G1 cells upon treatment, relative to that of control cells. This shows that treated cells fail to exit the S and G2-M phases properly (Chen and Beck 1995). Similarly, 1µg/mL CPDE treated cells at 4 days showed a higher percentage of cells in the G2-M (26.33 %) phase; a concomitant decline in the percentage of G1 cells upon treatment compared to control cells was observed. This indicates that there is an arrest in the cell cycle at the G2-M phase in CPME and CPDE treated cells (Figure 3.48).

Figure 3.48 Showed accumulation in G2/M phase of cell cycle in CPDE and CPME treated HL-60 cells.

Cells were treated with 1µg/mL CPDE and CPME for 4 days and 3 days. Cells stained with propidium iodide were subjected to flow cytometric analysis for cell distributions in each phase of cell cycle.
3.5.7 Fate of Differentiated Cells

3.5.7.1 Induction of differentiation mediated apoptosis in HL-60 cells

(a) Propidium iodide staining

The results from propidium iodide staining showed a clear fragmentation of nucleus in treated cells. Propidium Iodide dye binds to the DNA and hence PI staining can be used to find out if the nuclei of the drug treated cells have been fragmented. Since, 1µg/mL concentrations of CPME and CPDE induces differentiation at the 3 and 4 day time points respectively, the fate of the differentiated cells in apoptosis were studied using preliminary (PI staining and DNA fragmentation assay) and molecular level studies. For these studies, HL-60 cells were incubated with CPME and CPDE for an additional time period of 24 h (4 and 5 days respectively), from the time of observation of the differentiation peak. PI staining performed after 4 and 5 days of incubation, demonstrates that 1µg/mL concentrations of CPME and CPDE induces apoptosis. This is clearly seen by the presence of fragmented nuclei in the treated cells. IC₅₀ concentration of CPME (10µg/mL) and CPDE (5µg/mL) treated cells also showed fragmented nuclei and they were used for comparison with the 1µg/mL concentration of CPME and CPDE treated cells. Control cells showed intact non-fragmented nuclei (Figure 3.49).
Figure 3.49  Nuclear localisation of HL-60 cells

1. Untreated HL-60 cells (Control), 2. Curcumin 50\(\mu\)m, 3. CPDE 5\(\mu\)g/mL (1 day), 4. CPDE 1\(\mu\)g/mL (5 day), 5. CPME 10\(\mu\)g/mL (1 day) 6. CPME 1\(\mu\)g/mL (4 day) by propidium iodide staining. HL-60 cells were treated with crude extracts of \(C.\) episcens both the crude extracts at IC\(_{50}\) as well as 1\(\mu\)g/mL concentrations exhibited characteristic nuclear fragmentation when compared to control. Control-Untreated cells did not show any significant nuclear fragmentation.

(b) DNA fragmentation assay

DNA fragmentation assay performed on the 1\(\mu\)g/mL concentration of CPME or CPDE treated cells, at the 4 and 5 day time points respectively, showed ladder like DNA fragments. The fragmentation pattern was comparable to the results obtained from the cells treated with the IC\(_{50}\) concentrations of CPME or CPDE, at the 24 h time point. Control cells showed intact DNA. Presence of DNA ladder at the 4 and 5 day time points, in differentiated cells correlates with the results of PI staining and hence confirms that CPME and CPDE mediated differentiation terminated in apoptosis (Figure 3.50).
Figure 3.50 Showed the effect of CPDE and CPME on DNA fragmentation in HL-60 cells

Lanes represents 1. Untreated HL-60 cells (Control), 2. Curcumin 50µm, 3. CPDE 5µg/mL (1 day), 4. CPDE 1µg/mL (5 day), 5. CPME 10µg/mL (1 day), 6. CPME 1µg/mL (4 day), after treatment of the cells, DNA was isolated and separated on 1.5% agarose gels. DNA was stained with ethidium bromide and visualized under UV light. Similar patterns of gel electrophoresis were obtained in two separate experiments.

(c) Effect of CPME and CPDE on gene and protein expression of HL-60 cells

**CPME and CPDE modulate Bcl-2 expression:** Bcl-2 is an anti-apoptotic protein, which is over-expressed in many human cancers (Green and Evan et al. 2002). The permeabilization of the mitochondrial membrane is controlled by the members of the Bcl-2 family. During apoptosis, pro-apoptotic Bax monomer translocates to the mitochondria and forms dimer or higher order oligomers. The presence of an anti-apoptotic molecule such as Bcl-2 or Bcl-X\textsubscript{L} inhibits the activation of Bax following a death signal, thus
evading cell death. Decreased expression of Bcl-2 protein was observed in the 1µg/mL, 10 and 5µg/mL concentrations of CPME and CPDE treated cells, when compared to that of the control cells (Figure 3.51a). A drastic reduction in the intensity would mean significant apoptosis by decreasing the manifestation of Bcl-2. Thus it can be concluded that CPME and CPDE induces apoptosis via Bcl-2 down regulation, by removing the obstruction in the mitochondrial pathway.

**CPME and CPDE modulates Bax and caspase 3 expression:**
The gene and protein level expressions of Bax and Caspase-3 respectively were increased in the 1µg/mL (dissolved in Methanol), 10 and 5µg/mL (dissolved in DMSO) concentrations of CPME and CPDE treated cells, when compared to that of the control cells. These results confirm that the induced differentiation terminated in apoptosis. This is because caspases (Cytoplasmic aspartate-specific cysteine proteases of the ICE/CED-3 family) play a crucial role in controlling apoptotic cell death. Typical apoptosis pathways would increase the expression of Caspase-3 and decrease the levels of cell survival factors like Bcl-2, normally expressed in human B lymphocyte. Bcl-2 proteins, including Bcl-xL, Bcl-w, exert anti-apoptotic activity, while Bax protein exerts pro-apoptotic activity. A balance of anti and pro-apoptotic proteins appear to determine the fate of cells; those cells with an excess of anti-apoptotic proteins survive while an excess of pro-apoptotic proteins result in death. In the present study, the expression of Bcl-2 protein was decreased at the 1µg/mL concentration of CPME and CPDE treated HL-60 cells, while the expression of Bax protein was increased, as compared with that of untreated cells (Na-Young Kim et al 2001). Thus, these observations suggest that the differentiation mediated apoptosis exhibited by CPME and CPDE is dependent on decreased expression of Bcl-2 and the increased expression levels of Bax and Caspase-3 (Balkwill 2009). (Figure 3.51 b and c). GAPDH was used as internal control (Figure 3.51 h).
CPME and CPDE modulate PARP expression: The final pathway that leads to the execution of the death signal is the activation of a series of proteases termed caspases. The intrinsic and extrinsic apoptotic pathways converge to caspase-3, which cleaves the inhibitor of the caspase-activated deoxyribonuclease, leading to nuclear apoptosis. The downstream caspases (Caspase-3) induce cleavage of proteins such as poly-ADP-ribose-polymerase (PARP) and finally, destruction of cellular functions. An important factor in inducing apoptosis is the enzyme poly (ADP-ribose) polymerase (PARP), which has been widely studied in vitro. PARP has been proposed to act as a critical death substrate, because it is rapidly cleaved by Caspase-3 and mammalian ICE-related protease involved in apoptosis, resulting in its cleavage to 85- and 29-kD polypeptides. Caspase-3, a member of the caspase family of 13 aspartate specific cysteine proteases plays an important role in the execution of the apoptotic process (Hamid Boulares et al 1999), and is primarily responsible for the cleavage of PARP during cell death. This study investigated the expression levels of PARP protein to understand the possible involvement of PARP in the Centrosema pubescens induced apoptosis in HL-60 cells. HL-60 cells treated with 1µg/mL, 10 and 5µg/mL concentrations of CPME and CPDE treated cells (Figure 3.51d) showed significant cleavage of PARP, seen as an increase in band intensity compared to control.

CPME and CPDE modulate k-Ras expression: The role of Ras in the differentiation of HL-60 cells has been studied by analysing the changes in the level of expression of Ras gene by RT-PCR. 5µg/mL CPDE and actinomycin D treated cells exhibited down regulation of k-Ras oncogene when compared with untreated control cells. Interestingly 1µg/mL CPME and CPDE and 10µg/mL CPME treated cells showed up regulation of k-Ras gene levels (Figure 3.51e). Studies have shown that differentiation resulted in upregulated p21 Ras expression despite a marked decline in the number of
dividing cells (Naima Gueddari-Pouzols et al 2001). Our results indicate occurrence of differentiation leading to cell cycle arrest and further suggest p21 Ras has a role in the process of myeloid differentiation using *C. pubescens* extracts.

**CPME and CPDE lead to overexpression of tumor suppressor protein p27 and p16:** Cell differentiation is usually associated with exit of the cells from the cell cycle. Cell cycle progression in mammalian cells is regulated by a family of enzymes known as cyclin-dependent kinases (CDKs) whose activity is dependent on the binding to specific regulatory subunits called cyclins. The activity of the cyclin/CDK complexes is negatively regulated by specific CDK inhibitors like p16, p21, and p27 (Akihiro Muto et al 1999). They bind to cyclin-CDK complexes and prevent kinase activation and subsequently block the progression of cell cycle at Go/G1 or G2/M phase (Hsu et al 2004).

Since its discovery as a cyclin-dependent kinase inhibitor in 1993, the tumor suppressor p16 has gained widespread importance in cancer. The frequent mutations and deletions of p16 in human cancer cell lines suggests an important role for p16 in carcinogenesis. When compared with control cells (untreated HL-60 cells), p16 gene was observed to be upregulated significantly by 1µg/mL, 10 and 5µg/mL concentrations of CPME and CPDE treated cells (Figure 3.51f).

The level of p27 protein was also found to be increased at 1µg/mL CPME and CPDE treated cells in comparison to the control cells. These results show that subsequent to the occurrence of differentiation on the third and fourth day of treatment, there has been an elevation in the levels of p16 and p27 which in turn would have translated as inhibition of cell proliferation (Figure 3.51g).
Differentiation mediated apoptosis can also be related to the increased expression of p16 and p27, leading to cell-cycle arrest at the G2-M phase followed by apoptosis (Yasuhiro Terui et al 1998). Thus, differentiation mediated apoptosis can also be related to the increased expression of p16 and p27. β-actin (Figure 3.51i) was used as housekeeping gene (internal control).

![Figure 3.51](image)

**Figure 3.51 Effect of CPME, CPDE on differentiation mediated apoptosis**

(a) Bcl-2, (b) Bax, (c) caspase 3, (d) PARP (e) Ras, (f) p16, (g) p27, (h) GAPDH, (i) β-actin. The levels of GADPH transcripts and β-actin were determined in the sample as internal controls. The cell eluates were analyzed by RTPCR (Bax, Ras, p16, & GAPDH) and immunoblotting (Bcl-2, Caspase-3, PARP,p27 & β-actin) using specific primers and antibodies respectively. Lanes were labeled as 1.Control, 2.CPDE 5µg/mL (1day), 3.CPDE 1µg/mL (5day), 4. CPME 10µg/mL (1day), 5. CPME 1µg/mL (4 day).

In synopsis, [³H] - Thymidine incorporation assay proved CPME and CPDE has a high growth inhibitory effect on HL-60 cells. The time-course and dose-response analysis showed that 24 h and 10µg/mL and 5µg/mL respectively are the optimum time and doses required for producing 50% inhibition. Appearance of DNA fragments confirmed that the death of drug-treated cells was via apoptosis and not necrosis. NBT reduction assay revealed the cellular differentiation-inducing phenomenon by the *C.pubescens*
extracts at lower concentration (a dose at 24 h, did not induce apoptosis), that reached a peak at 3 and 4 days. Terminally differentiated cells permanently lost their proliferative potential which was confirmed with the cells induced to differentiation by thymidine incorporation and SOD assay. CPME and CPDE produced a remarkable up regulation of the CDK inhibitors p16 and p27 leading to cell cycle arrest at the G2-M phase. CPME and CPDE brought about a clear regulation of anti-apoptotic proteins, pro-apoptotic proteins, Caspase-3 and its substrate PARP, demonstrating the induction of apoptosis. The results confirm CPME and CPDE induced monocytic differentiation in HL-60 cells and arrest cell cycle at the G2-M phase, leading to apoptosis.

3.6 SUMMARY

Evaluation of _Centrosema pubescens_ extracts for differentiation therapy is interesting since to the best of our knowledge, this is the first report of its anti-proliferative, differentiation–inducing, cell cycle arresting and Apoptosis–inducing properties. The study has explored the anti-cancer potency of CPME and CPDE in human acute promyelocytic leukemia cell line HL-60, in an _in vitro_ model. The advantage of this therapy is it being non toxic to normal cells (least toxic doses were chosen), unlike other currently available therapies for cancer (Cytotoxic drugs). Our results indicate, CPME and CPDE induces monocytic differentiation in HL-60 cells, arrests cell cycle at G2-M phase, hence leading to apoptosis (Figure 3.52).
Figure 3.52 Schematic representation of the observed anti-cancer effect of CPDE, CPME and Lupenone from *Centrosea pubescens*.