Summary

In the present study, *Clerodendrum infortunatum* leaf and root material and their derived compounds have been evaluated for few pharmacological activities and in particular for their cytotoxic activity. *Clerodendrum infortunatum* L. belongs to the Verbenaceae family is a diverse genus that includes 560 to 580 species of small trees, shrubs, lianas, or occasionally perennial herbs, mostly in the tropics and subtropics of the old world. The genus *Clerodendrum* contains many plant species such as: *C. indicum, C. phlomidis, C. serratum, C. trichotomum, C. chinense*, *C. petasites, C. colebrookianum, C. calamitosum*, *C. trichotomum*, and *C. inerme* as that are being used in various health care systems for the treatment of various disorders including life-threatening diseases.

Primarily, both leaf and root material of the plant *C. infortunatum* were extracted using hexane, chloroform, ethyl acetate and ethanol solvents as per their polarity, from non-polar to polar solvents. The extracts so obtained from each of solvents were labelled and yield was calculated in terms of grams/weight of the powdered material. For leaf extraction 1000g of powder, whereas, for root extraction 500g root powder was used. In the leaf extraction process using various solvents such as hexane, chloroform, ethyl acetate and ethanol, the amount of extract obtained is 3.0, 2.0, 3.5 and 20% respectively. Similarly, the root material yielded 1.0, 1.5, 1.0 and 4% extracts with hexane, chloroform, ethyl acetate and ethanol respectively. It is observed that extraction with ethanol in both leaf and root yields the highest amount of yield.

Phytochemical screening of *C. infortunatum* extracts was performed for the qualitative detection of reducing sugars, terpenoids, flavonoids, saponins, tannins, alkaloids, phlobatannins, steroids, amino acids and glycosides. The presence of groups among all these different extracts include alkaloids, flavonoids, terpenoids, steroids, tannins and carbohydrates. In leaf material, flavonoids and carbohydrates are found to be present in all the extracts, and alkaloids and glycosides are also present in all the extracts except in ethanol extracts. Steroids, tannins, saponins and amino acids are present in only ethanol extracts. In case of root material, alkaloids are present in all the extracts except ethanol, and flavonoids are absent...
only in hexane. Tannins and saponins are present only in ethanol, whereas phlobotannins, glycosides and amino acids are completely absent in all the extracts. Carbohydrates are present in all the extracts.

In anthelmintic activity, test samples of each leaf extract were prepared at the concentration of 25, 50, 100 mg/ml DMSO. 10 mg/ml of standard piperazine citrate was used as the reference while normal saline served as the control. Treatment with ethanol extract induced loss of motility and consequent paralysis in 28, 24 and 16 min followed by death time of 59, 53, and 46 min respectively. In comparison, the 10 mg/ml dose of the reference drug caused paralysis to set in after 24 min of post incubation followed by the time of death in 51 min. Ethyl acetate extract has also shown significant results in terms of paralysis and death at similar concentrations attained paralysis in 34.33, 30.67 and 18.67 min followed by the time of death in 64, 55.67 and 48 min respectively. After the exposure of worms to hexane extract, it is detected that worms conquered by paralysis in 42, 35.33 and 30 min and death time is recorded as 67, 64 and 60 min which is reasonably more than the standard. When similar concentrations of chloroform extracts were operated on these worms, the paralysis seemed in 39, 33, 33.33 min and thereafter, death of the worms has been visualised in 66.67, 65, and 61 min that is slightly more than the standard used.

Antifungal activity was determined by the disc diffusion method. Muller Hilton and Saboured Dextrose Broth were used as a medium for fungal strains respectively. Four clinical strains Aspergillus niger, Aspergillus fumigates, Aspergillus flavorus and Candida albicans were used for assessing the antifungal activity and standard amphotericin (1 mg/ml) was used as positive control. The assessment of antifungal activity was based on the measurement of diameter of inhibition zone. The leaf extracts of C. infortunatum were prepared at a concentration of 50, 75 and 100 mg/ml in triplicates and one control was used for each extract. It is clear from the inhibition zones that most of the leaf extracts of C. infortunatum are effective against Aspergillus fumigate and ethanol extract has shown intermediate inhibition on Aspergillus flavorus. Hexane, chloroform and ethyl acetate extracts verified as ineffectual in contradiction of Aspergillus
flavorus. None of the extracts have shown substantial activity against *Aspergillus niger* and *Candida albicans*. The highest value of inhibition zone is recorded as 10.83 ± 0.44 mm in diameter in case of chloroform extract at a concentration of 75 mg/mL comparatively more than the standard amphotericin (7.50 ± 0.50) against *Aspergillus fumigates*. When this strain was exposed to hexane extract, it is detected that zone of inhibition has appeared to be 7.50 ± 0.29, 9.76 ± 0.44, and 8.50 ± 0.29 mm respectively. Ethyl acetate has also proven to be most effective against *Aspergillus fumigates* exhibiting inhibition zone of 9.33 ± 0.17, 10.33 ± 0.33, and 9.50 ±0.29 respectively. Among all these extracts ethanol extract proved to be incompetent towards *Aspergillus fumigates*, but has shown some promising action when acted upon *Aspergillus flavorus*.

- Cytotoxic activities of crude extracts of leaf and root from *C. infortunatum* were evaluated in T47D (Breast), PC-3 (prostate), A549 (lung) and HCT-116 (colon) cancer cells in order to scrutinize its potential as a cancer chemopreventive source. The growth inhibitory activity of the hexane, chloroform, ethyl acetate and ethanol extracts of both leaf and root were evaluated against four cell line panels using the SRB assay (Houghton et al., 2007). Several standard anticancer drugs were used as positive control for comparison. The drugs include adriamycin (10µM) against T47D, mitomycin (1µM) against PC-3, paclitaxel (1 µM) against A549 and 5-Fluorouracil (20µM) against HCT-116 were added as a positive control against desired cancer cell lines per well and incubated for 48 h. The best antiproliferative activity has been exerted by hexane of root exhibiting growth inhibition of 72.83 ± 0.44, 85.50 ± 0.29 and 68.17 ± 1.36 % against PC-3, A549 and HCT-116 at a concentration of 100µg/mL. At a similar concentration the chloroform extract is also effective against these three cell line showing 61.50±0.76, 67.00 ± 0.58 and 68.53 ± 0.80 % growth inhibition against PC-3, A549 and HCT-116 respectively. The results have indicated that all the leaf extracts as well as ethyl acetate and ethanol root have exhibited a poor response (≤40%).

- The human cancer cell line A549 (lung) has been used to evaluate the effect of hexane and chloroform root extracts at 50, 100 and 200µg/mL along with vehicle DMSO using clonogenic assay as these two extracts proved to be cytotoxic in
SRB assay. In this investigation, Staurosporine (20 nM) was used as a standard. Interestingly, at 200 g/ml of HR and CR extracts treatment, the number of soft agar colonies was reduced by ~4-fold \((p \leq 0.05)\) compared to the untreated control cells and appears to be equal to the effect of standard. The decrease in the colony formation ability of A549 cells after the treatment with HR, CR and standard in standard drug is found to be statistically significant \((p \leq 0.05)\).

The in vitro Scratch motility assay has been employed to study cell migration of A549 cells in vitro after the treatment with hexane and chloroform root extracts of C. infortunatum. Cells were successively treated in medium containing low serum (1.0%) in presence of different concentrations of HR and CR extracts (50, 100, 200 µg/ml) along with vehicle DMSO for 24 h. Wounded areas were progressively photographed with microscope attached with camera (100x magnification). It was observed that HR and CR effectively inhibited the migration of cells in a dose- and time-dependent manner compared to the untreated control cells. Fascinatingly, at 200 µg/ml, HR and CR extract treatment significantly decreased (~4-fold; \(p \leq 0.01\)) the migration rate of A549 cells thereby affecting their migration capability. Present investigation clearly suggests that HR and CR extracts significantly inhibited motility of A549 cells in statistically significant manner \((p \leq 0.05)\).

The effect of HR and CR extracts was envisioned by staining the cells with DAPI to detect morphology of the nucleus that had undergone apoptosis. A549 cells were incubated in 6-well plates and treated with varying concentrations of HR and CR (50, 100, and 200 µg/mL) for 24 h. Untreated cells (DMSO as vehicle) served as control. It is evident from the results that HR and CR extracts have direct influence on the nucleus of A549 cells. These extracts have malformed the nucleus of the cells in a dose-dependent manner compared to the untreated control cells. Interestingly at a concentration of 200μg/mL, nuclei of the cells are considerably aborted with the treatment of HR and CR extracts.

Among the extracts tested for cytotoxicity, the hexane and chloroform extracts of C. infortunatum roots showed in vitro cytotoxic activity. Hence, these extracts were selected for in vivo anti-tumour activity screening using EAT induced mice.
An average body weight of 25-30g Swiss albino mice were used for the experiment. These cells were maintained in Swiss albino mice weekly by intraperitoneal inoculation of $1 \times 10^6$ cells/mouse i.e., the viable EAT cells were counted (Trypan blue indicator) under microscope and were adjusted at $2 \times 10^6$ cell/ml. 5-Fluorouracil (5-FU) was used as standard. Among the two extracts of the roots of *C. infortunatum* hexane extract indicated by the increase in mean survival time, increase in lifespan and decrease in body weight to 24.50 ±0.40 days, 63.63 ± 0.32 % and 27.52±1.29% respectively. When compare to EAT control 20.00 ± 0.44 days of mean survival time and 38.08 ±1.29% increase in body weight. Treatment of EAT tumor cell growth cells by chloroform extract specified an increase in mean survival time, increase in lifespan and decrease in body weight to 21.91 ± 0.68 days, 60.55 ± 0.32 % and 24.48 ± 1.24% respectively. The tumor cell volume of EAT control was recorded at 4.38±0.11 mL which was significantly decreased by the induction of hexane and chloroform extracts at 1.40±0.03 and 1.55±0.04 mL respectively which is closely equal to standard at 1.10±0.02 mL. Similarly, packed cell volume has decreased to 4.19±0.26 and 5.05±0.37 mL of hexane and chloroform extract from 9.15±0.28 mL of EAT bearing mice. Administration of hexane and chloroform extracts showed a significant reversal of cell count (4.19±0.26 and 5.05±0.37 cells/mL) compared to EAT bearing mice (9.15±0.28 cells/mL).

- The hexane and chloroform extracts of *C. infortunatum* roots showed potent cytotoxic activity in both *in vitro* and *in vivo* methods. Hence, these were subjected to column chromatographic separation to isolate their bioactive constituents. Elutions carried out with hexane-methanol graded mixture (70:30) of hexane extract yielded a single compound. This compound was designated as KMB01. Fractions eluted with chloroform-methanol (90:10) yielded an amorphous residue (50 mg) and was monitored by TLC. After the evacuation of solvent a light yellow colour residue was obtained and was designates as KMB 02. The analysis of hexane derived KBM 01 and chloroform derived KBM 02 compounds involved spectroscopic studies such as IR, $^1$HNMR, $^{13}$CNMR and Mass Spectra analysis in order to characterize these isolated phytoconstituents.

From the interpretation of all these spectra, data suggested that the compound
KBM 01 is pentacyclic triterpene and identified as betulinic acid while as, KMB 02 is also a triterpenoid and recognized as lupeol acetate.

Subsequently, these bioactive compounds betulinic acid and lupeol acetate from hexane and chloroform root extracts were subjected for cytotoxic screening using MTT assay with an intention to get a clear picture about the cytotoxicity of C. infortunatum. The human cancer cell lines used in this study, comprising MCF-7 (Breast), HePG2 (Liver), A549 (lung) and HCT-116 (colon). Metformin acts as standard in this assay and all the results of isolated compounds were compared with respect to the cytotoxicity of this standard. Different concentrations of test drug betulinic acid (10, 20, 40, 60, 80 µg/mL) and lupeol acetate (50, 100, 150, 200, 250 µg/mL) were used to estimate the IC\textsubscript{50} values of these two derived compounds. After the treatment of MCF-7 cells with betulinic acid at the concentrations selected, response of the cells is found be significant and the IC\textsubscript{50} of betulinic acid against MCF-7 cells effects at 55.028 µg/mL. There is also a substantial effect on the viability of the HePG2 cells with IC\textsubscript{50} calculated as 50.912 µg/mL. Highest cytotoxicity activity of betulinic acid has been observed against HCT-116 cancer cell line at IC\textsubscript{50} value at 38.28 µg/mL, whereas, betulinic acid has not been proven effective against A549 cell line.

Lupeol acetate verified significant cytotoxicity against MCF7 cell line. The IC\textsubscript{50} value of lupeol acetate against MCF-7 is assessed as 118.656 µg/mL. This compound has also ascertained toxic to HePG2 cells with IC\textsubscript{50} at 27.27 µg/mL. This compound also proven to be potent against HCT-116 cancer cell line presenting IC\textsubscript{50} at 84.2 µg/mL. There is a also substantial effect on the viability of the A549 cells after the treatment with lupeol acetate at IC\textsubscript{50} concentration 75.307 µg/mL.

Based on above cytotoxic investigation it was found that the isolated compounds from Clerodendrum infortunatum exhibit strong anticancer activity against various cell lines which leads to cell growth inhibition. Therefore, to confirm the mechanism of cytotoxicity by betulinic acid and lupeol acetate related to apoptotic cell death was confirmed by a staining technique using Annexin V PI staining by selecting HCT-116 cell line because of its sensitivity towards both of...
these compounds based on their IC$_{50}$ values of 38.28 µg/mL and 84.2 µg/mL. Untreated, 5-Fluoro uracil (standard), betulinic acid (drug 1), and lupeol acetate (drug 2) treated HCT-116 cells were stained using the Annexin V FITC apoptosis detection kit. In the untreated (control) HCT-116 cells, 97.33% of cells have been found viable and non-apoptotic. Cells treated with 5-FU have shown 90.37% of cells viable and non-apoptotic. After the treatment of HCT-116 cell with betulinic acid, it leads to 7.57% of apoptotic cell death indicates adequate apoptotic response of HCT-116 against this compound after comparison with standard 5-FU. Upon the treatment of HCT-116 cell with lupeol acetate demonstrated 17.8% of apoptotic cell death. This compound has proved to be remarkably significant apoptotic agent.

- Later steps in apoptosis of HCT-116 cell line by betulinic acid and lupeol acetate based on their IC$_{50}$ values of 38.28 µg/mL and 84.2 µg/mL. was confirmed by DNA fragmentation, a process which results from the activation of endonucleases during the apoptotic program. Camptothecin was used as standard in this assay. The APO-DIRECT™ assay was used in this study. It is a single-step method for labelling DNA breaks with FITC-dUTP, followed by flow cytometric analysis. Untreated HCT-116 cells have displayed 1.23% of DNA fragmentation. Camptothecin treated HCT-116 cells presented 10.57% of FITC-dUTP. There is a rise in DNA fragmentation of cell populations from 1.23% of untreated cells to 10.57% of camptothecin treated cells. After the exposure of HCT-116 cells to betulinic acid, 1.96% DNA fragmentation of cancer cells has been recorded which displays a poor response of betulinic acid. In contrast, lupeol acetate has verified considerable DNA fragmented compound by demonstrating 33.06% FITC-dUTP labelled HCT-116 cells.

- Confirmation of the mechanism of apoptosis regulation by betulinic acid and lupeol acetate related to cell death, Bcl-2 expression was analysed using the anti-bcl-2 antibody. The protein is a regulator of the apoptotic process, and elevated levels can provide resistance to cell death. The Bcl-2 protein is expressed in the interior of cells, frequently localized to the mitochondrial membrane. Untreated, camptothecin (standard), betulinic acid, and lupeol acetate treated HCT-116 cells...
were examined by FACS from the histogram analysis in presence of concentration of betulinic acid (38.28 µg/mL) and lupeol acetate (84.2 µg/mL). In the untreated (control) samples, the intensity of fluorescence is lower as its percentage is recorded at 1.37%. When cells were treated with camptothecin, the intensity of fluorescence is intensified to 14.58%. This increase entails that Bcl-2 expression is under down regulation from untreated to camptothecin treated HCT-cell line as cells may have attained apoptotic condition. After the treatment of HCT-116 cells with betulinic acid, it detects 1.80% of fluorescence. This specifies Bcl-2 expression is normal resulted in less apoptosis of HCT-116 cells. Upon the treatment of HCT-116 cells with lupeol acetate, it demonstrates 93.13% intensity of fluorescence. This compound has proven to be remarkably uttermost apoptotic agent as there is enormous reduction in the Bcl-2 expression.

The pro-apoptotic mechanism by betulinic acid and lupeol acetate related to cell death, caspase-3 expression was analysed using the anticaspase-3 antibody. Caspase-3 is a key protease that becomes activated during the early stages of apoptosis. In its active form, it proteolytically cleaves and activates other caspases, as well as relevant targets in the cytoplasm (e.g., D4-GDI) and nucleus (e.g., PARP). Untreated, camptothecin (standard), betulinic acid, and lupeol acetate treated HCT-116 cells were examined by FACS from the histogram analysis in presence of concentration of betulinic acid (38.28 µg/mL) and lupeol acetate (84.2 µg/mL). In the untreated (control) samples, the intensity of fluorescence is lower as its percentage is recorded at 1.24%. when cells are treated with camptothecin, the intensity of fluorescence is intensified to 33.03%. This increase entails that caspase-3 expression is under up regulation from untreated to camptothecin treated HCT-cell line as cells may have attained apoptotic condition. After the treatment of HCT-116 cell with betulinic acid, detection of 1.74% fluorescence is observed which specifies caspase-3 expression is down regulated and it may be reason of less apoptosis of HCT-116 cells by betulinic acid. Lupeol acetate treated HCT-116 cells have revealed 55.93% intensity of fluorescence, which confirms activation of pro-apoptotic caspase-3 protein and can considered substantial apoptotic compound.