Anti-tumor studies with extracts of *Calotropis procera* (Ait.) R.Br. root employing Hep2 cells and their possible mechanism of action

Rajani Mathura*, Suresh K Gupta*, Sandeep R Mathur† and Thirumurthy Velpandian‡

*Department of Pharmacology, Delhi Institute of Pharmaceutical Sciences and Research, Pushp Vihar, New Delhi 110 017, India
†Department of Pathology, and ‡Department of Ocular Pharmacology, RP Centre, All India Institute of Medical Sciences, Ansari Nagar, New Delhi 110 029, India

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Anti-tumor potential of root extracts of *Calotropis procera*: methanolic extract (CM), hexane extract (CH), aqueous extract (CW) and ethylacetate extract (CE) and its possible mechanism against Hep2 cancer cells has been investigated. Cellular proliferation activities were assayed by tetrazolium bromide (MTT) colorimetry. Morphological changes of cancer cells were observed under inverted microscope and cell cycle parameters were determined by flow cytometry following propidium iodide staining. Treatment with the extracts at various doses of 1, 5, 10 and 25 µg/ml revealed that CM, CH and CE possessed cytotoxicity, whereas CW did not have cytotoxic effect. CE (10 µg/ml) showed strongest cytotoxic effect (96.3%) on Hep2 at 48 hr following treatment, whereas CM and CH showed cytotoxicity of 72.7 and 60.5%, respectively. Extract-treated cells exhibited typical morphological changes of apoptosis. Results of flow cytometric analysis clearly demonstrated that root extracts initiated apoptosis of Hep2 cells through cell cycle arrest at S phase, thus preventing cells from entering G2/M phase. Results of the study indicate that the root extracts of *C. procera* inhibit the proliferation of Hep2 cells via apoptotic and cell cycle disruption based mechanisms.

**Keywords:** Anti-tumor, Apoptosis, *Calotropis procera*, Cell Cycle, Hep2, Root

Species of Calotropis are commonly known as ‘The Swallow-Wort’ or ‘Milkweed’ and belong to the family Asclepiadaceae. Calotropis is regarded as useful medicinal plant and used in folk medicine¹-³. Although the latex of *Calotropis procera* has been extensively studied and found responsible for cytotoxic, procoagulant, anti-inflammatory, abortifacient activities¹-⁵; the medicinal properties of the roots of this important plant are not well explored.

Root bark of *C. procera* is reported to contain akundarol isovalerate, mundarol isovalerate and quercetin-3-rutinoside. The roots contain compounds called oxypregnaneoligoglycosides or steroidal lactones. These compounds are collectively called as ‘calotroposides’ and the roots contain calotroposides C, D, E, F, G.⁶

Alcoholic extract of root has been investigated for anti-cancer activity against a panel of human and mouse cell lines⁷-⁹. A potent cytotoxicity of the roots has been established against human but not mouse cell lines⁹. However, there is a paucity of reports suggesting the mechanism(s) underlying this property. Therefore, the present study was designed to evaluate the effect of the root extract of *C. procera* on cancer cell proliferation and cell cycle.

**Materials and Methods**

**Drugs**—Dulbecco’s Modified Eagles Medium, Propidium iodide, [3-(4,5-dimethylthiazolyL-2)-2,5-diphenyl tetrazolium bromide] or MTT, Trypsin, were purchased from Sigma-Aldrich (St.Louis, MO, USA); Fetal Calf Serum (Gibco Laboratories, USA). Solvents for HPLC were purchased from s.d.fine-chem Ltd (Mumbai, India). All other reagents were of analytical grade.

**Preparation of root extract**—Roots of *C. procera* grown in natural habitat and purchased from an authorized dealer were air-dried in shade and finely powdered. Chief botanist at NISCAIR(CSIR), New Delhi, India, identified the roots and a voucher specimen (accession number NISCAIR/RHM/F-3/2003/Consult/373) has been deposited at the herbarium of NISCAIR.

Root extracts of *C. procera* were carried out by the method of Kitagawa **et al.**¹⁰ Briefly, an exhaustive methanolic extract of the root powder was prepared
under reflux for 8 hr. The refluxate was filtered and concentrated using vacuum rotary evaporator. The concentrate was further lyophilized to give methanol extract of *C. procera* roots (CM). CM was then serially partitioned thrice into equal volumes of n-hexane and water (1:1). The n-hexane phase was concentrated using vacuum rotary evaporator followed by lyophilization to obtain the n-hexane extract (CH). The aqueous phase was concentrated by lyophilization to obtain CW and was further fractionated by shaking with equal volumes of ethyl acetate. The ethyl acetate phase was concentrated using vacuum rotary evaporator followed by lyophilization and labelled as CE. All the lyophilized powders were weighed and stored at -20°C, until used.

**Phytochemical evaluation**—Using standard reagents and protocols the methanolic root extract was subjected to phytochemical evaluation to test the presence of alkaloids (Mayer’s, Hager’s, Wagner’s and Dragendorff’s test), reducing sugars (Fehling’s test), deoxy sugars (Keller-kiliani test), steroids (Dragendorff’s test), and tannins (FeCl₃ test).

**HPLC analysis**—The HPLC analysis was performed on a Waters HPLC system (Milford, MA, USA) equipped with solvent delivery system, pumping system (510, Waters) and photodiode array detector (996, Waters), coupled with a Waters Millennium® Chromatography Manager and run on an Intel Pentium II workstation. Best separation and resolution was achieved on an octadecyl column (Nova Pak C₁₈, 3.9 x 150 mm, 4 μm particle size) and a mobile phase of potassium-di-hydrogen orthophosphate (50 mM) and acetonitrile (3:7 v/v), pumped at a flow rate of 1.5 ml for a run time of 30 min with constant photodiode array detection. For analytical evaluation, the wavelength of 354 nm was used. The HPLC analysis was documented by means of photomicroscopy (40x).

**Cell culture conditions**—Cancer cell line (Hep2) was generated from human epidermoid laryngeal carcinoma and grown under aseptic conditions using Dulbecco’s modified eagle’s medium (DMEM) enriched with 10% fetal calf serum and 100 U/ml penicillin, 75 U/ml streptomycin, at 37°C, pH 7.2 and 5%CO₂. After 60-80% confluency, the cells were trypsinized with 0.25% trypsin (dissolved in PBS, pH 7.4), counted and placed down at the needed density for treatment.

**MTT colorimetric assay**—Assessment of *in vitro* cytotoxicity was determined by MTT assay using standard protocol. Briefly, the Hep2 cells in exponential growth were placed down at a final adjusted density of 7-8 x 10³/100μl in a 96-well plate. After 40% confluence, the cells were treated either with CM, CH, CW, CE at varying concentrations of 1, 5, 10, 25 μg/ml, or vehicle or standard drug (cisplatin, 10 μg/ml). The Hep2 cells were exposed for 3 hr to 100 μl of MTT dye (1mg/ml), which was added at 24 hr or 48 hr after treatments. The supernatant was discarded and DMSO (200 μl) was added to solubilize the dye. The 96-well plate was vibrated on the micro-vibrator for an additional 10 min. The percentage of viable cells in each well was calculated from absorbance of the purple colored formazan crystals read at 560 nm using ELISA microtiter plate reader (Anthos Labtec, Austria). The growth inhibitory ratio of each extract was calculated using the following formula:

Growth inhibitory ratio (%)= (average A₅₆₀nm of control group-average A₅₆₀nm of treated group)/average A₅₆₀nm of control group x 100%

**Morphologic analysis by inverted microscope**—The morphology of the Hep2 cells was monitored using an inverted microscope equipped with digital camera (Nikon, Japan). Following 24 hr and 48 hr treatment with root extracts (CM, CH, CE, CW), or vehicle or control, changes in cellular morphology of Hep2 cells was observed and documented by means of photomicroscopy (40x).

**Flow cytometry**—The DNA content and the cell cycle of Hep2 cells were determined by flow cytometry. Briefly, the Hep2 cells were placed down at an adjusted density of 1x10⁷ cells/ml of medium, in a 96 well plate. After 40% confluence, the Hep2 cells were exposed to CM, CH, CE or CW at a concentration of 10 μg/ml for additional 48 hr. The rounded-off non-viable cells were pipetted out, pelleted and washed twice with ice-cold PBS buffer (pH 7.4), fixed using 70% ethanol. Within one week, the fixed cells were processed and cell DNA stained with propidium iodide (PI) (1 mg/ml) in the presence of 1% RNAase A at least for 30 min before analysis by flow cytometry at 488 nm (Coulter EPICS XL™, USA). A minimum of 10,000 cells/ sample were collected and System II™ software (Coulter, USA) was used for data analysis.

**Statistical analysis**—The results were expressed as mean±SD. The statistical analysis involving two
groups was performed by means of Student’s t-test. All data were processed with Graphpad prism ver5 software. The results were considered significant when \( P < 0.05 \); significance levels were reported as follows: * \( P < 0.05 \), ** \( P < 0.01 \), *** \( P < 0.001 \).

**Results**

**Phytochemical evaluation**—The percentage yield of the dried extracts was calculated from the starting weight of the dried roots. Accordingly, the percentage yield of CM, CH, CE and CW was calculated as 4.56, 0.71, 0.58 and 0.30%, respectively. CM tested positive for the presence of alkaloids, reducing sugars, deoxy sugars, steroidal moieties but not tannins.

Under the given HPLC conditions, well-resolved base separated peaks were generated in the chromatograms. The method allowed optimum separation (selectivity factor, \( \alpha > 1 \)) and high resolution (HETP>2000) within reasonable run time (30 min). Repeated analysis of the samples under the same conditions yielded identical elution patterns, which were characteristic of each extract. Twelve major representative peaks of CM were identified according to peak area, retention time and spectra (Fig. 1).

**Growth inhibitory effects of CM, CH, CE on Hep2 cells by MTT assay**—MTT assay was used to determine the viability of Hep2 cells exposed to CM, CH, CE and CW. CM, CH and CE caused cell death in a concentration and time dependent manner (Figs 2 and 3). CE (10 µg/ml) showed the strongest growth inhibitory effect (96.3%) on Hep2 at 48 hr following treatment, whereas in comparison, CM and CH demonstrated milder cytotoxic effect of 72.7 and 60.5 %, respectively. In contrast, CW did not show cytotoxic effect at any given time or concentration. The IC\(_{50}\) of CM, CH and CE was calculated as 10 µg/ml.

**Apoptotic death of Hep2 cells induced by CM, CH, CE: Morphological and Flow cytometry evidence**—C.procera root extract-induced changes in cellular morphology were documented by means of photomicroscopy (Nikon, Japan). Microscopically, the normal Hep2 cells appeared healthy, polygonal in shape and attached to the well plate. 24 hr after the addition of CM, CH and CE, but not CW, noticeable changes in the morphology and density of Hep2 cells were observed. Morphological assessment of treated Hep2 cells clearly indicated the play of apoptotic mechanisms leading to cell death. Morphological changes including cell shrinkage and loss of colony formation ability were observed. The treated cells appeared rounded off, shrunken and detached. 48 hr after treatment, extensive blebbing, presence of apoptotic bodies, dense cytoplasm, chromatin aggregates could also be seen in these cells (Fig. 4).

Data from flow cytometry revealed that Cisplatin treatment affected over 60% of the cell population apoptotic whereas in contrast, only 7% population was apoptotic in normal control wells. The normal, cisplatin and extract treated cell population was
calculated to be in diploid state with no indication of polyploidy. The percent apoptosis, and DNA index (DI) of G1 cells in control and treated cells is depicted in Table 1.

Cell cycle disruption of Hep2 cells—2-D histogram of normal control cells showed two peaks with a plateau in between corresponding to cells in G1, G2/M and S phase of the cell cycle, respectively. Cisplatin (10 μg/ml) treatment blocked the cell cycle at S phase and the cell population appeared to be predominantly distributed in G1 and SPF with only 1% in G2/M phase. Similarly, treatment with CM, CH and CE (10 μg/ml) increased the sub G0/G1 population, implying an increase in apoptotic cell population. Results of cell cycle distribution established that Hep2 cells were arrested at S phase and thus prevented from entering G2/M phase (Table 1). Consequently the cell population in G2/M phase declined, whereas a concomitant rise in G0/G1 was recorded. A typical 2-D histogram following treatment with CM is depicted (Fig. 5).

Discussion

The limitations of the available cancer management modalities create an urgent need to screen and generate novel molecules. Despite, well-documented illustrations of phytochemicals being used for prevention and treatment of cancer, their importance in modern medicine remains underestimated. Plants are storehouse of “pre-synthesised” molecules that act as lead structures, which can be optimized for new drug development. In practice, a large number of cancer chemotherapeutic agents that are currently available in the market can be traced back to their plant source

Traditionally, *C. procera* has been identified as a very potent and poisonous plant. Several reports associate it with intoxication and toxicities. In
Ayurveda the plant is attributed with important therapeutic properties, such as abortifacient and anti-cancer, amongst others. Although the latex of *C. procera* has been extensively investigated for its medicinal properties, there are very few studies on the potential use of the roots of this plant.

Preliminary reports have attributed the roots of *C. procera* with *in vitro* antitumor, cytotoxic, anti-inflammatory, analgesic and antifertility activities. The anti-cancer activity has been evidenced against a panel of cell lines including human epidermal carcinoma of the nasopharynx, COLO 320 and HeLa. Quaquebeke and co-workers have reported the presence of the cardenolide (2''-oxovoruscharin) in the roots of *C. procera*. The compounds from the root of *C. procera* hold a great potential as future cancer chemotherapeutic agents and are currently being hailed as one of the most potent drugs. Against a panel of 57 human cancer cell lines, hemisynthesised congener of this cardenolide demonstrated a potent *in vitro* antitumor activity which was similar to taxol but higher than SN-38. However, there is a paucity of studies that elaborate the mechanism underlying the activity of the roots of *C. procera*.

In the present study, fresh extracts of the roots of *C. procera* were prepared and investigated for their anti-proliferative activity. The study confirms the *in vitro* anti-proliferative property of roots of *C. procera* against human Hep2 cancer cell line. A comparison of the anti-proliferative activity of the extracts reveals that CE was the most effective in inducing cytotoxicity. Morphologic and flow cytometric analysis of the cells, indicated that the treatment had initiated apoptotic mechanisms to trigger cell death. Flow cytometry revealed that at 10 μg/ml over 50% of the cell population had been rendered apoptotic by CE. Further, studies are being conducted in our laboratory to purify and characterize the CE.

For the first time this study demonstrates that cell cycle disruption is the basis of anti-proliferative action of the roots of *C. procera*. The inhibitory concentrations of extracts acted by specifically blocking the cell cycle at S-phase and prevented the cells from entering the proliferative phase. Lack of evidence for polyploidization further confirms that initiation of DNA synthesis in Hep2 cells had been inhibited following *C. procera* treatment. Cell cycle regulation and its modulation by various plant-derived agents are gaining widespread attention in recent years. A large number of phytochemicals have been shown to inhibit cell cycle progression of various cancer cells. Especially, S phase transition provides an effective checkpoint in cell cycle progression that can severely affect the proliferation the cell population.

To conclude, the present study suggests the mechanism by which the root bark of *C. procera* exerts anti-proliferative action. Taken together, a putative explanation is provided in terms of pro-apoptotic and cell cycle disruption. However, further studies are required to confirm the proposed mechanism of anti-proliferative action.

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