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ANTIPROLIFERATIVE AND APOPTOSIS INDUCING ACTIVITIES OF LEAF ORGANIC SOLVENT EXTRACT FRACTIONS OF *CLERODENDRUM VISCOSUM* VENT.

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ABSTRACT

The genus *Clerodendrum* is used for therapeutic activities like anticancer, antimicrobial, hypoglycaemic, immunomodulatory and antioxidant activities. *Clerodendrum viscosum* Vent., a perennial shrub, (Family: Lamiaceae, Common name: Ghetu), is being commonly used in traditional systems of healthcare. Anti-tumor efficacy of this plant has become reputed particularly with respect to benign tumors and also for the treatment of cancer. The aim of the present study was to evaluate the antiproliferative and apoptosis inducing potentials of the leaf extract fractions of *Clerodendrum viscosum*. *In vitro,* antiproliferative and cytotoxic effects were determined by MTT assay and apoptotic cell death inducing effect was analyzed by staining with ethidium bromide and acridine orange under the fluorescence microscope after treatment with the organic solvent extract fractions of *C.viscosum* leaf on Dalton's lymphoma (DL) cells. MTT test results indicate dose-dependent increase in antiproliferative and cytotoxic effects of leaf organic solvent extracts of *C.viscosum* on DL cells. Similarly fluorescence microscopic analysis showed a significant increase in apoptotic cell death in a dose-dependent manner where methanolic extract fraction seemed to be the most effective fraction for apoptosis induction in DL cells. GC-MS data indicate methanolic fraction of *C.viscosum* leaf contains fatty acids and fatty acid esters as major bioactive compounds. In conclusion, this study explored antiproliferative and apoptosis-inducing effects of organic solvent extracts of *C.viscosum* and thus it validates the traditional use of this plant in tumor and cancer treatment.

KEYWORDS: *MTT test, Fluorescence microscopy, GC-MS analysis.*

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INTRODUCTION

Cancer is an important global health problem and it is considered as the second major cause of death in the human after cardiovascular disease both in the developed and developing countries.[1-2] To treat cancer recently greater emphasis has been given towards the complementary and alternative anticancer formulations from traditional medicines as chemotherapy and radiotherapy cause long-term toxicity to normal dividing cells.[3-4] Therefore, the search of new chemotherapeutic agents from natural products of plant origin has gained a renewed interest. Populations who use a high level of natural products have a reduced incidence of gastric cancer and the low incidence of colon cancer in Asian countries may be due to high consumption of soya bean products.[5-7]Soybeans are rich in saponins, which have been suggested as probable anti-carcinogens.[8] The active principle Curcumin, a yellow colored product of roots of *Curcuma longa*, is a phenolic compound possessing anticancer, antioxidant and anti-inflammatory activity.[9-10] Vinca alkaloids, vincristine, vinblastine and vinorelbine of *Catharanthus roseus* are proved to be effective for the treatment of Hodgkin's disease, Kaposi's sarcoma and breast, lung, germ cell and testicular cancer. Similarly, Taxol of *Taxus brevifolia* and Camptothecins (topotecan, irinotecan and etoposide) of *Camptotheca acuminate* are the leading agents to combat ovarian, breast, head, neck and lung cancer.[3] There is a renewed interest and urgency in isolating anticancer agents from natural sources, because of the many past successes in this endeavor.[11-12] Thus, large-scale testing of wide variety of natural products of plant origin is necessary to develop herbal anticancer drugs.[13] The genus *Clerodendrum* of Lamiaceae family (2n=24 to 2n=184) has been cited in many indigenous systems of health care for treatment of various diseases from ages. *Clerodendrum viscosum* Vent. (Ghetu, Bhat), small perennial shrub, is an important medicinal plant in Indian traditional practices.[14-15] It is used to prescribe for the treatment of worm infections, stomach pain, malarial fever and all types of skin diseases.[16] Crude leaf extract of *C. viscosum* is being used alone or in combination with the other vegetables for the treatment of diabetes, high blood pressure, asthma, stomach troubles and headache.[17] Leaf ethanolic extract of *C. viscosum* has shown significant anti-microbial activity and that was shown to resemble with the standard tetracycline type drugs.[18] Methanolic extract of *C. viscosum* leaves has shown to reduce necrosis in damaged liver indicating moderate hepatoprotective activity. Moreover, it also showed anti-inflammatory, antidiabetes, antinociceptive and neuropharmacological activities.[15,19-20]The antioxidant and protective effects against CCl_4 induced oxidative stress in rats have been found to be significantly high and apparently, it has reduced duration of seizures and has provided protection, in a dose-dependent manner, against leptazol-induced convulsions.[21-22] In some tribal communities, for the treatment of cancer, leaf juice of *C. viscosum* has been prescribed.[23] Anti-tumor efficacy of this plant has become reputed particularly with respect to benign tumors.[16] Cell cycle progression machinery may be affected by some novel anticancer plant extracts and antineoplastic agents.[24] In our earlier study, we have reported antiproliferative and apoptosis-inducing, cell cycle delay and metaphase arresting and mitotic abnormalities inducing activities of leaf aqueous extract of *Clerodendrum viscosum* (LAECV) in mouse bone marrow and root apical meristems of onion and wheat, by scoring metaphase frequency, mitotic index, transition of cells during cell cycle and mitotic abnormalities.[25-26] In onion and wheat root apical meristem cells mitotic index decreased, metaphase frequency increased and transition from metaphase to anaphase decreased. Experiment with mouse bone marrow cells indicated LAECV induced metaphase arrest (164.3% increased metaphase frequency with 300mg/kg body weight for 2.5h). Phytochemicals like glycosides, carbohydrates, tannins, saponins, and terpenoids were detected from LAECV and we concluded LAECV contains metaphase arresting active principles.[25]Tannins are polyphenolic compounds having anti-initiating, anti-promoting and cancer chemopreventive activity.[27] Reports are also available on the anticancer activity of the methanolic extract of *C. infortunatum* in Ehrlich's ascites carcinoma (EAC) in mice and it was also attributed to bioactive terpenoid, clerodinin A, and oleanolic acid.[28] However, detailed antitumor and anticancer potentials of the different extract fractions were not well studied. The aim of the present study was to evaluate antiproliferative and apoptosis potentials of the organic solvent extracts and to determine the active components. In the present study, chloroform, ethyl acetate, and methanolic extract fractions were tested on Dalton's Lymphoma (DL) cells for MTT and fluorescence microscopic analysis. GC-MS analysis was performed for phyto-chemical investigation of the different extract fractions.

MATERIALS AND METHODS

Chemicals

MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5 diphenyl-tetrazolium bromide dye),antibiotic solution 50X liquid, Fetal Bovine Serum (FBS) and Roswell Park Memorial Institute 1640(RPMI-1640) culture medium were obtained from Himedia, India. DMSO was obtained from Thermo Fisher Scientific Pvt Ltd., Mumbai. Ethidium bromide was obtained from Sigma, St Louis, MO, USA. Streptomycin and acridine orange were purchased from S.D.Fine-Chem. Limited, Mumbai, India. Proteinase K and RNAse were purchased from Bioline and Genie, Merck, India respectively. Other chemicals used in this study were of analytical grade from reputed manufacturers.

Plant leaf collection and authentication

Disease-free healthy fresh tender leaves of naturally grown *Clerodendrum viscosum* were harvested in the month of May-June, 2012 from in and around Golapbag campus of The University of Burdwan, West Bengal, India. Initially, during collection time, the plant species was identified by local people and later it was identified and authenticated by renowned senior plant taxonomist Prof. Ambarish Mukherjee, Department of Botany, The University of Burdwan, West Bengal, India as *Clerodendrum viscosum* Vent. that belongs to the family- Lamiaceae. A voucher specimen of *C.viscosum*

was retained at the Department of Zoology, Burdwan University having herbarium code No.BUTBSR011 for future reference.

Preparation of leaf extracts

Collected leaves of *C.viscosum* were washed thoroughly in running tap water, shed dried and then pulverized in a motor grinder (Philips Mixer Grinder HL 1605).With soxhlet apparatus 50 g dried ground leaf powder was extracted for 16h at boiling temperature successively with petroleum ether, chloroform, ethyl acetate and methanol and the extract fractions were coded as PECV (Petroleum ether extract of *Clerodendrum viscosum*) , CHCV(Chloroform extract of *C. viscosum*), EACV(Ethylacetate extract of *C. viscosum*) and MECV(Methanol extract of *C viscosum*) respectively. Extract fractions were filtered through Whatman filter paper (No.1) and were condensed using rotary evaporator and kept in hot air oven at 40^{o}C until complete dryness. The dried extracts were kept in airtight screw cap tubes and stored in a refrigerator at 4^{0}C for further use.

Dalton's lymphoma cell maintenance and culture

Dalton's lymphoma (DL), a T-cell lymphoma of spontaneous origin, is widely used as a tumor model. In 1947, the tumor was first developed in the thymus gland of a DBA/2 mouse at the National Cancer Institute Bethesda, USA. From that time DL cells are maintained by serial murine transplantations.[29] Healthy female adult albino mice aged 2-3 months and weighing 20-25 g were used for the study. The animals were housed in Tarsons animal cage in a controlled environment (temperature 25±2^{0}C and 12h light: 2h dark) with standard laboratory diet and water *ad libitum*. Rules of the International Animal care and use committee were strictly followed during the whole experiment and steps were taken to protect their welfare. DL cells were collected from DL bearing mice and cultured *in vitro* RPMI-1640 medium supplemented with 10% FBS and antibiotic solution for MTT, and fluorescence microscopic analysis of the treated and untreated samples.

MTT assay

Colorimetric MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide dye) assay reflects the number of viable cells present under defined conditions by assessing cellular metabolic activity. Antiproliferative and cytotoxicity evaluation were made by MTT assay in Dalton's lymphoma cells after treatment with the CHCV, EACV and MECV extract fractions of *C. viscosum* leaf. To make stock solutions, the extract fractions were first dissolved in dimethyl sulphoxide (DMSO) and then by using culture medium, it was diluted to yield extract solution with a final 0.1% DMSO concentration. In the preliminary experiments, it had been shown that 0.1% DMSO did not affect cell viability. DL cells were collected from DL bearing mice after 14 days of implantations. Cells were seeded onto 96-well microtiter tissue culture plates at 5 x 10^{3} cells per well and incubated for 24 h at 37 °C in humidified 5% CO_2 and 95% air atmosphere. The medium was then replaced with fresh medium containing different concentrations (100-1000µg/ml) of extracts or the vehicle. Then cells were further incubated for another 3 h at 37˚C after adding with 10µl MTT (5mg/ml in PBS) and were allowed for intracellular reduction of the soluble yellow MTT to insoluble purple formazan crystals.890 µl 100% DMSO was needed for incubation at 37˚C.[30] Then solution's absorbance was measured at 570 nm using a UV-VIS Spectrophotometer (UV-1800 Series, Shimadzu, Japan). Half maximum inhibitory concentration (IC_{50}) of the extract fractions of *C.viscosum* for cell viability and cytotoxicity were calculated.[31]

Fluorescence microscopic analysis of cellular and nuclear morphology alteration and apoptosis using acridine orange (AO) and ethidium bromide (EB) stains

DL cells were collected from DL bearing mouse and washed with PBS.DL cells (1 × 10^{6} cells/ml) were treated with 100-600 μg/ml of the *C.viscosum* leaf CHCV, EACV and MECV extracts in 0.1% DMSO solution for 24 h at 37^{0}C in 5% CO_2.The cells were then fixed with absolute ethanol at −20^{o}C for 15 minutes. After fixation, the cells were washed with 1xPBS and were stained with 20 μl ethidium bromide (100 μg/ml) and 20 μl acridine orange (100 μg/ml) in a ratio of 1:1.The cells were washed again and 10 μl cell suspensions were taken on a slide. Fluorescent images were scanned using fluorescence microscope (Nikon E800, Japan) and the images were captured by a digital camera. Acridine orange permeates all the cells and makes the nuclei appear green. Ethidium bromide is only taken up by dead cells when cytoplasmic membrane integrity is lost and the nucleus stains yellowish orange. Therefore, live cells have a normal green nucleus; early apoptotic cells show bright green/yellowish nucleus with condensed or fragmented chromatin; late apoptotic cells display condensed and fragmented orange/red chromatin while the cells that have died from direct necrosis have a structurally normal deep orange nucleus.[32] Quantitative analysis of apoptotic cell death was determined by calculating the apoptotic index (AI). At least 1000 cells were scored under fluorescence microscope for each experiment. Apoptotic Index or Percentage of apoptotic cells = (Total number of apoptotic cells/Total number of cells counted) ×100.

RESULTS

MTT assay

Effects of CHCV, EACV and MECV on Dalton's Lymphoma (DL) cells' proliferation and viability

DL cells were exposed with non polar phytofractions of *C.viscosum* (CHCV, EACV and MECV ranging 100-1000µg/ml) for 24 h and subsequently DL cells were exposed with MTT solution for 3 h and then IC_{50} values were calculated. All the extract fractions show dose dependent decrease in OD value.The half maximum inhibitory concentration, IC_{50} values were determined as 0.750, 0.600 and 0.575 mg/ml for CHCV, EACV and MECV respectively at 24 h (Figure 1.).

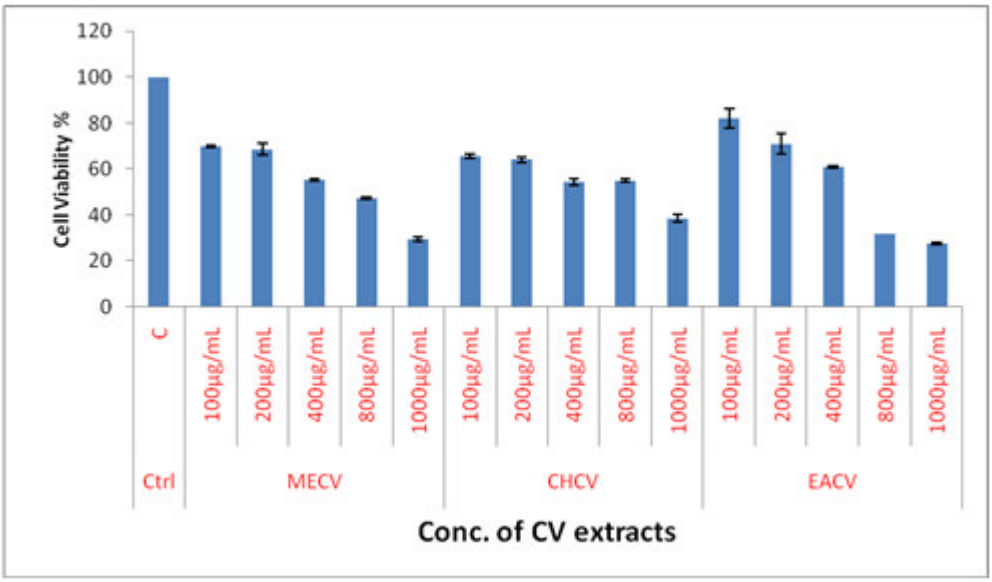

Figure 1
Showing effects of CHCV, EACV and MECV on Dalton's Lymphoma(DL) cells' proliferation and viability with MTT assay.

Fluorescence microscopic analysis of cellular and nuclear morphology alteration and apoptosis

DL cells' cellular and nuclear morphology were studied under a fluorescence microscope after staining with AO-EB. *In vitro* treatment with CHCV (200-600µg/ml), EACV (100-600µg/ml) and MECV (500-600µg/ml) for 24 h to DL cells could induce cellular and nuclear morphological alterations. A cell with green nucleus, yellowish-orange and red represents respectively live, early apoptotic and late apoptotic cells. As the DL cells were allowed to grow only for 24 h after treatment and before fluorescence microscopic analysis for cellular and nuclear morphological alterations, there were mostly the early apoptotic or intermediate cellular and nuclear morphological alterations towards the apoptotic cell death like cellular blebbing, nuclear condensation and fragmentation. Induced nuclear condensation (86.84%) and fragmentation (63.15%) data indicate EACV as the most effective and followed by CHCV and MECV. Data indicate the maximum cellular blebbing (17.56%) induced in MECV treatment and that was followed by EACV (15.78%) and CHCV (6.00%) treatment at the highest used concentration of 600µg/ml, whereas, apoptosis (31.08%) induced in MECV treatment and that was followed by CHCV (12.00%) and EACV (5.26%) (Table 1.).

Table 1
Effects of different extract fractions of C.viscosum on Dalton's lymphoma cell death and nuclear morphology

Extracts - [Concentrations (µg/ml)]	TC	CNC (%)	CNF (%)	CBL (%)	APC (%)
UNTREATED-[0]	1976	00	00	00	08 (0.40)
CHCV -[200]	1280	648^{c} (50.63)	320^{c} (25.00)	00	16^{a} (1.25)
-[400]	1040	864^{c} (83.07)	560^{c} (53.84)	40^{c} (3.84)	16^{b} (1.53)
-[600]	1000	780^{c} (78.00)	600^{c} (60.00)	60^{c} (6.00)	120^{c} (12.0)
EACV -[100]	1600	1120^{c} (70.00)	640^{c} (40.00)	40^{c} (2.50)	**12 (0.70)**
-[200]	1800	1480^{c} (82.22)	800^{c} (44.44)	80^{c} (4.44)	40^{c} (2.22)
-[400]	1680	1280^{c} (76.19)	640^{c} (38.09)	240^{c} (14.28)	80^{c} (4.76)
-[600]	1824	**1584^{c} (86.84)**	**1152^{c} (63.15)**	288^{c} (15.78)	96^{c} (5.26)
MECV-[500]	1008	656^{c} (65.07)	576^{c} (57.14)	168^{c} (16.66)	**80 (7.80)**
-[600]	1184	624^{c} (52.70)	520^{c} (43.91)	**208^{c} (17.56)**	368^{c} (31.08)

Significant at $^{b}p< 0.01$ and $^{c}p < 0.001$ 2 x 2 contingency $\chi 2$ analysis compared to respective control. (TC, Total Cells; APC, Apoptotic cell; CNC, Cells with nuclear condensation; CBL, Cells with blebbing; CNF, Cells with nuclear fragmentation).

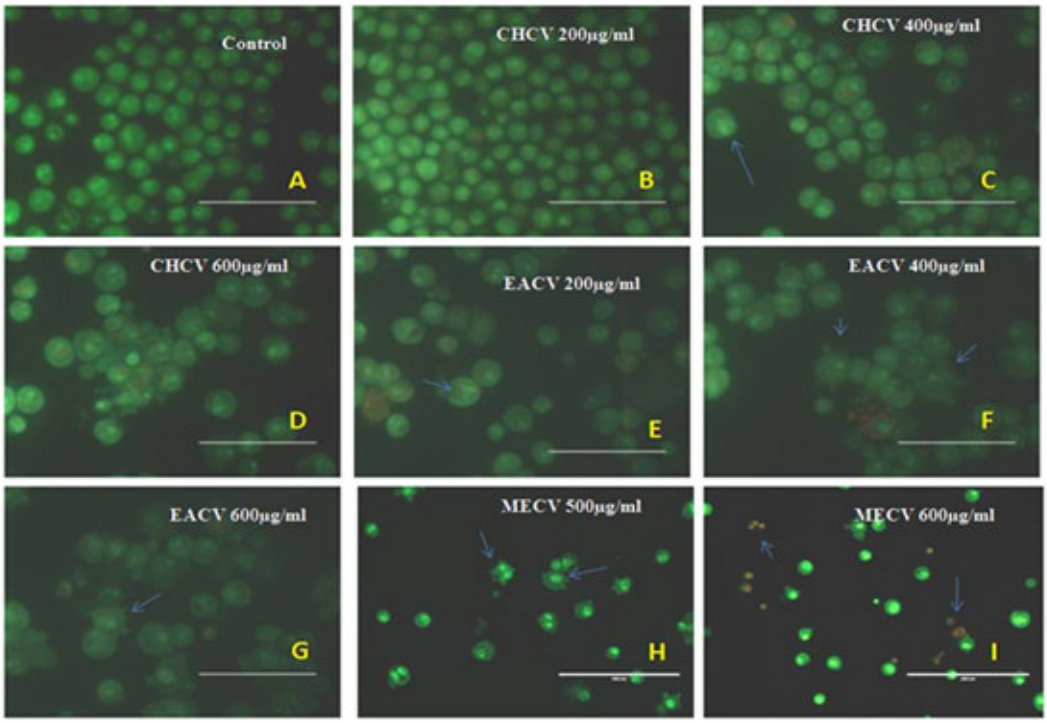

Figure 2

Fluorescence microscopic photographs (A) showing ethidium bromide and acridine orange stained untreated control Dalton's lymphoma cells, (B, C & D) CHCV, (E, F & G) EACV and (H & I) MECV treated cells where arrows indicate distinct apoptotic cellular and nuclear morphology, 400X.

GC-MS analysis

Gas chromatogram (Figure3.) of chloroform extract fraction contained seven (7) compounds with retention times 6.52, 6.78, 6.96, 7.27, 7.33, 10.04 and 10.02 minutes respectively. A peak with retention time 6.96 is dibutyl phthalate in accordance to mass spectral data analysis. Here, the most abundant compound is with 10.04 minutes retention time and mass spectral analysis revealed that the compound is a clerodane diterpenoid, Clerodin with molecular weight 434.256. Gas chromatogram (Figure4.) of ethyl acetate extract fraction showed sixteen (16) peaks at 5.42, 6.51, 6.58, 6.64, 6.77, 6.87, 6.95, 7.31, 7.37, 7.39, 7.43, 7.45, 7.50, 10.04,10.20 and 10.60 minutes where peaks with retention times 6.95 and 7.43 minutes are dibutyl phthalate and stearic acid ester. But mass spectral analysis clearly depicted that the most abundant compound having retention time 6.87 is the palmitic acid ester. The Gas chromatogram (Figure5.) of` the methanolic extract fraction indicates total fifteen (15) peaks at retention time 5.69, 6.26, 6.37, 6.50, 6.58, 6.76, 6.84, 6.87, 6.92, 7.32, 7.37, 7.43, 8.12, 10.05 and 10.59 minutes while the most abundant peak is with 6.87 minutes (methyl palmitate) and the second rise peak is at 7.43 minutes and mass spectral analysis it was identified as stearic acid and all the other peaks were of different fatty acids and conjugated fatty acids of C14 to C18.

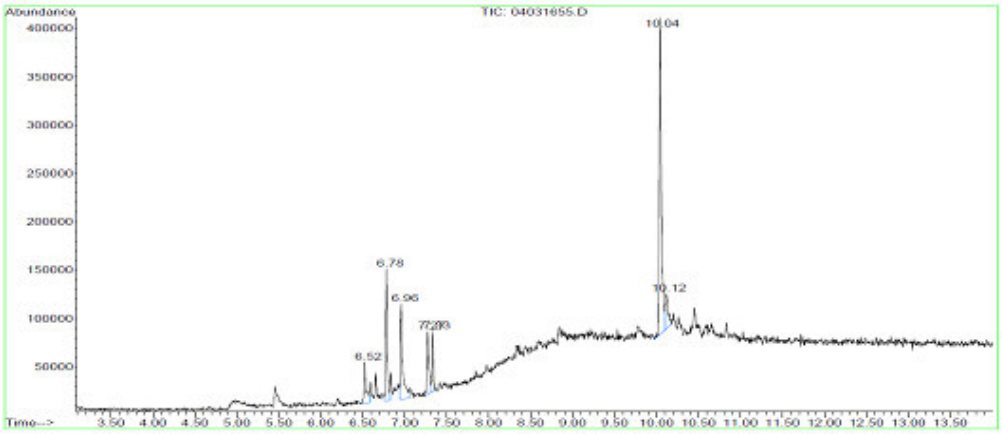

Figure 3

Gas chromatogram of chloroform extract fraction after successive extraction with petroleum ether solvent of C. viscosum leaf powder.

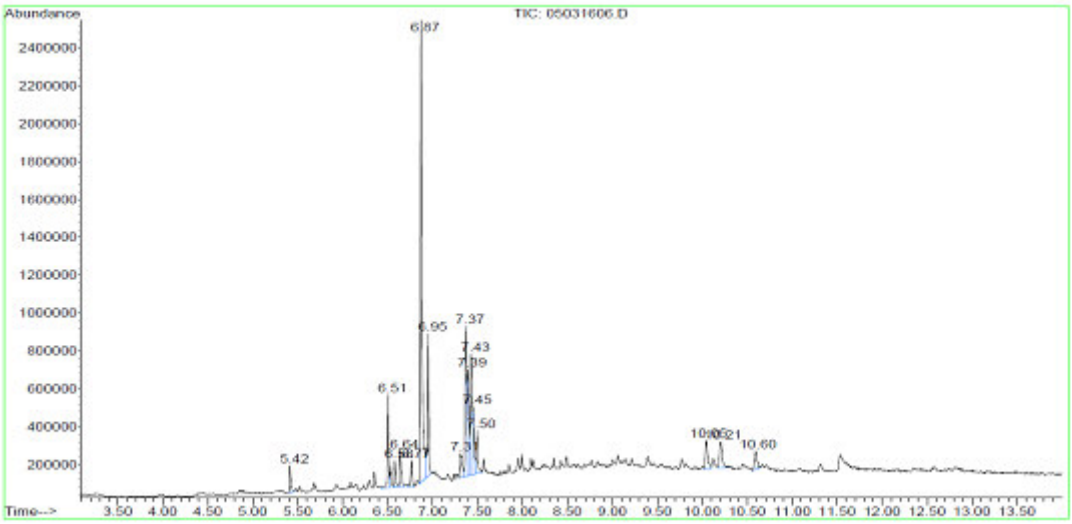

Figure 4
Gas chromatogram of ethyl acetate extract fraction after successive extraction with petroleum and chloroform solvents of C. viscosum leaf powder.

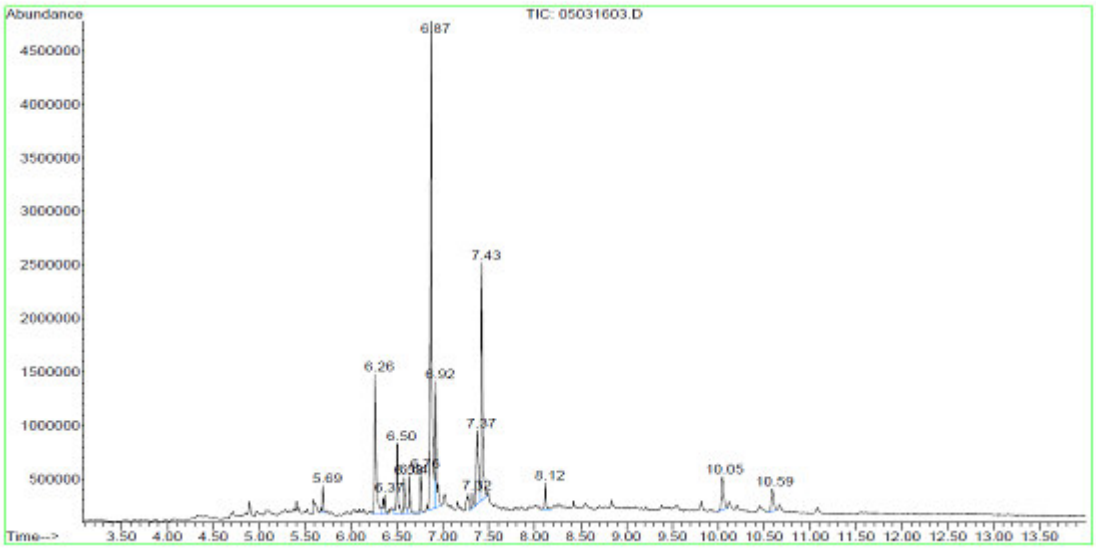

Figure 5
Gas chromatogram of methanol extract fraction after successive extraction with petroleum ether, chloroform and ethyl acetate solvents of C. viscosum leaf powder.

DISCUSSION

Antiproliferative and apoptosis inducing activities of leaf organic solvent extract fractions (CHCV, EACV and MECV) of *C.viscosum* were studied on DL cells respectively by MTT assay and fluorescence microscopic detection.The dose-dependent decrease in OD indicates antiproliferative and cytotoxic actions of extract fractions.Insoluble purple formazan crystal formation 50% inhibition (IC_{50}) values were determined as 0.750, 0.600 and 0.575 mg/ ml respectively for CHCV, EACV and MECV extract fractions at 24 h after treatment. Differences in IC_{50} values are may be due to the differential sensibility of the cell line and also may be due to differences in the phytochemical composition of the extracts. Earlier in cytotoxicity study, IC_{50} value of the methanolic extract of *C. infortunatum* (MECI) was recorded as 0.498mg/ml.[28] A study with *Allium cepa* root apical meristem and mouse bone marrow cells indicate LAECV treatment could induce MI% reduction and increase in micronuclei and were interpreted as a direct toxic effect of LAECV and correlated with its antiproliferative and apoptosis-inducing actions .[25,33] There are recent reports on anticancer activity of the active fractions of *Dillenia suffruticosa* that induce cell cycle arrest at G_2/M phase and apoptosis in MCF-7 cells.[34] The ethanol extract of *D. formosum* induce cell cycle arrest at G_2/M phase at concentrations 250µg/ml and 350µg/ml.[35] To observe fluorescence microscopic apoptotic cell nuclear morphology, DL cells were treated with the extracts and stained with acridine orange-ethidium bromide (AO-EB). In untreated samples, the majority of cells (99.6%) were found live, showing intact nucleus with green fluorescence of acridine orange.

Data indicate DL cells that were treated with the CHCV, EACV and MECV extract fractions could induce apoptotic cellular morphology (Table1. & Figure2.) like cellular shrinkage and membrane blebbing with corresponding changes from green to yellow to reddish, early and late apoptotic cells with condensed and fragmented chromatin respectively were found.[36] Earlier we have reported that LAECV could induce dose-dependent increased apoptotic cell death frequency in *Lemna* fronds and wheat root tip cells. Apoptosis-inducing activity of LAECV was further analyzed with DNA fragmentation assay that showed a typical apoptotic DNA ladder in wheat root tip and mouse bone marrow cells.[33] The apoptotic cell death inducing activity of CHCV, EACV and MECV extract fractions appears to explain the anticancer effects of their bioactive components like phenols, glycosides, terpenoids, fatty acids and saponins. The medicinal properties of plants have been claimed to lie in their phytochemical ingredients.[37] Plants have served as a good source of antitumor agents. Others and our previous study indicated that the presence of bioactive compounds like carbohydrate, glycosides, saponins, tannins and terpenoids in the LAECV.[33,38] Results of the present study indicate leaf chloroform, ethyl acetate, and methanol extracts have variation in chemical composition though fatty acid, dibutyl phthalate and terpenoids are common in all the extract fractions. Terpenoids have been shown to suppress the growth of a variety of cancer cell. Terpenoids are considered as plant antioxidant and the promising anticancer agents.[39-40]Terpenoids obtained from the plant and green algae exhibit potent anticancer activity against classical and a typical multidrug resistant cancer cells.[41] They inhibit carcinogenesis and induce tumor cell apoptosis.[42-43] MECV extract fraction could induce ideal apoptotic fluorescent microscopic nuclear alterations in DL cells and the major quantity of the phytochemicals were identified by GC-MS, indicate the presence of palmitic and stearic acids. Long-time fat rich diet ingestion has a strong relation in opposition to different types of cancer in human.[44-48] Fatty acids not only have inhibitory potency against tumor and metastasis on animals and cultured cells but also have anticancer property against breast cancer cell line.[49] Dietary fatty acids show a vital role against breast cancer cells during *in vivo* study.[50-51] A high concentration of stearic acid (18:0) in different dietary substances has antiproliferative effects on human breast cancer cells *in vitro* and breast tumor cells *in vivo*.[52-56] Dietary stearic acid has an aptitude to induce breast cancer cells apoptosis and cell cycle arrest.[57-58] On carcinogenesis models (A/ST mice) stearic acid reduces the configuration of tumor against mammary adenocarcinoma cells.[59] An epidemiological study reveals that breast cancer prevention and treatment by stearic acid shows successful results.[60] Cancer treatment potency can be enhanced by the additive action of the dietary substances and drugs.[61-62] Drug amalgamation along with dietary substances (lipophilic carrier) promotes cellular uptake by crossing plasma membrane to affect intracellular targets.[63-64] Fatty acid esters have therapeutic targets owing to their lipophilic nature, specificity, biopotency and less toxicity.[65]Anticancer effects of leaf methanolic extract of *C. infortunatum* (MECI) against EAC cells may be due to the presence of oleanolic acid and clerodinin A and their anticancer effects were may be due to the suppression of lipid peroxidation and increase in the content of the enzymatic defense system.[28] A large number of plants possessing anticancer properties have been documented. Many plants and their active principles, vinblastine, taxol, vincristine, the camptothecin derivatives, irinotecan and topotecan and etoposide derived from epipodophyllotoxin, act as antitumor and apoptotic inducer in cancer cells.[66-67] Paclitaxel, the most successful and efficient anticancer chemotherapeutic drug, is also an example of a diterpenoid type secondary metabolite that stabilizes microtubules and inhibits cell proliferation. The present study involving *in vitro* antitumor and anticancer assessment in Dalton's lymphoma cells clearly demonstrate potent antiproliferative and apoptosis-inducing activities of the successive extract fractions of *Clerodendrum viscosum* leaves where the methanolic extract fraction seems to be the most potent and its activities ate may be due to the fatty acids and their esters.

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CONFLICT OF INTEREST

Conflicts of interest declared none.

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