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Methanolic extract of *Phlogacanthus thyrsiflorus* Nees leaf induces apoptosis in cancer cells

Khangembam Victoria Chanu[1,3]*, Leishangthem Geeta Devi[2], Sandeep Kumar Srivastava[2], Meena Kataria[1], Dimpal Thakuria[3] & Satish Kumar[4]

[1]Division of Animal Biochemistry, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India
[2]Department of Pathology, All India Institute of Medical Sciences (AIIMS), Ansari Nagar, New Delhi, India
[3]ICAR-Directorate of Coldwater Fisheries Research, Bhimtal, Uttarakhand, India
[4]Emeritus Scientist, Division of Animal Biotechnology, ICAR-Indian Veterinary Research Institute, Bareilly, Uttar Pradesh, India

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Phlogacanthus thyrsiflorus Nees is a medicinal herb commonly used in traditional folk medicine, and it is known to possess anticancer activity. Here, we explored the anticancer properties of methanolic extract of *P. thyrsiflorus* leaves in HeLa and MCF-7 cell lines. We observed nuclear fragmentation as indication of apoptosis in the MPT treated cancer cells using haematoxylin and eosin (H&E) as well as fluorescent dye. DNA from the treated cells showed characteristic laddering of DNA fragments in agarose gel electrophoresis. Cell populations undergoing secondary necrosis following apoptosis could also be detected in FACS by annexin V/propidium iodide (PI) staining. Activated caspase-3 in the treated HeLa cells was detected by polyclonal anti-caspase-3 antibody utilizing immunocytochemistry. Using transmission electron microscopy, sub-cellular changes like rough endoplasmic reticulum, swollen mitochondria, distorted mitochondrial membrane, loss of cristae and matrix were observed in the treated HeLa cells. Extensive plasma membrane blebbing was also observed by scanning electron microscopy. Our findings support that *Phlogacanthus thyrsiflorus* leaves are natural source of potent anticancer agent.

Keywords: Annexin V/PI staining, Anticancer, Caspase-3, Cytotoxicity, DNA fragmentation, Electron microscopy, Nuclear fragmentation, Tumor

Plant derived natural products represent a good source of free radical scavenging molecules like phenolic compounds, flavonoids, steroids, terpenoids and other endogenous metabolites[1-4]. Despite advances in modern science and allopathic medicine, a large section of people in rural and remote areas depend on traditional medicine for their primary health care[5]. The World Health Organization (WHO) through its Traditional Medicine Strategy 2014-2023 support Member States in harnessing the potential contribution of traditional and complementary medicine to health, wellness and people-centred health care to promote its safe and effective use[6]. In North-Eastern states of India, a region rich in plant biodiversity, there are many plants which are used in traditional medicine. For this study, *Phlogacanthus thyrsiflorus*, an evergreen shrub, was chosen. This plant has the advantages of availability throughout the year and preference by the locals for consumption as vegetable and medication in the traditional healing practices. Whole plant is used for treatment of whooping cough and menorrhagia while burnt fruits and leaves are prescribed for fever[7]. The flowers and leaves are consumed as vegetable while the mixture of fruit and leaf ash are used to treat fever by tribal community of Meghalaya[8]. Because of its medicinal value, leaves are used in preparation of health promoting herbal recipe in Assam[9]. Leaves are used for curing skin disorders in Manipur while in Bangladesh; it is used for treatment of gout and rheumatism[10,11]. Leaves of *P. thyrsiflorus* possess analgesic, anti-inflammatory, and antioxidant activities which may be due to the presence of flavonoids, tannins and triterpenes[12]. The leaves are also reported to contain several bioactive compounds such as sitosterol, lupeol, betulin and diterpene lactones[13-15].

Apart from the above medicinal properties, *P. thyrsiflorus* is also known to possess anticancer property. Cancer is one of the major non-communicable diseases reported to be the leading cause of premature death worldwide[16]. It has caused

*Correspondence:
Phone: +91 8463083857.
E-Mail: drvictoriachanu@rediffmail.com

10 million deaths, and expected to affect 28.4 million by 2040[16,17]. In this context, it becomes necessary to study plants having anticancer property. Therefore, in the present study, we evaluated the potential anti-cancer property of *Phlogacanthus thyrsiflorus* leaves extract against cancer cell lines HeLa and MCF-7.

Materials and Methods

Chemicals and labware

All the reagents used in the study as mentioned in the text were of analytical or cell culture grade. The glasswares were procured from Borosil® (India) and the plastic wares including microfuge tubes, microtips were obtained from Tarsons Products Pvt. Ltd. (India). Cell culture plate, tissue culture flask, and cell scraper were from Techno Plastic Products AG (TPP, Switzerland) and 0.2 µm filter was purchased from Nalgene Labware (USA).

Cell lines

Cancer cell lines (viz. HeLa and MCF-7) were the kind gifts from Prof. AK Dinda, Department of Pathology, All India Institute of Medical Sciences, New Delhi, India, which were originally procured from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained at less than 20 passages from the original purchased vial from ATCC.

Preparation of plant extract

Leaves and flowers of *Phlogacanthus thyrsiflorus* Nees were collected from Manipur, India, and deposited at the herbarium of Botany Department, Bareilly College, Bareilly, Uttar Pradesh, India. The plant was identified by Dr. DK Saxena, Department of Botany and the identification number was 2011060359014A.

Leaves were dried under shade and powdered. The powdered leaves were then extracted with methanol in a Soxhlet extraction apparatus (Macro Scientific Works, Delhi) following the protocol of Jadhav *et al.*[18]. Evaporation of the solvent followed by freeze drying (Heto Power Dry LL3000 Freeze Dryer; Thermo Fisher Scientific, India) yielded the crude dry extract. The extract (MPT) was dissolved in 1% dimethyl sulfoxide (DMSO) in Dulbecco's Modified Eagle's medium (DMEM), filtered, and used for further analyses.

Determination of IC_{50} of MPT in HeLa and MCF-7 cells

Exponentially growing HeLa and MCF-7 cells were suspended at a density of 1,00,000 cells/mL in DMEM (Mediatech Inc, VA) and incubated at 37°C for 24 h. The cells were washed with sterile phosphate buffered saline (PBS) and then treated with various concentrations of MPT for 24, 48 and 72 h to observe the viability at different time period. Control cells were treated with 1% DMSO in DMEM (vehicle) alone. Fluracil (Biochem Pharmaceutical Industrial Ltd., Mumbai), a commercially available anticancer drug was used as positive control for cell death. The viability of the cells (both control and treated) was assessed by MTT [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide] tetra-zolium salt assay by measuring the absorbance at 570 nm using ELISA plate reader (BioRad, Model 680, India). Results were expressed as the percent growth inhibition with respect to vehicle treated cells and half maximal inhibitory concentration (IC_{50}) was determined[19]. Further assays were carried out using respective IC_{50} of MPT for the cancer cells.

Cytotoxicity assay of MPT on lymphocytes

Lymphocytes were isolated by density gradient centrifugation from human blood using histopaque (Density 1.077) according to the manufacturer's instructions. The isolated lymphocytes were re-suspended in PBS and counted by the trypan blue exclusion test and then diluted to get 10^6 cells /mL in Roswell Park Memorial Institute (RPMI; Cambrex, Walkersville, USA) growth media containing 10% fetal bovine serum (FBS) and 1% penicillin (10,000 IU) and streptomycin (100 µg/mL) and incubated for 24 h[20]. The cells were washed with sterile PBS and then treated with various concentrations of MPT for 24 h to determine the cytotoxic concentration of MPT to normal cells.

Morphological examination of cancer cells by Haematoxylin and Eosin (H&E) staining

Both the HeLa and MCF-7 cells were seeded at a density of 100000 cells/mL in 6-well plate containing cover slips. The cells were allowed to grow for 24 h and treated with respective IC_{50} of MPT for 24 h. The cover slips containing the cells were then processed for H&E staining[21]. The cover slips were then mounted on a clean glass slide and observed under microscope (Nikon Eclipse E600) fitted with camera (Olympus, DP71).

Fluorescent staining of nuclei

HeLa and MCF-7 cells (1,00,000 cells/mL) were seeded in six-well plates containing cover slips and incubated for 24 h. The cells were washed with sterile PBS and incubated with media containing MPT but

no FBS for 24 h. The media was removed from the well and 1.0 mL of PBS containing 0.2 µg of Hoechst S769121 (Invitrogen Bioservices, Bangalore) was added and kept for 5 min in dark at room temperature (25°C) following similar protocol as reported earlier[22]. The cover slip was then mounted on a clean glass slide with aqueous mountant (Vector Laboratories, Inc. Burlingame, CA) and observed under microscope.

DNA fragmentation analysis by gel electrophoresis

DNA fragmentation was assessed by agarose gel electrophoresis according to Herrmann and co-workers with little modifications[23]. The MPT treated cells (both adherent and non-adherent) were washed with cold PBS and pelleted by centrifugation. Cells were lysed with lysis buffer (1% Triton X-100 in 20 mM EDTA, 50 mM TrisHCl, pH 7.5) and centrifuged at 1,300×g for 20 s. The supernatant was removed, and extraction was repeated. DNA from the supernatants were recovered by adding sodium dodecyl sulfate (SDS) and treated with 5 µg/mL RNase A for 2 h at 56°C followed by proteinase K (2.5 µg/mL) for 2 h at 37ºC. DNA fragments in the solution were precipitated with 0.5 volume of 10 M ammonium acetate and 2.5 volume of cold absolute ethanol. The resulting DNA was pelleted by centrifugation followed by rinsing with cold 75% v/v ethanol and then absolute ethanol. The DNA pellet was dissolved in 30 µL Tris EDTA (TE) buffer. The extracted DNA was electrophoresed in a 1.5% agarose gel containing ethidium bromide and visualized under ultraviolet illumination (Alpha Imager Gel Documentation System, Alpha Inno Tech Corporation, USA).

Assessment of apoptotic and necrotic cells by FACS

HeLa and MCF-7 cells were treated with MPT for 12 h and analysed for apoptotic and necrotic cell populations by fluorescence-activated cell sorting (FACS) Calibur (BD Biosciences, San Jose, CA) using annexin V-FITC/PI staining. Briefly, after incubation with MPT, the cells were harvested, washed with PBS and suspended in 100 µL binding buffer (1X). Cells were incubated with 5 µL annexin V-FITC (Calbiochem, EMD Chemicals Inc., CA) and 5 µL PI (Sigma Chemicals Co., St. Louis, MO) for 15 min at room temperature in darkness and 400 µL binding buffer (1X) was added to each microtubes. The fluorescein isothiocyanate (FITC) and PI fluorescence were measured through FL-1 filter (530 nm) and FL-2 filter (585 nm), respectively, and more than 50,000 events were acquired. Annexin V/PI dot plots were positioned into quadrants and live cells (Annexin V-/PI-), early apoptotic/primary apoptotic cells (Annexin V+/PI-), late apoptotic/secondary apoptotic cells (Annexin V+/PI+), and necrotic cells (Annexin V-/PI+) were distinguished[24].

Immunocytochemistry for detection of activated caspase-3

Activated caspase-3 in the treated HeLa cells undergoing apoptosis was detected by immuno cytochemistry using anti-caspase-3 polyclonal antibody (Cayman Chemical Company, Ann Arbor, MI) as per Duan *et al*.[25] with some modifications. Briefly, the cells were grown on cover slips and treated with MPT for 24 h. The cells were then washed with PBS and fixed by adding 4% paraformaldehyde (PFA) for 15 min. The cells were then washed three times with PBT (0.05% Tween-20 in PBS) and incubated for 30 min with 1% bovine serum albumin (BSA). The cells were again washed with PBT three times and then incubated with 0.2 mL of working concentration of caspase-3 polyclonal antibody (1:1,000) diluted in 0.1% BSA for 60 min at room temperature. The cells were washed again with PBT three times for 5 min each followed by incubation with secondary antibody (PK–8800, Vectastain® Universal Quick Kit, Burlingame, CA) for 40 min at room temperature. After washing with PBT, the cells were incubated with three drops of Streptavidin-horseradish peroxidase (HRP) for 40 min at room temperature followed by washing again. The cells were then incubated with 3,3`-diaminobenzidine (DAB) for 5 min at room temperature and washed. Haematoxylin was used to counterstain the cells for 5 min and washed with PBT and fixed again with 0.5 mL of 4% PFA. The coverslip was mounted on glass slide using aqueous mountant and observed under microscope.

Transmission electron microscopy (TEM)

HeLa cells were fixed in Karnovsky's fixative, pelleted, and processed for TEM[26]. Briefly, cells pellet was post-fixed with 1% osmium tetroxide for 1 h and then processed and embedded with epoxy-resin. Ultrathin sections were cut and collected on the grid and were stained with uranyl acetate and lead citrate. The stained sections were viewed under transmission electron microscope (Morgagni 268D, FEI Electron Optics Co., Eindhoven, The Netherlands).

Scanning electron microscopy

HeLa cells were grown on cover slip and treated with MPT for 24 h. Vehicle (1% DMSO in media) treated cells were kept as control. The cells were then processed for scanning electron microscopy[27,28].

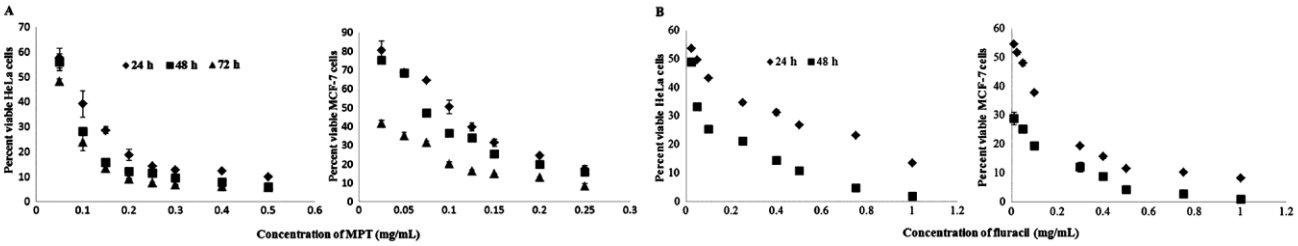

Fig. 1 — Effect of (A) MPT; and (B) Fluracil on viability of HeLa cells and MCF-7 cells at different concentration and treatment period. [Values are presented as mean±SE (standard error), n=3]

Briefly, the cells were washed with PBS to remove biological medium and fixed with 2.5% glutaraldehyde in 0.1 M sodium phosphate buffer for 1 h at room temperature. Cells were washed again to remove glutaryldehyde, and dehydrated gradually by treating with 30, 50, 70, 90 and 100% ethanol for 10 min each followed by critical point drying in hexamethyl-disilazane (HMDS). Gold sputtering of the sample was carried out under vacuum at 12.5 kV and less than 7 mA current for 5 min. The cells were examined under scanning electron microscope (JEOL, Tokyo, Japan).

Results

Growth inhibitory and cytotoxicity assay

The percent cell death in HeLa and MCF-7 cells treated with MPT for 24 h at different concentrations was determined by MTT assay. MPT produce 50% cell death in HeLa and MCF-7 (Fig. 1A) cells at a concentration of 58.97 and 89.18 µg/mL, respectively, at 24 h. The percent cell death induced by MPT also increases with increased incubation period. Fluracil (a known anticancer drug, 5-fluoro-uracil) was utilized as a positive control for cell death assessments. It caused 50% cell death in HeLa and MCF-7 (Fig. 1B) cells at 46 and 25.7 µg/mL, respectively, at 24 h. Percent cell death increased with longer treatment period with fluracil and almost all cells died by 72 h. For further assays, the IC_{50} value of MPT was set and rounded to 60 and 90 µg/mL, respectively, for HeLa and MCF-7 cells. The concentration of MPT required to cause 50% cell death (IC_{50}) of lymphocytes at 24 h was found to be 0.625 mg/mL, much higher than that of HeLa and MCF-7 cells (Fig. 2).

Morphological examination of cells

Morphological changes like nuclear condensation, fragmentation, and emergence of apoptotic elements were observed in the MPT-treated HeLa cells (Fig. 3). In the treated MCF-7 cells, the morphological changes though present were not as prominent as in case of HeLa cells.

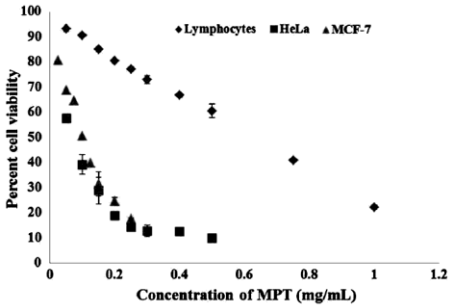

Fig. 2 — Effect of the extract (MPT) on viability of normal lymphocytes, HeLa and MCF-7 cells after 24 h of treatment. [Values are presented as mean±SE (standard error), n=3]

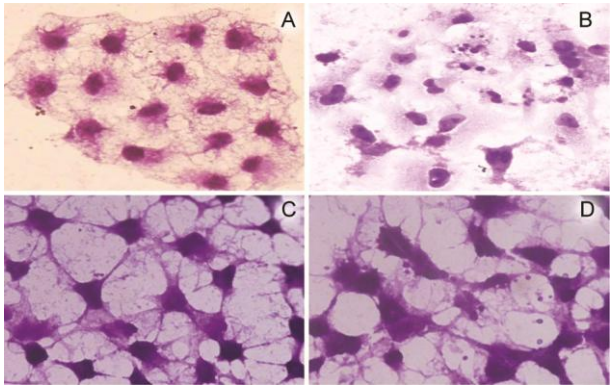

Fig. 3 — Haematoxylin and Eosin staining of HeLa and MCF-7 cells. (A) Control HeLa cells; (B) MPT treated HeLa cells at 24 h; (C) Control MCF-7 cells; and (D) MPT treated MCF-7 cells at 24 h.

Fluorescent staining of nuclei

Fragmented condensed nuclei were clearly visible in the MPT-treated HeLa and MCF-7 cells after staining with Hoechst S769121 (Fig. 4). Control cells showed rounded, intact greenish yellow nucleus whereas many pyknotic and fragmented nuclei were observed in the treated cells.

Analysis of DNA fragmentation by gel electrophoresis

Ladder formation by DNA fragments could not osberved in the DNA isolated from the negative

control cells (Fig. 5). The treated HeLa cells showed DNA fragmentation as laddering in agarose gel electrophoresis. In MCF-7 cells, the laddering was not clear, however, could be observed as smear away from the well.

FACS analysis of apoptosis and necrosis by Annexin V-FITC/PI staining

Utilizing FACS system, the MPT-treated HeLa and MCF-7 cells showed different populations stained with either annexin V-FITC or PI or both (Fig. 6). Control HeLa cells showed 95.1% annexin V-/PI- and 3.74% annexin V+/PI- populations whereas cells treated with MPT for 12 h showed 9.85% annexin V+/PI-, 7.69% annexin V+/PI+, and 7.65% annexin V-/PI+ populations. Control MCF-7 cells showed more than 91% annexin V-/PI- populations along with 2.3% annexin V+/PI-, 1.88% annexin V+/PI+, 4.2% annexin V-/PI+ cells. MPT treated MCF-7 cells showed 74.8% annexin V-/PI-, 3.59% annexin V+/PI, 6.45% annexin V+/PI+, 15.2% annexin V-/PI+ populations.

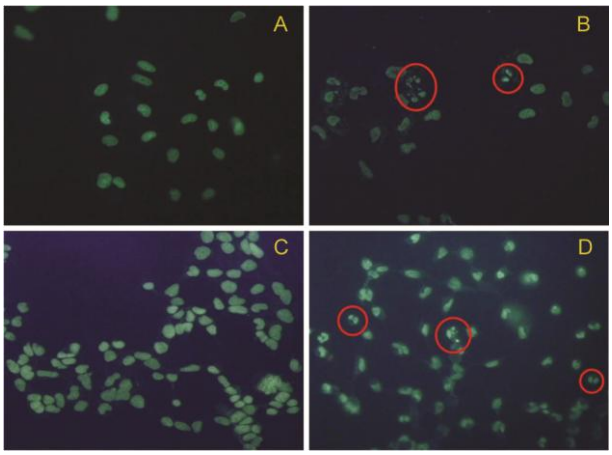

Fig. 4 — Fluorescent staining of nuclei with Hoechst S769121. (A) Control HeLa cells; (B) MPT treated HeLa cells at 24 h; (C) Control MCF-7 cells; and (D) MPT treated MCF-7 cells at 24 h. [Some of the fragmented nucleus in the treated group is encircled red]

Immunocytochemistry for the detection of caspase-3

Caspase-3, a key enzyme of apoptosis, was detected in the treated HeLa cells by immuno cytochemistry using caspase-3 polyclonal antibody. The nucleus of the treated HeLa cells was dark hematoxylin stained showing typical condensed chromatin pulled away from the nuclear envelope (Fig. 7). Some of the dark stained nucleus was showing fragmentation into small nuclear elements.

Transmission electron microscopy

The MPT-treated HeLa cells were degenerated with either the loss of mitochondria or irregular

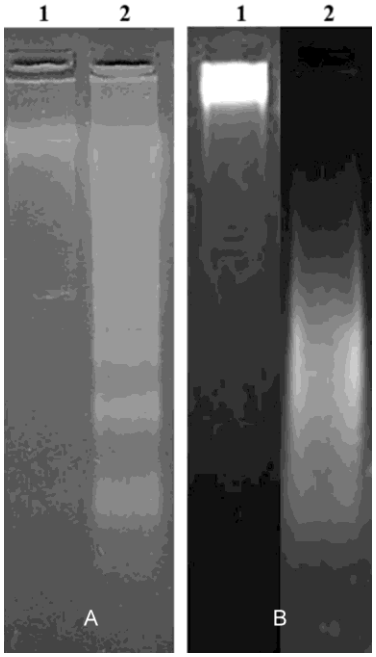

Fig. 5 — DNA fragmentation analysis by agarose gel electrophoresis (A) HeLa cells (B) MCF-7 cells. [Lane 1, control cells; and Lane 2, MPT treated cells]

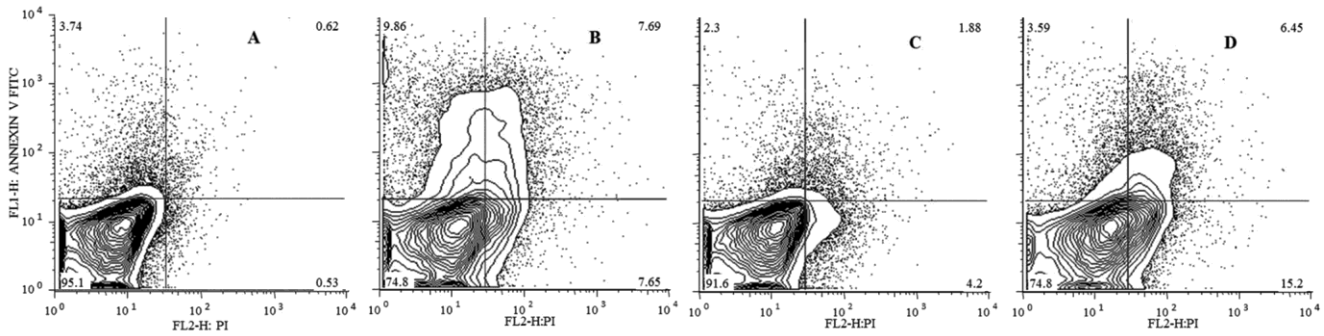

Fig. 6 — FACS analysis of apoptotic and necrotic cell population using Annexin V-FITC and PI. (A) Control HeLa cells; (B) MPT treated HeLa cells; (C) Control MCF-7 cells; and (D) MPT treated MCF-7 cells

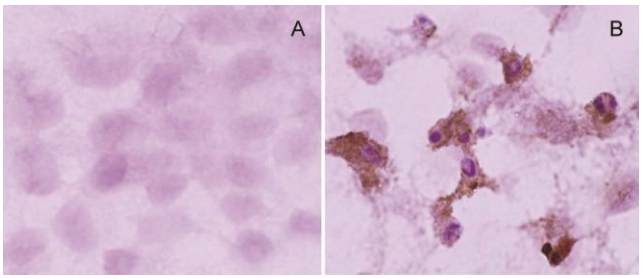

Fig. 7 — Immunocytochemistry using anti caspase-3 polyclonal antibody. [Caspase-3 (bown spots) were detected in the MPT treated cells. Dark haematoxylin stained and fragmented nucleus can be seen in the treated cells]

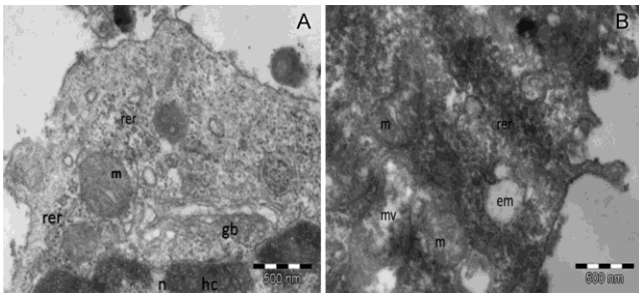

Fig. 8 — Transmission Electron Microscopy of HeLa cells. [Control HeLa cells showed nucleus (n) with prominent electron dense heterochromatin (hc) and round doubled membrane mitochondria (m) with fine cristae and dense matrix, rough endoplasmic reticulum (rer) with attached ribosomes, free ribosomes (r) and golgi bodies (gb) in the cytoplasm. MPT treated cells showed irregular shaped mitochondria (m) with distorted membrane and enlarged or loss of cristae along with loss of matrix and few rough endoplasmic reticulum (rer), empty mitochondria (em) and mitochondria vacuolization (mv)]

swollen mitochondria and few rough endoplasmic reticulum. The mitochondria showed distorted membrane with either distended or loss of cristae leading to the formation of vacuole within the mitochondra and less or no matrix (Fig. 8). Loss of matrix and cristae are indicated by the empty mitochondria. Control HeLa cells showed normal features where the nucleus has electron dense heterochromatin along with normal doubled membrane mitochondria showing fine cristae and dense matrix, rough endoplasmic reticulum and Golgi bodies in the cytoplasm.

Scanning electron microscopy

Control HeLa cells could be seen spread over the cover slip with projections for cross-linking the cells with one another. The MPT-treated HeLa cells showed a rounded structure with numerous blebbing indicating the formation of apoptotic elements (Fig. 9).

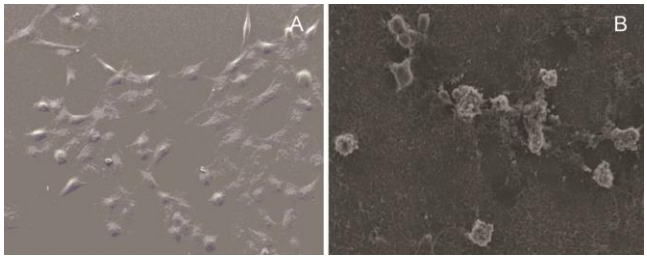

Fig. 9 — Scanning Electron Microscopy of HeLa cells. (A) Control; and (B) MPT treated cells. [Control cells looked more flattened with many projections. Treated cells are rounded with numerous blebs]

Discussion

Cancer is one of the major non-communicable diseases and leading cause of deaths in the world[29,30]. The estimated number of new cases of cancer in 2020 worldwide is 19,292,789 as per the Global cancer statistics[31]. In India, the projected number of patients with cancer for the year 2020 is 1,392,179, and the common 5 leading sites are breast, lung, mouth, cervix uteri, and tongue[32]. Breast and cervical cancer are among the leading causes of cancer related deaths in women worldwide[33,34]. For treatment of cancer, various bioactive compounds of natural origin are being studied to find new drugs with less toxicity[30]. In the present study, methanolic extract of *P. thyrsiflorus* leaves (i.e., MPT) was found to produce 50% cell death in HeLa and MCF-7 cells at a concentration lower than that of the healthy lymphocytes indicating the selective cytotoxic effect of MPT on cancer cells[35]. Selective activity of anticancer agent on cancer cells at concentrations that do not significantly affect non-malignant cells is essential in order to be clinically effective[36]. Lymphocytes were used as healthy cell control because they are relatively easier to isolate from blood and directly related to the immune origin/system and represent a closely relevant model for studying the potential for anti-cancer drug toxicity *in vitro*[37,38]. The effect of MPT against HeLa and MCF-7 cells was found to be comparable to that of fluracil (fluorouracil), one of the most commonly used chemotherapeutic agent for treatment of cancer[39]. Fluorouracil is known to produce antitumor effect by binding to DNA and RNA, thereby blocking DNA synthesis and cell proliferation[31].

Morphological changes in the MPT treated cells for 24 h could also be observed in the H&E staining. Control HeLa cells have intact nucleus and projections of diverse shape which cross-linked the cells into one another. The treated HeLa cells showed

fragmentation of nucleus and loss of cross-linkage between the cells indicating apoptosis. Nuclear fragmentation was also observed in the treated MCF-7 cells which was not as clear as that of HeLa cells. Nuclear fragmentation was better observed when the cells were stained with fluorescent dye Hoechst S769121, a nuclear yellow fluorophores used to stain DNA[40]. Control cells have rounded intact nucleus whereas the treated cells showed fragmentation of nucleus. Nuclear fragmentation is a morphological hallmark of apoptosis, which can be observed under the light or electron microscopy and staining with fluorescent dye like Hoechst simplifies the detection of apoptosis in nucleus[41].

In agarose gel electrophoresis, DNA isolated from the control cells formed large band near the well. The treated HeLa cells showed laddering of the DNA fragments, a characteristic feature of DNA fragmentation in apoptotic cells[42]. DNA laddering was not clear in the treated MCF-7 cells but there was smearing away from the wells. It may be due to the irregular size of the DNA fragments which can be observed in case of necrosis[43].

Apoptotic cells undergo secondary necrosis when there is no intervention of scavengers and complete the apoptotic cell death[44]. Since the study was carried out with *in vitro* culture of the cancer cells with no scavenger cells, the apoptotic cells might have undergone necrosis to complete the programme. To determine the apoptotic and necrotic cell populations, FACS analysis of the cells was carried out using annexin V/PI staining. Annexin V detects phosphatidylserine which is translocated to the outer leaflet during the early stage of apoptosis; whereas PI detects the increased permeability of the dead cells[24,45]. Both the MPT-treated HeLa and MCF-7 cells showed live cells, early apoptotic, late apoptotic and necrotic cells as indicated by the annexin V/PI staining pattern which suggest that the apoptotic cells were undergoing secondary necrosis.

In apoptosis, there is activation of a series of caspase enzymes, especially the caspase-3, which is responsible for the cleavage of a number of substrates that lead to biochemical and morphological changes to the apoptotic cells[46]. In the MPT-treated HeLa cells, activated caspase-3 could be detected by immunocytochemistry using polyclonal caspase-3 antibody. Caspase-3 in the MCF-7 cells was not detected as these cells do not express caspase-3[47]. The treated HeLa cells also showed deep haematoxylin (counter-stain) stained small nucleus. It may be due to the condensed chromatin which is pulled away from the nuclear envelope during apoptosis[48]. This phenomenon indicates that MPT induced apoptosis in HeLa cells through the activation of caspase-3 whereas MCF-7 cells might have adopted other caspase pathway(s).

Further electron microscopic examination was also focussed only on HeLa cells as MPT was found to be more effective against HeLa cells as observed in growth inhibition assay. The sub-cellular changes in the MPT-treated HeLa cells were better defined using transmission electron microscopy where swollen mitochondria with distorted membrane, loss of cristae, and matrix leading to the formation of vacuole within mitochondria and rough endoplasmic reticulum were observed. Mitochondrial swelling with cristae depletion and outer membrane rupture has been observed in mitochondria-mediated cell death through both apoptosis and necrosis[49,50]. These changes were not observed in the control cells. Using scanning electron microscopy, extensive plasma membrane blubbing were also observed in the treated HeLa cells, which precede the separation of cell fragments into the apoptotic elements during budding[44].

Conclusion

The above results demonstrated that the methanolic extract of *Phlogacanthus thyrsiflorus* leaves caused 50% cell death in HeLa and MCF-7 cells at a concentration much lower than that of healthy lymphocytes. The apoptotic cell death induced by the extract in the cancer cells was confirmed by morphological and biochemical changes, such as nuclear and DNA fragmentation, membrane blebbing, translocation of phosphatidylserine and activation of Caspase-3 in HeLa cells.

Acknowledgement

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Conflict of interest

Authors declare no conflict of interests.

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