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APOPTOSIS INDUCING ACTIVITY OF *PHYLLANTHUS POLYPHYLLUS* ON HUMAN CANCER CELL LINES

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ABSTRACT

The aim of this study was to investigate the apoptosis inducing property of methanolic extract of *Phyllanthus polyphyllus* (MPP) *in vitro* using human cervical cancer cells (HeLa) and human breast carcinoma cells (HBL-100). First, the anti-proliferative activity of MPP was determined using Tryphan blue dye exclusion method. Then, the apoptosis of cells was evidenced in MPP (500 µg/ml) by using propidium iodide (PI) staining and fluorescent microscopy. The Tryphan blue dye exclusion assay showed that MPP had induced a dose- dependent decrease in cell viability. The IC_{50} of MPP was found to be 340 and 250 µg/ml. Among the tested cell lines, MPP was more selective cytotoxic against HBL-100 than HeLa. Extensive apoptotic changes were found in HBL-100 and HeLa cells. The morphology of cancer cells induced by MPP after staining with PI and examined under a fluorescence microscope was condensed chromatin and fragmented nuclei.

KEYWORDS: **Cytotoxic potential, *Phyllanthus polyphyllus*, HeLa, HBL-100, Fluorescent microscopy, Apoptosis.**

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INTRODUCTION

Cell lines are generally used to evaluate antitumor drugs since a correlation exists between *in vitro* and *in vivo* activities. Cytotoxicity testing in tissue culture is scientifically justified as animal models are inadequate in projecting the effects of chemicals on humans. Inhibition of the zone of outgrowth or morphological damage produced by the chemicals tested are analysed in cytotoxicity studies [1,2]. Tryphan blue dye exclusion method is a simple precise method for cytotoxic studies in which Tryphan blue stain is used to selectively color the dead cells or tissues blue. The underlying theory is that viable (live) cells actively efflux the dye whereas non-viable (dead) cells do not [3]. Apoptosis, the programmed cell death, is regulated by following pathways (a) mitochondrial pathway and (b) death receptor pathway. Mitochondrial pathway is specified by mitochondrial transmembrane potential loss and the release of cytochrome C [4]. Death receptor pathway is characterized by serial activation of Fas, which is a cell surface death receptor of the TNF(tumor necrosis factor) family of cytokines. Apoptosis is an innate suicide mechanism to remove excess or infected cells of metazoans. Apoptosis is characterized by several structural and biochemical changes, including cell shrinkage, chromatin condensation, phosphatidylserine (PS) externalization, genomic DNA fragmentation, and plasma-membrane blebbing [5,6]. Inappropriate control of apoptosis will occur in several diseases such as acute myocardial infarct and cancer[7]. This induction of apoptosis is an effective strategy for cancer therapy. To induce apoptosis in cancer cells, various stimuli like toxins, cytokines, ionizing radiation and anticancer drugs have been used in many studies [8,9]. Recently, plant extracts have been recognized to exert apoptosis [10,11]. Our initial study found that MPP exhibited antitumor activity against Dalton's Ascitic Lymphoma (DAL) in mice [12]. In this study, apoptosis induction in HeLa and HBL-100 cells was investigated with regard to its potential utility as cytotoxic agent.

MATERIALS AND METHODS

Plant material and Extraction

Fresh leaves of *Phyllanthus polyphyllus* were obtained from kolli hills of Tamilnadu and validated by the Botanical Survey of India, Coimbatore (Tamil Nadu, India). The leaves were dried, powdered using a mechanical grinder and sieved. Then the powder was treated with petroleum ether in a Soxhlet apparatus and then was extracted using methanol as solvent. The extract was then stored in a desiccator (5.2% w/w yield).

Cell lines

Human Breast Carcinoma cells (HBL-100) and Cervical Carcinoma cells (HeLa) used in this study were acquired from National Centre for Cell Sciences, Pune.

Trypan Blue dye exclusion assay [13]

Cell suspensions were prepared, transferred to 24 well plate and incubated in 5% CO_2 for 24 h. 300 µl of MPP (62.5-1000µg/ml) were added and incubated for 48 h. Cell suspension (100 µl) and 0.4% Trypan Blue solution (100 µl) were added to Eppendorf tube, stirred well and kept for 15 min. With the cover-slip in place, a little quantity of Trypan Blue-cell suspension mixture was transferred to both chambers of hemocytometer using a Pasteur pipette. Each chamber was allowed to fill through capillary action by touching delicately the cover-slip edge with a pipette tip. All the cells were counted and % inhibition was calculated using the below formula.

$$\% \text{ Inhibition} = \frac{\text{Number of non viable cells (Stained)}}{\text{Total Cells (Stained and Unstained)}} \times 100$$

PI Fluorescence assay: morphological assessment of apoptosis

2.5 X 10^5 cells/well of each cell line were incubated with 2ml of cell culture media for 24 h. Then treated with MPP (500 µg/ml). Cell suspensions were trypsinized and transferred into micro centrifuges for centrifugation at 5000 rpm for 10 min. Cells were washed and resuspended in phosphate-buffered saline (PBS) for fluorescence staining using PI (500 ng/ml). The sample plates were covered with aluminium foil for protection from light. The Coverslip was kept on the microscope slide and the cells were viewed under a fluorescent microscope for the detection of morphological changes. The peak excitation and peak emission for PI are 536 nm and 620 nm respectively. Apoptotic cells (condensed nuclei and fragmented cells) were counted from a total of 200 cells per slide [14].

Statistical analysis

All experiments were performed in triplicate and repeated twice to validate the similarity in results. All values were expressed as mean ± SEM.

RESULTS

In the Trypan Blue dye exclusion assay, the IC_{50} of MPP was found to be 340 µg/ml & 250 µg/ml against the HeLa and HBL-100 cell lines respectively (Table 1). MPP was found to be more selective cytotoxic against HBL-100 cell line than HeLa among the tested cell lines. Following the induction of apoptosis, extensive apoptotic alterations were found in MPP (500 µg/ml) that increased over time. Nuclear morphological changes were assessed by PI staining. It was found that more than 30% of HeLa cells and 37% of HBL-100 cells underwent apoptosis (Table 2 and Figure 1). The viable (live) cells were uniform, whereas the apoptotic cells were red and contained bright red dots in the nuclei, indicating nuclear fragmentation. Condensed chromatin and fragmented nuclei were found, that are classic characteristics of apoptotic cells.

Table 1
Cytotoxicity study of MPP on HeLa & HBL-100 by Tryphan Blue Dye Exclusion Assay

Concentration (µg/ml)	HeLa % cell death	HBL-100 % cell death
1000	72.5 ± 1.54	82.3 ±2.0
500	63.8 ± 2.71	69 ±1.10
250	46.36 ± 0.73	49.5 ±1.4
125	31.84 ± 0.84	29 ± 2.5
62.5	11.7 ± 0.50	17.5 ±3.0
IC_{50} (µg/ml)	**340**	**250**

Readings are expressed as mean ± SEM.
Average of 3 determinations, 3 replicates
IC_{50}, Drug concentration inhibiting 50% cellular growth following the drug exposure

Table 2
Percentage of apoptosis on HeLa & HBL-100 cells by treatment with PP

	control	HeLa	HBL-100
percentage death	07	32	36
	10	30	35
	08	29	40
Avg	8.33	30.33	37.0
SD	1.52	1.52	2.64

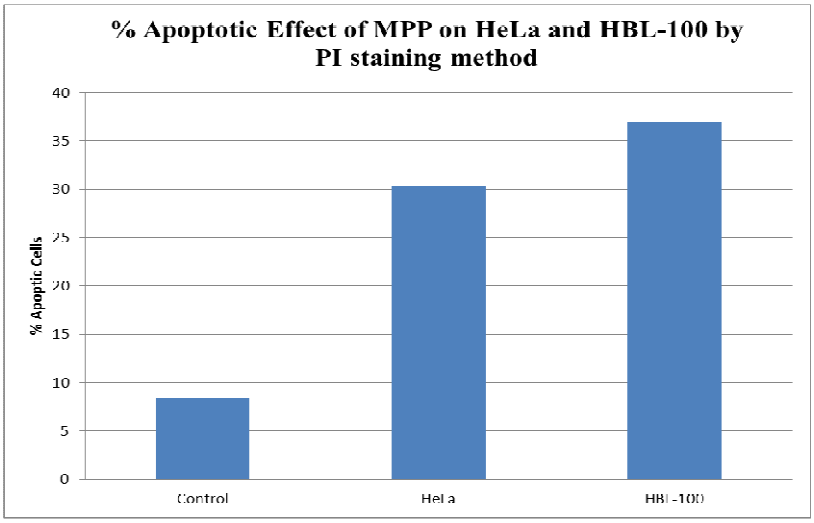

Figure 1
Percentage of apoptic cells after staining with PI.

DISCUSSION

This is the first study, as per our knowledge, MPP exhibiting the potential to induce apoptosis in Human Cancer Cell lines (HeLa and HBL-100). Cytotoxic studies by Trypan Blue dye exclusion is a precise method which takes both viable and dead cells into account in the estimation of IC_{50} concentration [15]. The Trypan Blue assay revealed a significant decrease in the total number of viable cells and significant increase in the number of dead cells. This observation suggests that MPP has a profound effect on the cancer cell viability. One of the characteristics of tumor cells is rapid multiplication along with DNA synthesis and cellular replication [16]. MPP significantly obstructed the DNA synthesis of Hela and HBL-100 cells, that provides the complete profile of the inactive status of the cell due to the effect of the extract. These results are inconsistent with the presence of apoptotic cells. The apoptosis induction has been regarded as a standard and the best strategy in anticancer therapy [17, 18]. Onset of apoptosis is indicated by condensation of nuclear chromatin into delineated masses that get localized on the nuclear membrane [19]. These morphological changes can result from abnormalities in chromatid packing and genetic material destabilization [20]. PI fluorescence assay was employed to detect typical DNA condensation and nuclear fragmentation. PI binds with DNA by means of intercalation between the bases with slight or no sequence preferences and with a stoichiometry of one dye per 4 to 5 DNA base pairs. Also PI binds with RNA, requiring treatment with nucleases to differentiate between RNA staining & DNA staining. Upon binding of the dye with nucleic acids, its fluorescence excitation maximum is shifted ~30 to 40nm towards red and fluorescene emission maximum is shifted ~15nm towards blue [21]. Although the extinction coefficient (molar absorptivity) is relatively low, PI displays large stokes shift to allow concurrent identification of nuclear DNA and fluorescein labeled antibodies, provided proper optical filters are used. PI is used for fluorescence microscopy, flow cytometry, confocal laser staining and fluorometry. PI is membrane impermeable and usually excluded from viable cells. PI is often used for detecting nonviable cells and as a counter strain in multicolor fluorescent techniques. This technique is helpful for detection and characterization of cells in various stages of apoptotic process.

The cytotoxic effect of MPP may be due to the induction of cell death or the inhibition of cell proliferation. To establish the antitumor property of MPP was due to the induction of cell apoptosis, DNA fragmentation, which is the hallmark of cell apoptosis, was analysed in HeLa and HBL-

100 cells after MPP treatment. The active ingredients in MPP that exerted an anticancer effect may be flavonoids [22], which are abundant in *P. polyphyllus*. The plant flavonoids have also shown to stimulate mitochondria Ca2þ uniporter, thus precipitating apoptosis [23]. Flavonoids also possess the classical feature of activating apoptotic transcription factors [24, 25] that might have contributed to the antitumor properties of MPP. Overall, this study exhibits anticancer activities for flavonoid-rich MPP. However, the detailed mechanisms responsible for anticancer activity of *P. polyphyllus* and the definite functional constituents need to be further investigated in the near future. It can be concluded from the present study that the antitumor effects of MPP on Human HeLa and HBL-100 cancer cells are associated with induction of apoptosis.

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