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Antiproliferative and apoptosis inducing activity of PHB (polyhydroxybutyrate) from *Bacillus Cereus* against HeLa and L929 cell lines

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ABSTRACT

Natural product research has employed well known applications in pharmaceutical industries, human healthcare, nutrition and therapeutic application. Polyhydroxybutyrate (PHB) is a synthesized macromolecule from bacteria that is commonly used due to its rapid degradation in natural environment and their abundant occurrence from natural resource. Our aim is to isolate potential PHB producing bacteria from marine invertebrates and its cytotoxic effect against L929 and HeLa cell lines and apoptotic effects of PHB. Cytotoxic effect of PHB was more effective against HeLa cell lines by inhibiting 48.90% for 100µg.ml^{-1} and showed good IC_{50} value. Apoptotic effect against HeLa cell line revealed late apoptosis by orange-stained nuclei with chromatin condensation. Flow cytometric analysis showed inhibitory effect on cell cycle progression at G0/G1 phase. These findings could open a new possibility to use PHB as potential therapeutics from marine origin to treat cervical cancer.

Key words: Polyhydroxybutyrate, cell line, apoptosis, HeLa, L929.

INTRODUCTION

Some bacteria synthesized Polyhydroxybutyrate (PHB) is the intracellular granule and it acts as energy storage. PHB provides energy for sporulation in *Bacillus* sp. (Slepecky and Law, 1961). Microorganism's synthesis granules when it has unbalanced growth condition such as limited concentration of oxygen, nitrogen, phosporous, sulphur or trace elements, such as magnesium, calcium, ferrous and high carbon concentration (Sudesh *et al.,* 2000; Kessler and Witholt, 2001). The nutrient sources are utilized for synthesis of proteins which are used for the growth of bacteria when favourable conditions.

Some microbes accumulate PHB and store as reserve food materials such as *Ralstonia eutrophes*, *Azotobacter beijerinckia*, *Bacillus megaterium*, *Pseudomonas oleovorans* and some nitrogen fixing bacteria's which are found on root nodules of plants (Singh *et al.,* 2011). Researchers had found prominent applications of PHB in their individual and combined forms, bioplastic production (Tripathi *et al.,* 2013), drug delivery system (Perveen *et al.,* 2020). Microbes which are employed for PHB production can be grown at low cost even in sugar industry waste water (Singh *et al.,* 2013).

PHB is closely related to oxidative stress, mitochondrial dysfunction and also shows the

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treatment of inflammatory bowel disease, myocardium injury, diabetes, obesity and cancer (Theiss and Sitaraman, 2011). PHB helps to activate down-stream signal transduction in plasma membrane it acts as transmembrane adaptor (Zhou and Quin, 2013). In nucleus it regulates cell cycle and transcriptional activation (Peng *et al.,* 2015).

In worldwide, cancer causes major morbidity and mortality among human community and stands top three causes of death. Chemotherapy is one of the potential treatment handled to recover cancer (Senthilraja and Kathiresan, 2015). About 60 % of anticancer drugs are isolated from natural origin such as plants (vincristine, irinotecan, camptothecines) and microorganisms (doxorubicin, dactinomicines, mitomycin and bleomycin) (Grever, 2001). Natural product drug has major role in providing substitutes for existing drugs (Kosanic *et al.,* 2016)

However, usage of many chemotherapeutic drugs has become toxicity towards normal cells by causing side effects to the patients. Some drugs poses no activity against tumor cells due to development of resistance (Peters *et al.,* 2002). Thus alternative methods and potential drug development are under research trial to find efficient drugs which is active against cancer cells and non-toxic towards normal cells. In this work, we focus to find cytotoxic activity of PHB against L929 and HeLa cell lines by MTT assay and apoptotic analysis by flow cytometry method.

MATERIALS AND METHODS

Isolation of marine bacterial strain for PHB production

Samples were collected from different coastal places in and around Colachel, Muttom, China Muttom and Kadiyapattinam of kanyakumari district. Invertebrates collected were used for further colony isolation. Cultured plates were used for further isolation of pure colonies for PHB production. The purified colonies were preserved on nutrient agar slants at 4°C for future use.

Analysis of PHB production

The PHB obtained from the strain will be confirmed through Nile red staining. PHB extracted from *Bacillus cereaes* will be purified by sodium hypochloride method demonstrated by Law and Slepecky, (1961).

In-vitro cytotoxic effect determination by MTT assay

To determine the cytotoxic effect of PHB, cell viability study was done with the MTT reduction assay. L929 (Fibroblast) cells and HeLa (Cervical carcinoma) cells were seeded in a 96-well plate at the density of 5×10^3 cells/well. The cells were allowed to attach and were grown in a 96-well plate for 24 hours, in 200 µl of DMEM (Dulbecco's Modified Eagle's Medium) with 10% FBS (Fetal bovine serum).

Appropriate concentrations of PHB (6.25, 12.5, 25, 50 and 100 $\mu g.ml^{-1}$) dissolved in DMEM media high glucose (Cat No. AL111, Himedia) were added and incubated for 24 hours at 37°C in a 5% CO_2 atmosphere. Camptothecin with the concentration of 25 µm is used as a positive control for the study. After the incubation period, remove the spent media and add 100 µL of MTT reagent (Cat No: 4060, Himedia) was added and incubated for 3 hours at 37°C. After incubation period, the formed formazan crystals were dissolved with 100 µL of DMSO (Cat No.1309, Sigma) and the absorbance readings were taken by ELISA Reader (ELX 800, Biotek) at 570 nm and the IC_{50} value is

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calculated using linear regression equation i.e. Y =Mx+C derived from the cell viability graph. The viability of the cells was determined by the following formula.

Percentage of viability = (OD of test compound treated cells/OD of untreated cells) x 100

Apoptotic effects by Acridine Orange (AO) and Ethidium Bromide (EtBr) stain

To differentiate quiescent and actively proliferating cells AO/EtBr staining technique is used. It is also used to measure apoptosis. To the treated cells, 10 µl of AO/EtBr were added and spread by placing a cover slip over it. The stained slides were incubated at room temperature for 5 minutes. The apoptotic cells with condensed chromatin and fragmented nuclei were identified by their red fluorescence and the normal cells were visualized by their green fluorescence which was counted by using an upright fluorescent microscope at 40× magnification with excitation filter at 510-590 nm.

Analysis of cell cycle profile

Cells were washed with a Phosphate Buffer Saline (PBS) (Sigma Aldrich, St. Louis, MO, USA), fixed with ice-cold 70% ethanol (Sigma Aldrich, St. Louis, MO, USA) and stored at 4^{o}C for at least 12 hours. Cells were then centrifuged for 5 min at 290 × g and pellets were resuspended in a solution of PBS containing 5 µg/mL propidium iodide (Sigma Aldrich, St. Louis, MO, USA) and 0.1 mg/mL RNase A (Sigma Aldrich, St. Louis, MO, USA). Cellular DNA content analysis was performed by flow cytometry (BD Accuri™ C6 Flow cytometer, USA) and the percentage of cells in the G0/G1, S and G2/M phases of the cell cycle was determined by cell debris and aggregates exclusion and plotting at least 10,000 events per sample, (Vasconcelos *et al.*, 2000). All the results were analyzed using the FlowJo software (version 7.6.5, Tree Star, Inc., Ashland, USA).

RESULT AND DISCUSSION

Bioactive compounds from plants, microorganisms, vertebrates, invertebrates are indispensable natural resources which are available in nature. Compounds from natural environment is less cost effective and more active. Large number of active compounds were isolated from natural products are effective against many diseases (Amador *et al.*, 2003). Thus strains isolated from different localities of marine invertebrates revealed fifty-three bacterial strains. Active PHB accumulating strains was selected and compared for their ability to accumulate PHB granules inside their cells.

The PHB yielding strain was confirmed through Nile red staining. The cells stained with Nile red were analyzed by fluorescence microscopy and the presence of fluorescence cells confirmed the production of PHB. Accumulated PHB was extracted by sodium hypochlorite chloroform method was shown in figure 1.

Nile red stained cells showed numerous bright orange-red granules within the cells under fluorescence indicating the accumulation of PHB figure 2. Microbial metabolites have a major role as anticancer agents, metabolites such as daunorubicin, doxorubicin, bleomycin and pentostatin are active against cancer (Senthilraja, and Kathiresan, 2015).

As concentration of PHB increased, the cell viability of the HeLa cervical carcinoma cell line significantly decreased to 48.90 at 100µg.ml^{-1} concentration. This implies PHB has able to inhibit the proliferation of HeLa cells. This induced cell cytotoxicity was concentration dependent manner. This

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is consistent with findings of Abdelfadil *et al.* (2013) against T28 oral cancer treated by thymoquinone. Inhibitory concentration (IC50) to compete HeLa cells was 82.941µg.ml^{-1}. Resulted data implies that PHB was more toxic towards HeLa cancer cells than normal L929 cells. Similar to our study IC50 values for hymoquinone against MCF-7, MDA-MB-231 and BT-474 was 32, 11 and 21 µM (Woo et al., 2011). Non-toxic behavior of PHB against L929 cells was noted by MTT assay. Microscopic observation of the cells treated against isolated compound was shown in figure 3.

The cells treated against PHB was observed for apoptotic study by double staining method showed significance of compound against HeLa cells. Apoptosis on cancer cells is a crucial activity of some chemo preventive agents (Bauer *et al.,* 1995). The result revealed living cells which is normal green nucleus, early apoptotic of bright green nucleus with condensed or fragmented chromatin, late apoptotic stage showed orange-stained nuclei with chromatin condensation or fragmentation and necrotic cells observed with uniformly orange-stained cell nuclei. Similarly extracts from *Chromohalobacter salexigens* (P3-86A, K-30, P3-86B (2)), *Halomonas meridian* (P3-37B), *Idiomarina loihiensis* (P3-37C) and *Chromohalobacter israelensis* (K-18) was found to be most active by showing apoptosis against HeLa cells (Sagar *et al.,* 2013). The untreated non-apoptotic cells showed low fluorescence, smooth, flattened nuclear morphology and normal nuclei, as well as uniformly dispersed chromatin. Results obtained by staining was compared with control and displayed in figure 4.

Cells treated with PHB showed a significant increase in apoptosis percentage of in their differential stages. Anticancer agent alters the cell cycle regulation machinery it leads to arrest of the cell in different phases of cell cycle which reduces the growth and proliferation of cancer cell (Kim *et al.,* 2004). The result implies cells lagged or retained in their cell cycle at initial stages when compared with control cells. The result showed that 57.3% of the cells were retained or accumulated in G0/G1 phase, followed by 26.8% at S phase and 9.5% of the cells in G2/M phase in PHB treated HeLa cells. Similarly Fe-MIL-101 exhibited G0/G1 phase cell cycle arrest in ovarian cancer cells (Wang *et al.,* 2016). Sathiyamoorthy and Sudhakar, (2018) also confirms that the ethanolic leaf extract also provides anticancer activity against human colorectal cancer (HT-29 cell line) during the GO/GI phase. Whereas in control 49.1% accumulated in G0/G1 phase, 33.8% in S phase and 10.2% in G2/M phase. The result clearly implies that PHB possessed a notable inhibitory effect on cell cycle progression at G0/G1 phase figure 5.

To assess the ROS levels in HeLa cells by DCFDA dye showed significant increase in emission of fluorescence (green) than the control. The increased fluorescent intensity of (22,308.88 AU) was obtained in PHB treated HeLa cells and the control cells showed (6,764.78 AU). Observance of no ROS generation by cancer cells during treatment with marine derived endophytic fungus *Talaromyces purpureogenus* by DCFH-DA staining in flow cytometry (Kumari *et al.,* 2018). Thus tumor cell lines were susceptible to PHB this indicating the broad spectrum anticancer activity. Further studies to be carried out at *in vivo* and clinical trials needs to be conducted to establish PHB as a safe agent for cancer therapy.

CONCLUSION

The present study was carried out to explore the antiproliferative and apoptosis possible of PHB obtained from Bacillus species obtained from marine invertebrates. Its cytotoxic effect against

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L929 and HeLa cell lines indicates it has good anticancer property with 48.9% of inhibition. Apoptosis study against HeLa cells indicates orange-stained nuclei with chromatin condensation with fragmentation. Flow cytometric analysis implies 57.3% of HeLa cells got accumulated in G0/G1 phase. PHB isolated has induced apoptosis in HeLa cell line which indicates programmed cell death of tumor cells. Thus PHB could be advance to get novel compounds which could be exploited in the treatment of cancer.

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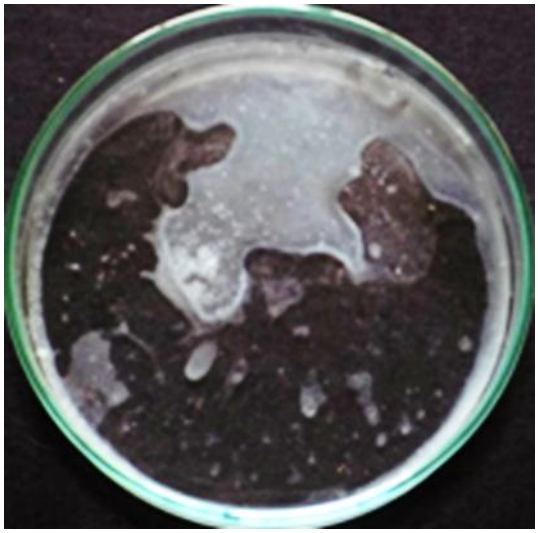

Figure 1. PHB extracted by sodium hypochlorite chloroform method

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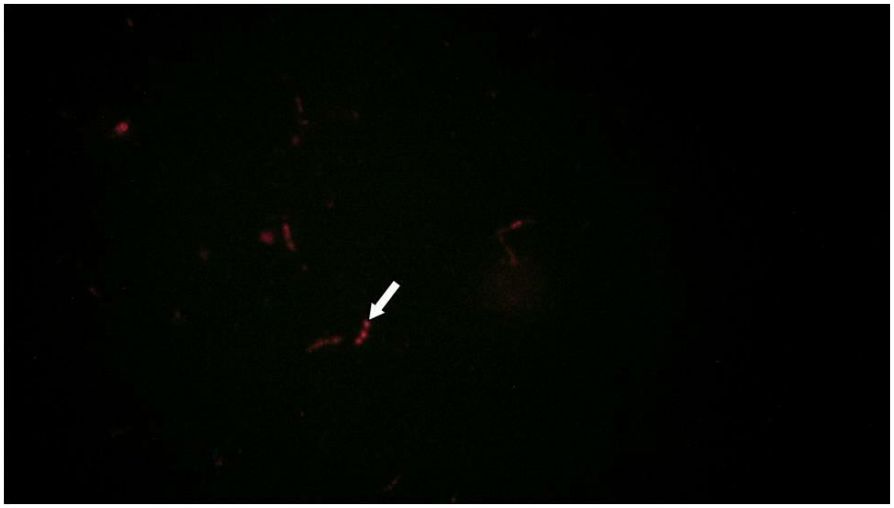

Figure 2. PHB accumulation by Nile red staining

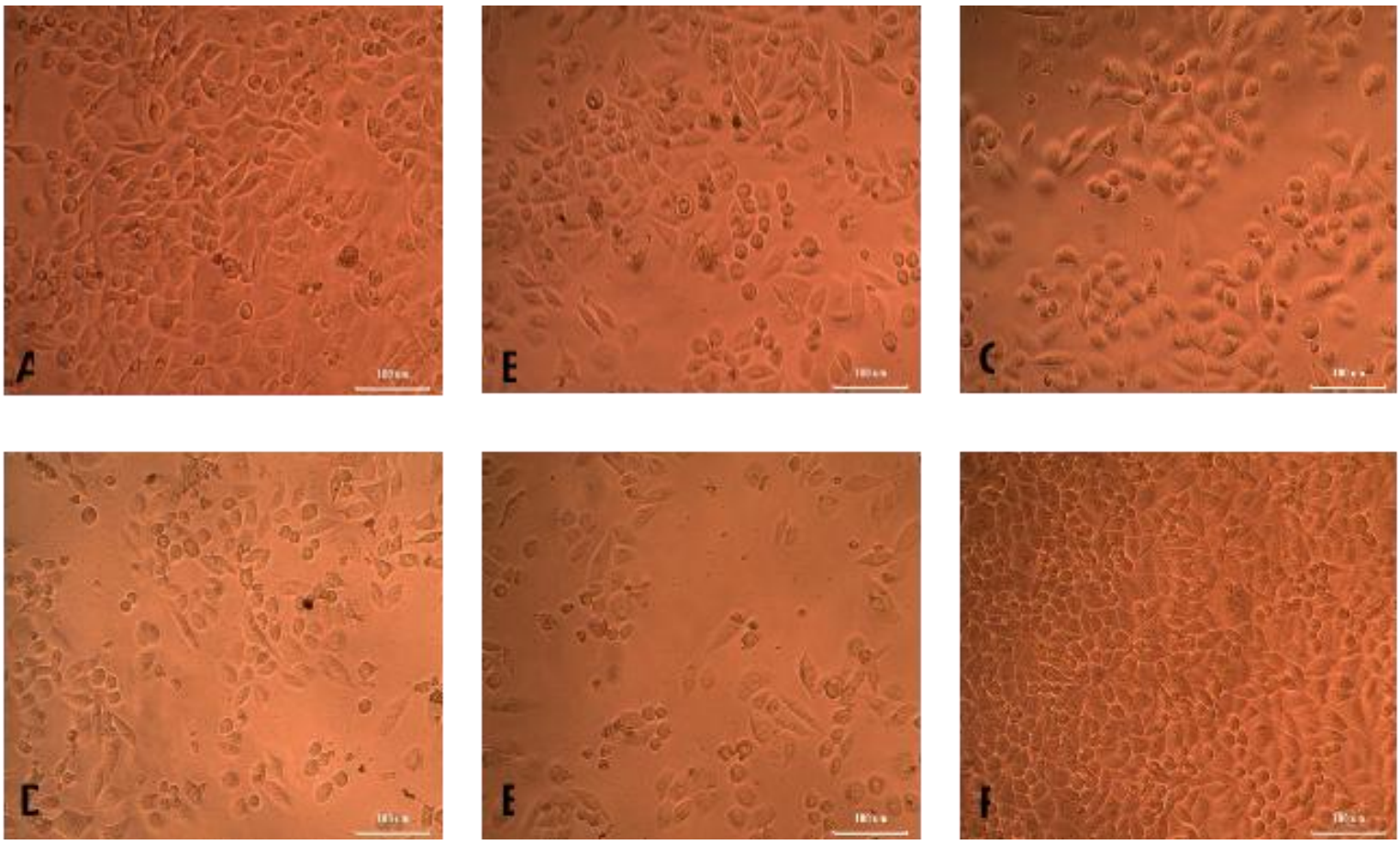

Figure 3. MTT assay of HeLa cell line at concentrations (A) 6.25 (B) 12.5 (C) 25 (D) 50 (E) 100 (F) control

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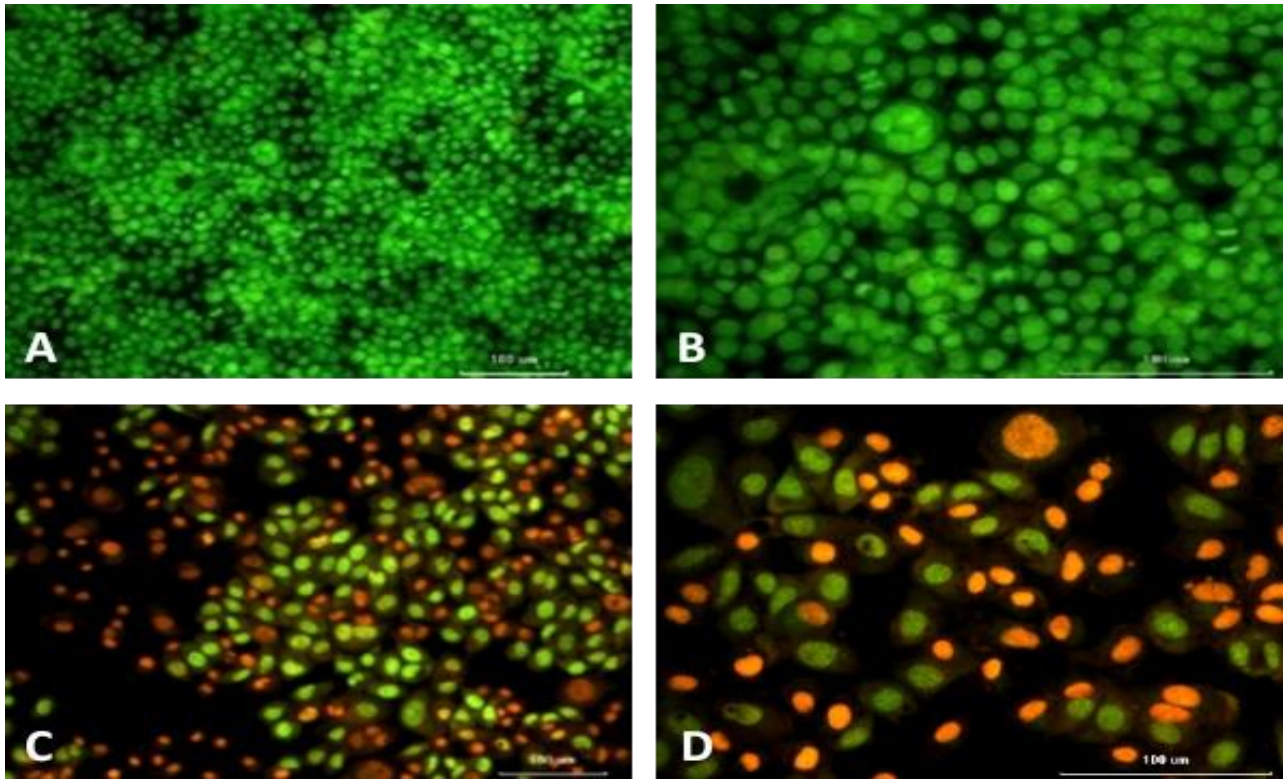

Figure 4. Apoptosis (A) Control cells at 10 X (B) Control cell at 20 X (C) HeLa cells at 10 X (D) HeLa cells at 20 X

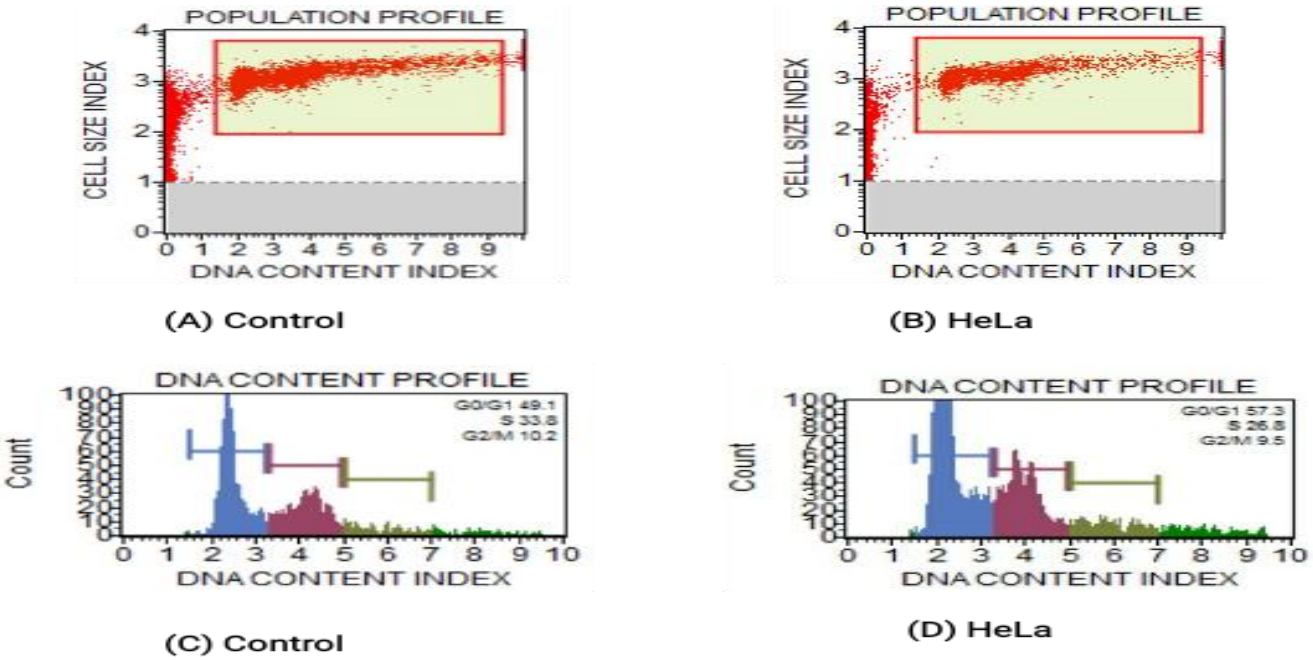

Figure 5. Flow cytometric analysis of HeLa cell line