RESEARCH ARTICLE

Apoptosis Induction, Cell Cycle Arrest and in Vitro Anticancer Activity of Gonothalamin in a Cancer Cell Lines

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Abstract

Cancer is one of the major health problems worldwide and its current treatments have a number of undesired adverse side effects. Natural compounds may reduce these. Currently, a few plant products are being used to treat cancer. In this study, goniothalamin, a natural occurring styryl-lactone extracted from *Goniothalamus macrophyllus*, was investigated for cytotoxic properties against cervical cancer (HeLa), breast carcinoma (MCF-7) and colon cancer (HT29) cells as well as normal mouse fibroblast (3T3) using MTT assay. Fluorescence microscopy showed that GTN is able to induce apoptosis in HeLa cells in a time dependent manner. Flow cytometry further revealed HeLa cells treated with GTN to be arrested in the S phase. Phosphatidyl serine properties present during apoptosis enable early detection of the apoptosis in the cells. Using annexin V/PI double staining it could be shown that GTN induces early apoptosis on HeLa cells after 24, 48 and 72 h. It could be concluded that goniothalamin showing a promising cytotoxicity effect against several cancer cell lines including cervical cancer cells (HeLa) with apoptosis as the mode of cell death induced on HeLa cells by Goniothalamin was.

Keywords: Goniothalamin - HeLa cervical cancer cell line - fluorescence microscopy - cellular DNA content - apoptosis.

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Introduction

Cancer is uncontrolled growth of cells. It can affect almost any part of the body. The growths often invade surrounding tissue and can metastasize to distant sites (WHO, 2011). Cancer is caused by mutations in the DNA. Normal cells repair the mutation or simply die when a mutation occurs whereas cancerous cells continue to survive with the mutations and they grow in an uncontrolled manner until a mass of cells known as tumor is created. Often the tumor interferes with the normal functioning of healthy tissues and can spread to other parts of the body (Tompa, 2007).

Cervical cancer is a malignant neoplasm of the cervical area. It is an important women's health problem in developing countries, killing 270,000 women each year. It is the third most common cancer overall and the leading cause of death from cancer among women in developing countries. At least 370,000 new cases are identified each year (WHO, 2010). Current cancer chemotherapy can damage or kill the rapid dividing and healthy cell but causes serious side effects such as nausea, anemia, and hair loss. In addition, the cost of chemotherapy drug is high as compared to the natural compound from medicinal plants.

Goniothalamin, a natural occurring styryl-lactone and extract from Goniothalamus SPP. it is a novel compound with putative anti-cancer properties (Lin and Pihie, 2003; Chen et al., 2005; Al-Qubaisi et al., 2011). Goniothalamin extracted from Goniothalamus andersonii had been able to induce cytotoxicity in a variety of cancer cell lines including cervical (HeLa), gastric (HGC-27), kidney (768-0), breast carcinomas (MCF-7, T47D and MDA-MB-231) and leukemia (HL-60, Jurkat and CEM-SS) (Rajab et al., 2005; Inayat-Hussain et al., 2010). Goniothalamin has been proved to be only cytotoxic to ovarian cancer cell line (Caov-3) without causing cell death in normal kidney cell (MDBK) as happened in tamoxifen or taxol treated cells (Lin and Pihie, 2003). In addition, goniothalamin showed lower toxicity to normal liver Chang cell line as compared to doxorubicin (chemotherapy drug) (Al-Qubaisi et al., 2011). Goniothalamin is a promising antitumor agent against cancerous cell lines (Wattanapiromsakul et al., 2005). Cytotoxicity of goniothalamin in human leukemia (HL-60 and Jurkat) and human breast carcinoma (MDA-MB-231) occurs via apoptosis after treated with goniothalamin (Chen et al., 2005; Inayat-Hussain et al., 2010).

In this study, goniothalamin, a natural occurring styryl-lactone and extract from root of Goniothalamus macrophyllus is used to investigate the cytotoxic

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properties against several cancer cell lines. Furthermore, this study carried out to study the mechanism of apoptosis induction of goniothalamin on HeLa cells by determines the DNA content and Phosphatidyl Serine properties.

Materials and Methods

Goniothalamin extract

Dried and powdered root (500g) of Goniothalamus macrophyllus were extracted with dichloromethane and concentrated. Fifty g of brown resin was subjected to silica gel chromatography with gradient of hexane/ethyl acetate (8:2) which gave goniothalamin 5g (colorless crystal), structurally confirmed by comparing 1H and 13C-NMR data with those reported. 13C NMR: δ 29.87, 77.95, 121.60, 125.72, 126.72, 128.71, 133.10, 135.80, 144.76 and 163.90.

Cells and cell culture

cervical cancer (HeLa), breast carcinoma (MCF-7), colon cancer (HT29) and Normal mouse fibroblast cells (3T3) obtained from animal tissue culture laboratory, UniSZA. cell lines were grown in 25 cm^2 tissue culture flasks (Nunclon TM, Nunc) at 37°C, 5%CO_2 and 90% humidity in RPMI -1640 medium (Sigma Chemical Company), containing 10% fetal bovine serum (Culture lab), penicillin (100 IU/ml) and streptomycin (100 μg/ml). The cells were grown confluence, which could be observed under an inverted microscope and sub – cultured at three to four days interval.

MTT Cytotoxicity Assay

All cell lines were trypsinized and counted using hemocytometer then were seeded in 96-well micro plate at 5×10^5 cells/ml and then incubated at 37°C in 5%CO_2 to allow cells attachment. The medium was removed and replaced with fresh medium containing various concentrations of goniothalamin starting with the highest concentration of 60 μg /ml (two folded dilution). Cells were incubated at 37°C, 5%CO_2 for 72 hours. Each concentration was assayed in triplicates (n=3). Seventy-two hours later, 20 μl of MTT (5 mg/ml) solution was added to each well and then the plate was further incubated for 4 h. All remaining supernatant were removed and 150 μl of DMSO was added to dissolve the formed crystal formazan. MTT assay reading was performed using ELISA plate reader (Tecan 200, USA).

The MTT Cell Proliferation Assay

To confirm anti-proliferative effects of goniothalamin on HeLa cells, MTT cell proliferation assay was carried out. In this assay, two different concentrations of compound with cells were prepared together with control. The concentration chosen were IC_{25} and IC_{50} concentrations (3.2 and 1.2 μg/ml). Each sample was assayed in triplicate, and control samples include cells without goniothalamin. The cells were treated by goniothalamin for 24, 48, and 72 hours. At the end of incubation periods, 20µl of MTT solution (5 mg/ml MTT dissolved in PBS) were added to each well containing cells and the plate was incubated at 37ºC in an atmosphere of 5%CO_2 for 4 hours. After that, most of the medium was removed, then a volume 100 μl of DMSO (dimethyl sulfoxide) was added into the wells to soluble the crystals. Finally the absorbance was measured by ELISA reader at a wavelength of 570 nm. Graphs (OD of samples against time) were plotted to determine the growth rates of cells in a given values.

Acridine Orange (AO) and Propodium Iodide (PI) Double Staining using Fluorescent Microscopy

HeLa cells were quantified using propidium iodide (PI) and acridine-orange (AO) double staining according to standard procedures and examine under fluorescence microscope (Lieca attached with Q-Floro Software) (Mishell et al., 1980; Ali, 2011).

Cells suspension was mixed with an equal volume of staining solution (1 : 1) containing 10 µg/mL acridine orange and 10 µg/mL propium iodide (dissolved in PBS) and observed under fluorescence microscope within 30 minutes. The viable (green intact cells), apoptotic (green shrinking cells with condensed of fragmented nucleus), and necrotic (red cells) were the morphological changes that were examined under fluorescence microscope.

HeLa cells were seeded in six-well plate and incubated at 37 °C in 5%CO_2 atmosphere. Twenty-four hours later, the medium in each well was removed and replaced with Goniothalamin at IC50 concentration dissolved in medium and incubated at 37°C in 5%CO_2 atmosphere for 24, 48, and 72 h. After incubation period, Cells suspension was mixed with an equal volume of staining solution (1 : 1) containing 10 µg/mL acridine orange and 10 µg/ml propium iodide (dissolved in PBS) and observed under fluorescence microscope within 30 minutes. The viable (green intact cells), apoptotic (green shrinking cells with condensed of fragmented nucleus), and necrotic (red cells) were the morphological changes that were examined under fluorescence microscope (Leica, Germany). Each experiment was assayed three times (n=3) to provide a useful quantitative evaluation. Viable, apoptotic and necrotic cells was quantified in a population of 200 cells. The results were expressed as a proportion of the total number of the cells examined.

Analysis of Cellular DNA Content Using Propidium Iodide

HeLa cells at a concentration of $1x10^6$ cells/ml were seeded into 6-well plate in 2 ml culture medium with a concentration of IC_{50} value of goniothalamin and were incubated at 37ºC in an atmosphere of 5%CO_2 for 24,48 and 72 hours. Some wells were left with no treatment to be used as a control. After the incubation period, the cultured cells were harvested using trypsin and centrifuged. After incubation, the cells were detached and stained by using the Cycle TEST TM PLUS DNA Reagent Kit. Cell cycle was read using the Cell Quest software within 3 hours.

Flow cytometry (Annexin V/PI double staining): HeLa cells at a concentration of 1 X 10^6 cell/ml were seeded into the 6-well plate and treated with IC_{50} concentration of Goniothalamin. After 24, 48 and 72 h incubation, the cells were detached and stained by using PE Annexin V Apoptosis Detection Kit I. All samples were read by the flow cytometer.

Statistical Analysis

Data was expressed as mean±SD. Statistical analysis was performed with Student's t-test using the independent t-test (SPSS version 15). Differences were considered significant at P=0.05.

Results

MTT Cytotoxicity Assay

Cytotoxicity of goniothalamin was evaluated using MTT assay. The IC_{50} values of gonoitahalamin concentrations that kill 50% of treated cell lines compared to untreated cells were 3.2±0.72, 6.6±0.92, 3.8±1.10 and >10 μl/ml for (HeLa), breast carcinoma (MCF-7), colon cancer (HT29) and Normal mouse fibroblast cells (3T3), respectively (Table 1).

The MTT Cell Proliferation Assay

The effect of goniothalamin on cells proliferation was studied in vitro, by using the MTT proliferation assay with HeLa cell lines. In the assay, both concentrations of GTN, IC_{50} and IC_{25}, were used. Untreated cells were used as control. To determine the changes in the numbers of cells in the wells during the experiment, cells proliferation had to be measured 24, 48 and 72 hours after the start of the incubation period. GTN treatment on HeLa cells showed that the optical density was lower in both concentrations, IC_{50} and IC_{25}, than controls. Whereas GTN treatment on HeLa cells with the IC_{50} values showed that the optical density was lower than inoculation with the IC_{25} values. This optical density is in proportion to the number of variable cells. Figure 1 shows that the growth rates decreased in the treated cells as compared with the untreated cells whereas inoculation with a higher concentration (IC_{50}) decreased the growth rate more than low concentration (IC_{25}). On the other hand, the percentage of non-viable cells treated with IC_{25} value was 28% (day 1), 38% (day 2) and 45% (day 3). But the percentage of non-viable cells treated with IC50 values were 38% (day 1), 45% (day 2) and 48% (day 3) (Figure 2).

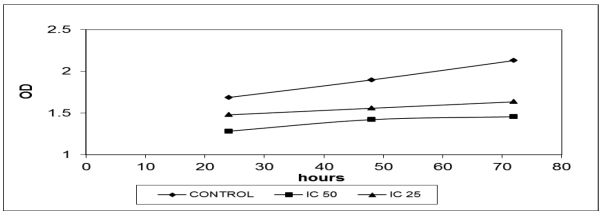

Figure 1. MTT Proliferation Assay for IC_{50} and IC_{25} Goniothalamin Concentrations (3.2 and 1.2 μg/ml) Against HeLa Cells at 24, 48 and 72 Hours Post-Treatment. The growth rates decreased in the treated cells as compared with the untreated cells whereas inoculation with a higher concentration of virus (IC_{50}) decreased the growth rate more than low concentration (IC_{25})

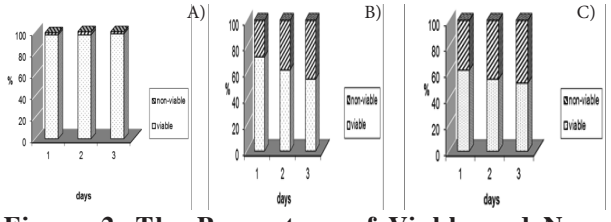

Figure 2. The Percentage of Viable and Non-viable HeLa Cells in Population after Treated with Goniothalamin after 24, 48 and 72 h. A) untreated cells, B) HeLa cells treated with IC_{25} Goniothalamin concentration (1.2 μg/ml), C) HeLa cells treated with IC_{50} Goniothalamin concentration (3.2 μg/ml)

Acridine Orange (AO) and Propodium Iodide (PI) Double Staining using Fluorescent Microscopy

fluorescent microscope was conducted to study of morphological changes of cell death mode induced by goniothalamin after 24, 48 and 72 h. Acridine orange (AO) and propidium iodide (PI) staining was used. Viable cells displayed green fluorescence with the appearance of circular cell; intact DNA and nucleus give a round and green nuclei. The early apoptotic cells have fragmented DNA which gives several green colored nuclei and cell blebbing. Late apoptotic and necrotic cell's DNA would be fragmented and stained orange and red (Figure 3)

Besides the study of morphological changes, the percentage of viable, apoptotic and necrotic cells also recorded in Table 2 and plotted as a graph in Figure 4. The percentage of apoptotic cells in untreated cells slightly increased from 0.67% after 24 h to 5.33% and 7% after 48 and 72h, respectively (Figure 5). Whereas, cells treated with goniothalamin at IC_{50} concentration, the percentage of apoptotic cells increased rapidly from 37% after 24h to 53% and 63% after 48 and 72h, respectively.

Analysis of Cellular DNA Content Using Propidium Iodide

The DNA Content of HeLa cells were monitored by

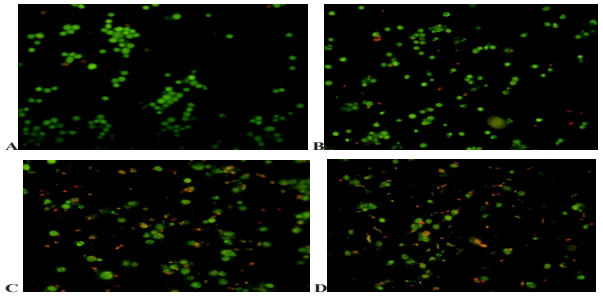

Figure 3. Fluorescence Microscopy Examination of HeLa Cell Line (Magnification 200X). A) Untreated HeLa cells, B) HeLa cells treated with Goniothalamin after 24 h, C) HeLa cells treated with Goniothalamin after 48 h, D) HeLa cells treated with Goniothalamin after 72 h.

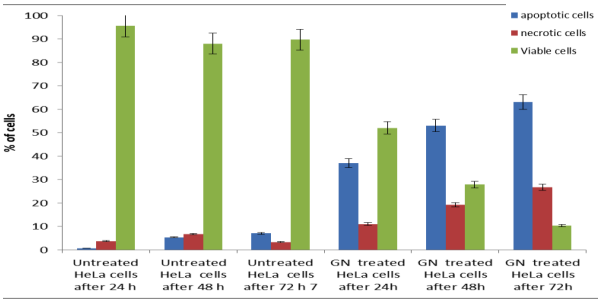

Figure 4. Flourecent Microscopy Examination. Percentage of apoptotic cells, necrotic cells and viable cells in HeLa cell population with gonithalamin treatment after 24 48 and 72 h. HeLa cell death via apoptosis increased significantly (*P < 0.05) in time-dependent manner

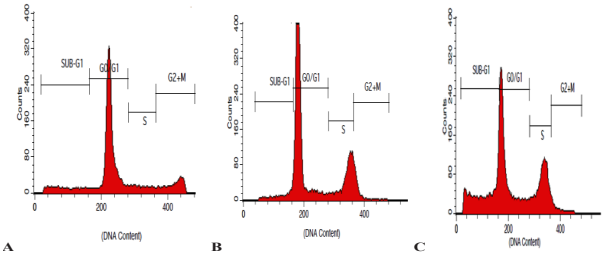

Figure 5. Cell Cycle Analysis of Cervical Cell Cancer Treated with Goniothalamin at IC_{50} Concentration. A) Untreated cells, B) Treatment after 24h, C) Treatment after 48 h.

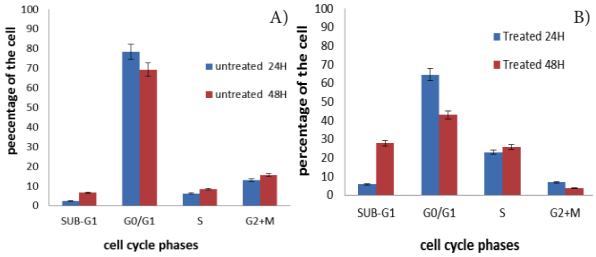

Figure 6. Analysis of the Cell Cycle on Cervical Cancer Cells (HeLa) after 24h and 48h. A) Untreated HeLa cells, B) HeLa cells treated with Goniothalamin.

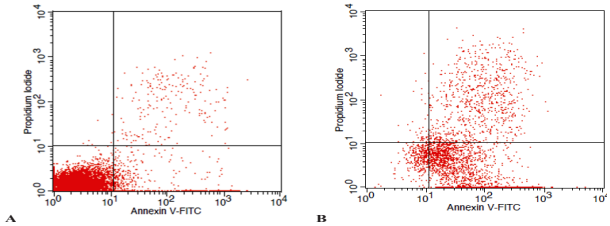

Figure 7. Contour Diagram of Annexin V/PI Flow Cytometry. A) untreated HeLa cells, B) HeLa cells at 24 h post-inoculation of IC_{50} value of Goniothalamin. Lower left quadrants show viable cells, excluding PI and negative for Annexin V binding. The upper right quadrants contain the non-viable, necrotic cells, positive for Annexin V and PI uptake. Lower right quadrants represent the apoptotic cells, Annexin V positive and PI negative.

flow cytometry after propidium iodide staining nuclei. Goniothalamin induced a significant time-dependent increase in the proportion of sub-G1 in HeLa cell population. However, a slight increase was observed at Sub G1 phase of untreated cervical cell (HeLa) over time. Tables 3 and 4 show that the percentages of the treated cells in Sub-G1 increased from 5.91% at 24 hours to 27.99% at 48 h while the percentages in untreated cells increased from 2.44% at 24 hours to 6.62% at 48 h ($p<0.005$).

On the other hand DNA histogram showed that goniothalamin increased the population of cells at S phase in a time-dependent manner (Figure 6). The S population increased significantly from 6.17% and 8.53% in the untreated cells to 23.17% and 25.92% in cells treated with IC50 goniothalamin for 24 and 48h, respectively Tables 3 and 4. While concomitantly the G1 population decreased from 78.38 %and 69.27% in the untreated cells to 64.66% and 43.10% in the treated cells for 24 and 48h, respectively Tables 3 and 4. Similarly the G2/M population decreased from 12.98% and 15.58% in the untreated cells to 7.08% and 3.76% in the treated cells for 24 and 48h, respectively Tables 3 and 4.

Table 1. Cytotoxicity of Goniothalamin against Various Cell Lines

Cell line	IC_{50} value (μg/ml)
Cervical cancer (HeLa)	3.2±0.72
Breast carcinoma (MCF-7)	6.6±0.92
Colon cancer (HT29)	3.8±1.10
Normal mouse fibroblast cells (3T3)	>10

Table 2. Percentages of Apoptotic, Necrotic and Viable HeLa Cells after 24 and 48 h

HeLa cells and Treatment	Apoptotic cells %	Necrotic cells %	Viable cells %
Untreated HeLa cells after 24 h	0.67	3.67	95.66
Untreated HeLa cells after 48 h	5.33	6.67	88.00
Untreated HeLa cells after 72 h	7.00	3.33	89.67
GN treated HeLa cells after 24h	37.00	11.00	52.00
GN treated HeLa cells after 48h	53.00	19.20	27.80
GN treated HeLa cells after 72h	63.00	26.67	10.33

Table 3. Percentages of Untreated Cervical Cancer Cells at 24h and 48 h

Cell cycle phase	HeLa cells (%)	
	24H	48H
SUB-G1	2.44	6.62
G1	78.38	69.27
S	6.17	8.53
G2/M	12.98	15.58

Table 4. Cell Cycle Analysis of Cervical Cancer Cells (HeLa) at 24h and 48h Treated with Goniothalamin

Cell cycle phase	Treated with IC_{50} Goniothalamin (%)	
	24H	48H
SUB-G1	5.91	27.99
G1	64.66	43.10
S	23.17	25.90
G2/M	7.08	3.76

Flow cytometry (Annexin V/PI double staining)

Apoptotic cells exclude all dyes which are in use for cell viability assays, such as PI, while necrotic cells do not. In cells with a damaged cell membrane PI induces a red fluorescence on the DNA, whilst it is excluded by cells with a preserved cytoplasm membrane. Hence during the initial phase of apoptosis, the cells are still able to exclude PI and therefore do not show any red fluorescence signal, similar to that of living cells. Figure 7 showed the results of Annexin V/PI flow cytometry of HeLa cells after treatment with IC_{50} value of goniothalamin. Untreated cell was found in the lower left quadrant of the cytograms, these viable cells excluded PI and were negative for Annexin V binding. The upper right quadrant represents the non-viable, necrotic cells, positive for Annexin V binding and showing PI uptake. The lower right quadrant represents the apoptotic cells, Annexin V positive and PI negative, demonstrating Annexin V binding and cytoplasmic membrane integrity (Figure. 11). The Annexin V/PI – apoptotic cell population for HeLa cell line increased from 6.4%in untreated cells, to 26.45% in treated cells at 24 h post-infection.

Discussion

Cell death in mammalian cells are divided into two morphologically and biochemically distinct modes namely apoptosis and necrosis (Doyle and Griffiths, 1998). Apoptosis is an organized, pre-programmed response of cell to shifting of environmental conditions. Characteristics of apoptotic cell include cell shrinkage, nuclear and DNA fragmentation and breaking up of the cell into membrane-bounded vesicles, termed 'apoptotic bodies', which are subsequently ingested by macrophages (Doyle and Griffiths, 1998). Apoptosis plays a vital role in regulating growth, development and immune response, and also clearing abnormal cells (Fan et al., 2005). This apoptosis program becomes important in medical study in order to cure the cancerous cell without give the inflammatory effect. Aberrant cell death processes may underlie many human diseases including cancers, autoimmune, neurodegenerative and immunodeficiency disorders (Baehrecke, 2002).

Cytotoxic has been defined as the cell killing property of a chemical compound independent from the mechanism of death (Graham-Evans et al., 2003). Cytotoxicity assay is an appropriate method for screening new substances within a short time in order to determine cytotoxicity on cancer cells (Alley et al., 1988). The effective dose for a 50% reduction in cell number for plants products to be considered cytotoxic should be less than 20 μg/ml (Geran et al., 1972).

MTT cytotoxicity assay used to measure the cytotoxic effect of goniothalamin (GTN) on cervical cancer (HeLa), breast carcinoma (MCF-7), colon cancer (HT29) and Normal mouse fibroblast cells (3T3) measure of cytotoxic effect and The IC_{50} concentration that kill 50% of the cells was determined graphically after 72 h. In screening result, GNT has shown broad spectrum cytotoxicity and It had most active cytotoxic activity cervical cancer (HeLa) but not on Normal mouse fibroblast cells (3T3). These results conducted to other studies investigated the cytotoxic effect of Goniothalamin towards human breast cancer, vascular smooth muscle cells (VSMCs), Jurkat leukemia cells, HL-60 leukemia cells, Chinese hamster ovary (CHO) and hepatoblastoma HepG2 cells (Ali et al., 1997; Pihie et al., 1998; Inayat-Hussain et al., 1999; Inayat-Hussain et al., 2003; Nasir et al., 2004; Chen et al., 2005; Chan et al., 2006; Al-Qubaisi et al., 2011).

In this study, GTN have indicated significant growth inhibition in HeLa cell line at low concentration of IC_{50} values. MTT proliferation assay was carried out to determine the growth rate of cells. A linear relationship between the formazan generated and the number of viable cells was demonstrated, together with time-dependent growth characteristics for HeLa cells (Ferrari et al., 1990). GTN treatment on HeLa cells cell lines showed significant decrease in growth rate compared with control. Whereas treatment with high concentration (IC_{50} value) showed that the growth rates of the cells were more decreased than of low concentration (IC_{25} values). On the other hand the percentage of non-viable cells on both cell lines increased with the increasing period of treatment.

However, MTT cytotoxic results of GTN on HeLa cells have been further supported with morpgological study using fluorescent microscopy Acridine Orange Propidium Iodide staining assay and flow cytometric analysis of cell cycle.

The apoptotic features were confirmed and the percentage of apoptotic cells was determined from at least 300 counted cells observed under fluorescent microscope. The calculation of apoptotic cells is described as the percentage of apoptotic cells and apoptotic bodies within the overall population of cells. The percentage of apoptotic cells and the graph showed that the percentage of apoptotic cells treated with goniothalamin was increasing among the time. These distinctive morphological features form the basis of some of the most widely used techniques for the identification and quantification of apoptosis, and thus morphologic description using Phase Contrast microscopy and fluorescence microscopy remains one of the best ways to define apoptosis (Doonan and Cotter , 2008).

The quantitative analysis of cell cycle is very important in the study of molecular mechanism of cell death and cell cycle progression (Tao et al., 2004). Untreated and treated HeLa cells were evaluated for apoptosis by measuring the amount of apoptotic cells using of DNA flow cytometry (FCM). Flow cytometric analysis of cell cycle measures the apoptotic changes in cells by staining them with DNA dyes (Telford et al., 1994). Apoptotic cells, due to a change in membrane permeability, showed an increased up-take of the vital dye, PI, compared to live cells (Nicoletti et al., 1991; Telford et al., 1994). This method is useful for quantitative estimates of the fractions of cells in the different phases of the cell cycle (Ali et al., 2011). In this study goniothalamin treatment on HeLa cells produced S phase cell cycle accumulation with a large increase in the sub-G1 which mean there was a relationship between goniothalamin-induced S phase arrest and apoptosis($p<0.001$). A study of cell cycle pattern has been documented that goniothalamin treatment causes cell cycle arrest and cell death maximally at G2/M phase (Chen et al., 2005). Another study demonstrates that GTN arrested cell cycle at G0/G1 in SK-Hep1, and at G2/M in Hep-3B cells (Cheng-Hui , 2008). These results concurred with the previous results to suggest that goniothalamin induce apoptosis on HeLa cells more extensively with increasing in time.

Change in plasma membranes is the earliest features of apoptosis. In apoptotic cells, the membrane phospholopid, phosphotidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane thereby exposing PS to the external cellular activity (Lawen, 2003). Annexin binding assay is a method permits the detection of the early phases of apoptosis before the loss of cell membrane integrity (Vermes et al., 1995; Aubry et al., 1999). The principle of Annexin V staining method used is the conjugation of Annexin V to phosphotidylserine of the apoptosis cells and in conjunction of dye Propodium Iodide which binds to cells at different stage and distinguishes apoptosis cells with necrotic cells (Tao et al., 2004). Apparently, the results indicate that the percentage of cells in early apoptosis of the cervical cancer cell (HeLa) treated with Goniothalamin appeared after 24 hr.

The percentage of the cells treated with Goniothalamin were decreased in early apoptosis phase and increased in late apoptosis over time.

In summary, goniothalamin (GTN) showed selective cytotoxic towards cervical cancer (HeLa), breast carcinoma (MCF-7), and colon cancer (HT29) but is not normal mouse fibroblast cells (3T3). The compound is potentially a good anti-cancer drug since it is non-toxic towards healthy cells. Our results indicate that GTN inhibits HeLa cell proliferation via apoptosis and causes cell cycle arrest and cell death at S phase.

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