

RESEARCH ARTICLE

Induction of Caspase-9, Biochemical Assessment and Morphological Changes Caused by Apoptosis in Cancer Cells Treated with Goniothalamin Extracted from *Goniothalamus macrophyllus*

Aied Mohammed Alabsi^{1,2*}, Rola Ali³, Abdul Manaf Ali³, Hazlan Harun⁴, Sami Abdo Radman Al-Dubai⁵, Kurubaran Ganasegeran⁶, Mustafa Ahmed Alshagga⁷, Sameer Dirhim Salem⁸, Noor Hayaty Binti Abu Kasim⁹

Abstract

Goniothalamin, a natural compound extracted from *Goniothalamus* sp. belonging to the Annonaceae family, possesses anticancer properties towards several tumor cell lines. This study focused on apoptosis induction by goniothalamin (GTN) in the Hela cervical cancer cell line. Cell growth inhibition was measured by MTT assay and the IC₅₀ value of goniothalamin was 3.2±0.72 µg/ml. Morphological changes and biochemical processes associated with apoptosis were evident on phase contrast microscopy and fluorescence microscopy. DNA fragmentation, DNA damage, caspase-9 activation and a large increase in the sub-G1 and S cell cycle phases confirmed the occurrence of apoptosis in a time-dependent manner. It could be concluded that goniothalamin show a promising cytotoxicity effect against cervical cancer cells (Hela) and the cell death mode induced by goniothalamin was apoptosis.

Keywords: Goniothalamin - hela cervical cancer cell line - fluorescence microscopy - DNA fragmentation - DNA damage

Asian Pac J Cancer Prev, **14** (11), 6273-6280

Introduction

Currently, almost 510,000 cases of cervical cancer are reported each year with nearly 80% of the cases are reported in the developing countries; 68,000 in Africa, 77,000 in Latin America, and 245,000 in Asia. In Malaysia, cervical cancer is the most common gynecological malignancy and the second most prevalent cause of death among female cancer patients (Zvavahera et al., 1995). Through the recent development of technologies, cervical cancer patients can be treated by surgery, radiotherapy, chemotherapy and immunotherapy. However, the side effect caused by this treatment is severe and affect their quality of the life (WHO, 2012).

The side effects from the cancer therapy are that the treatment can also damage healthy cells and tissue. Apoptosis is the cell death mode that can be seen in multicellular organisms which kills the specific cells and removed it through phagocytosis for the benefit of the organisms. It involved biochemical and physical changes

in the cytoplasma, nucleus and plasma membrane. Due to it programmed death mode, the apoptotic bodies which undergo physiological changes in the plasma membrane will enable the recognition of the apoptotic bodies by the phagocytic cells. Since the apoptotic bodies are surrounded by intact plasma membrane, apoptosis usually occur without the leakage of the cell content and without inflammation (Lawen, 2003).

Several medicinal plants had been used traditionally for the prevention and treatment of cancer (Madhuri and Govind, 2009). According to the latest reports, there are about 1200 species of plants in Malaysia that potentially had pharmaceutical or medicinal value (Khatijah, 2006). *Goniothalamus* is one of the medicinal plants that contain substance which possess the ability to control growth of cancerous cell through increasing the cancerous cell death without give the inflammatory effect to the normal cells (Ali et al., 1997; Nasir et al., 2004).

This plant is commonly found in Malaysia and Thailand is from the family Announce. Goniothalamin

¹Dental Research and Training Unit, ²Oral Cancer Research and Coordinating Centre, ³Department of Molecular Medicine, ⁴Department of Restorative Dentistry, Faculty of Dentistry, University of Malaya, Kuala Lumpur, ⁵Department of Biotechnology, ⁶Department of Chemistry, Universiti Sultan Zainal Abidin (UniSZA), Kuala Terengganu, ⁷Community Medicine Department, International Medical University, Kuala Lumpur, ⁸International Medical School, Management and Science University (MSU), Shah Alam, Selangor, ⁹Newcastle University Medicine Malaysia , Johor Bahru, Malaysia *For correspondence: aied_absi@yahoo.com

is a biologically active styrylpyrone derivative isolated from various Goniothalamus sp. Styryl-lactones is the active compound that able to kill the cancerous cell. Currently, there are approximately 100 styryl-lactones either discovered from natural products or synthetic analogs. These compounds have been demonstrated to be cytotoxic with prefential killing of cancer cells (Lin and Pihie, 2003; Chen et al., 2005; De Fátima et al., 2006; Al-Qubaisi et al., 2011).

Goniothalamin had been able to induce cytotoxicity in a variety of cancer cell lines including gastric (HGC-27), kidney (768-0), breast carcinomas (MCF-7, T47D and MDA-MB-231) and leukemia (HL-60, Jurkat and CEM-SS) (Chen et al., 2005; 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). It also represents a novel class of anticancer drug that induces apoptosis in Hepatocellular carcinoma (Kuo et al., 2011). Goniothalamin is a promising antitumor agent against cancerous cell lines (Wattanapiromsakul et al., 2005). On the other hand a study by Vendramini-Costa et al , 2010 demonstrated that the antiproliferative activity of goniothalamin in some solid tumor experimental model with no evidence of toxic effects in the animals after single and repeated doses.

This study is carried out to detect apoptosis accrue in Hela cervical cell line induced by goniothalamin via observing the morphological and biochemical features and to study the mechanism of GTN toxicity on Hela cells particularly on the mode of cell death, cell proliferation, DNA damage and caspases activity.

Materials and Methods

Goniothalamin extract. Dried and powdered root (500g) of Goniothalamus macrophyllus were extracted with dichloromethane and concentrated. 50g 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. Hela cell line obtained from animal tissue culture laboratory, Faculty of Agriculture and Biotechnology, Universiti Sultan Sultan Zainal Abidin. Hela cells were grown as a monolayer in 25 cm² tissue culture flasks (Nunclon TM, Nunc) at 37°C, 5%CO₂ 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 cultured till confluence and sub – cultured at three to four days interval.

Cell proliferation assessment

MTT Cytotoxicity Assay. Hela cells were trypsinized

and counted using hemocytometer then were seeded in 96-well micro plate at 5×10⁵ cells/ml and then incubated at 37°C in 5%CO₂ 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) and were incubated at 37°C, 5%CO₂ 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).

Trypan Blue Exclusion Assay. This assay was employed to determine the number of viable cells in cultures. Hela cells were incubated with different IC₅₀ and IC₂₅ concentrations of both Goniothalamin (3.2 and 0.9µg/ml) at 37°C. The viability of the cells was then determined at the designated time interval. Cells were analyzed by viable cell counts and the percentage cell viability was obtained. The results were expressed as the mean percentage of cell viability±SD of triplicate cultures.

Morphological assessment

Phase Contrast Microscopy. Hela cells at concentration of 1×10⁵ cell/ml in 2 ml culture medium containing 10% FBS was seeded into 6 wells plate (Nunclon TM, Nunc) and was treated with Goniothalamin at IC₅₀. Then, the plates incubated in an atmosphere of 5%CO₂ at 37°C. The morphological changes were observed after 72h by using a phase contrast microscope.

Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining. 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). Hela cells were seeded in 6-well plate and incubated at 37°C in 5%CO₂ atmosphere. Twenty-four hours later, the medium in each well was removed and replaced with Goniothalamin at IC₅₀ concentration dissolved in the culture medium and incubated at 37°C in 5%CO₂ atmosphere for 72 h. After the incubation period, detached cells in the medium were collected and added back to trypsinised adherent cells. The cell suspensions were washed with PBS and then incubated with 5µl of acridine orange (10µg/ml) and 5µl propidium iodide (10µg/ml) at a ratio of 1:1 in 1ml of cells and recentrifuged at 1000rpm/5min. A volume of 10µl of pellet was pipetted on a slide before putting on the cover slip. Within 30 min, the slide was analyzed using fluorescent microscope (Leica, Germany). Each experiment was assayed three times (n=3). 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. In apoptotic bodies, blebbing of plasma membrane and condensation of chromatin were seen. In contrast, necrotic cells fluorescent red after propidium iodide staining with tiny fraction of chromatin dispersed around the nucleus. This assay provides a useful quantitative evaluation (n=3).

Biochemical assessment

DNA Fragmentation Assay. Cells at a concentration of 5×10^6 cells/ml were seeded into 6-well plate (NunclonTM, Denmark) in 2ml culture medium with a concentration of IC_{50} value of goniothalamin. Some wells were left without treatment to be used as a control. After the 72h of incubation, detached cells in the medium were collected and added back to trypsinised adherent cells. The cells were spun down and the pellet was washed with PBS twice. The DNA extraction from treated and untreated cells was carried out according to the protocol of a kit for Blood and Cultured Cells from QIAGEN.

Single-cell gel electrophoresis Comet assay. The Comet assay was performed under alkaline conditions using the method adapted from Singh et al. (1980) as described previously by Gedik et al. (1992) and Collins et al. (1997). In brief, Hela cells with goniothalamin treatment were suspended in 1% low melting point agarose in PBS, pH 7.4, at 37°C, and 100 µl were pipetted onto a frosted glass microscope slide pre-coated with an 100 µl layer of 1% normal melting point agarose. The agarose was allowed to set on ice for 10 min and the slide immersed in lysis solution (2.5 M NaCl, 100 mM Na2EDTA, 10 mM Tris, NaOH to pH 10.0, and 1% Triton X-100) at 4°C for 1h to remove the cellular proteins. Slides were then placed in an electrophoresis tank containing 0.3 M NaOH and 1 mM Na2EDTA pH>12 for 30 min before electrophoresis at 25 V (1 V/cm, 300 mA) for 30 min at an ambient temperature of 4°C. The slides were then washed 3 times for 5 min, each with 0.4 M Tris-HCl, pH 7.5, at 4°C before staining with 5 µg/ml DAPI. The scoring for DNA damage was based on the length of the formed tail which represents the migration of DNA with strand breakage during electrophoresis following treatment (Chan et al., 2006). The images were captured using fluorescence microscope.

Caspases activity assay. The apoptosis assay, employing the caspase kits, was performed according to supplier's protocol (Promega, USA). Briefly, Hela cells were plated in a 96 well-plate at 5×10^5 cell/ml and treated with IC_{50} concentration of Goniothalamin in triplicate. Luminescence emission spectra were measured for the samples. Untreated cells were used as a negative control. This procedure was done to detect caspases 3/7, 8 and 9.

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

Effects of goniothalamin on Cell Cycle Distribution. HeLa cells at a concentration of 1×10^6 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 37°C in an atmosphere of 5%CO₂ for 24, 48 and 72 h. 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 h.

Flow cytometry (Annexin V/PI double staining): HeLa cells at a concentration of 1×10^6 cell/ml were seeded into the 6-well plate and treated with IC_{50} concentration of Goniothalamin. After 24, 48 and 72h 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±SE. Statistical analysis was performed with Student's t-test for data from AO/PI staining assay, Comet assay and Caspase activity assay. Differences were considered significant at p=0.05.

Results

Cell proliferation assessment

MTT cytotoxicity assay: Cytotoxicity of goniothalamin was evaluated using MTT assay. As shown as in Figure 1, the IC_{50} value of goniothalamin concentration that reduced the growth of treated cells by 50% compared to untreated cells was $3.2 \pm 0.72 \mu\text{g}/\text{ml}$. Table 1 determined the percentage of cell viability after treatment with different concentrations of goniothalamin, cell viability decreased with the increasing of goniothalamine concentration.

Trypan blue exclusion assay: Based on the results of the trypan blue dye exclusion assay, increasing goniothalamine exposure time had a significant effect on Hela cells viability. Treatment of Hela cells with IC_{50} and IC_{25} concentrations of goniothalamine (3.2 and 0.9 µg/ml) resulted in a dose-dependent inhibition of cell growth. As observed in Figure 2, at high concentration IC_{50} , cell viability had reduced from 78% at 24h to 61% and 55% at

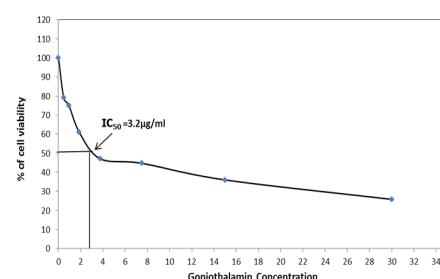


Figure 1. Percentage of Hela Cell Viability Against Goniothalamin Concentration. The IC_{50} value is the concentration required for 50% growth inhibition. Fifty percent of cell viability (IC_{50}) was obtained at $3.2 \pm 0.72 \mu\text{g}/\text{ml}$

Table 1. Percentage of Hela Cells Viability Treated with Goniothalamin in Different Concentrations

Concentration	% Cell viability
30	25.79867
15	36.01634
7.5	44.7142
3.75	47.11773
1.875	60.99233
0.9375	75.09763
0.46875	79.23344
0	100

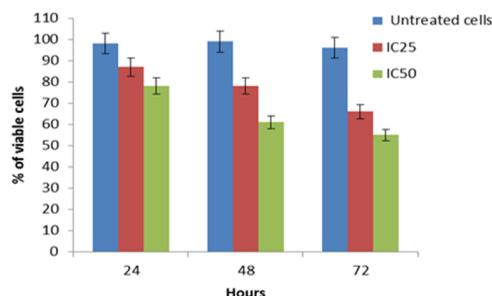


Figure 2. Trypan Blue Exclusion Assay. The percentage of viable cells in HeLa cell population after treatment with different concentrations of Goniothalamin at various time intervals

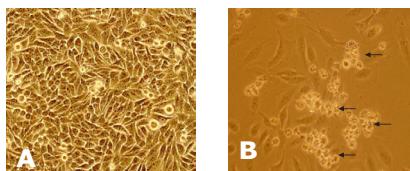


Figure 3. Phase Contrast Microscope. A) HeLa cells without any treatment after 72 h; **B)** Hela Cells Treated with IC₅₀ of Goniothalamin Compound after 72h. Appearance of membrane cell blebs (arrow) and also has some part of cell membranes disrupted. The decreasing number of cells signified that cell death had occurred (Magnification 200×)

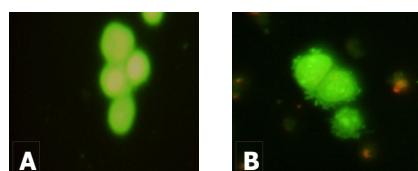


Figure 4. Cells Under Fluorescent Microscope after Staining with AO/PI Dye. A) Untreated cells stained bright green fluorescence with intact membrane and nucleus; **B)** Cells treated at IC₅₀ concentration of goniothalamin after 72h, cells appear with membrane blebbing (Magnification 400×)

48h and 72h, respectively. Whereas, at IC₂₅, cell viability reduced from 87% at 24h to 78% and 66% at 48h and 72h, respectively. Compared with the untreated cells, there were no significant differences in cell viability with time.

Morphological assessment of HeLa cell

Phase Contrast Microscopy. Morphological study of HeLa cell treated with goniothalamin compound was observed using the Phase Contrast Microscopy. The characteristics of apoptosis can be recognized by the presence of cell membrane blebbing, condensation of nuclear and fragmentation. Furthermore the cells in culture lost contact with adjacent cells. Besides that, apoptotic bodies and oval masses of cytoplasm, which were smaller than the origin cells were observed in the plasma membrane. The cytoplasmic organelles became compact from plasma membrane as observed in a small portion of the cell. These morphological changes were found to characteristics of apoptotic cell death (Figure 3).

Quantification of Apoptosis Using Propidium Iodide and Acridine Orange Double Staining. Fluorescent microscope was conducted to study of morphological changes of cell death mode induced by goniothalamin. Acridine orange (AO) and propidium iodide (PI) staining was used. Viable cells displayed green fluorescence

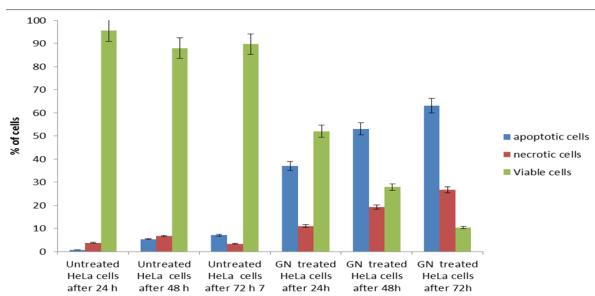


Figure 5. Fluorecent Microscopy Examination. Percentage of apoptotic cells, necrotic cells and viable cells in HeLa cell population with goniothalamin treatment after 24, 48 and 72h. HeLa cell death via apoptosis increased significantly ($p<0.05$) in time-dependent manner

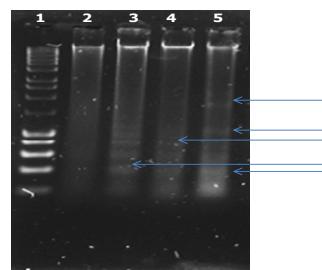


Figure 6. Agarose-gel-electrophoretic Patterns showing DNA Fragmentation (arrows) of Hela Cells Treated with Goniothalamin at IC₅₀ Concentrations. From left to right: lane 1: 1000 bp DNA markers; lane 2: untreated Hela cells; lane 3-5: Hela cells treated with goniothalamin after 24, 48, and 72 h, respectively

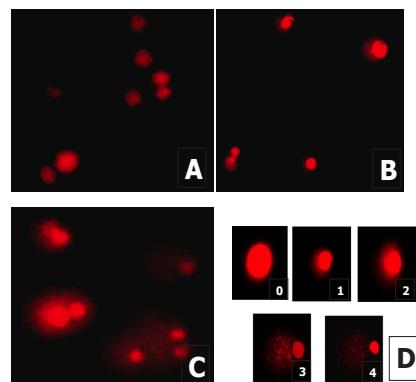


Figure 7. Hela Cells Under Fluorescent Microscope with Different DNA Damage Score after 2h Goniothalamin Treatment. **A)** Untreated cells; **B)** Cells treated at IC₁₀ value; **C)** Cells treated at IC₂₅ value. The DNA damage in Hela cells treated with goniothalamin increased in a concentration dependent manner (Magnification 400X)

with the appearance of circular cell with 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 4). Besides the study of morphological changes, the percentage of viable, apoptotic and necrotic cells plotted as a graph in Figure 5. The percentage of apoptotic cells in untreated cells slightly increased from 0.67% after 24h to 5.33% and 7% after 48 and 72h, respectively (Figure 5). Whereas, cells treated with goniothalamin at IC₅₀ concentration, the percentage

of apoptotic cells increased rapidly from 37% after 24h to 53% and 63% after 48 and 72h, respectively.

Biochemical assessment

DNA Fragmentation Assay. The DNA ladder assay is generally accepted as specific for apoptosis because it detects oligonucleosomal cleavage rather than artificial DNA cleavage or necrosis. The DNA of Hela cells treated for 24, 48 and 72h with goniothalamin was extracted and analyzed by agarose gel electrophoresis. Untreated cells contained only high-molecular-weight genomic DNA. The inoculation of goniothalamin on Hela cells showed the characteristic pattern of nucleosomal laddering specific to apoptosis which was visible as faint bands on the gel (Figure 6). Goniothalamin produce DNA fragments of low molecular weight consisting of multimers of 180-200 base pairs on Hela cell line.

Single-cell gel electrophoresis Comet assay. In this study, the alkaline comet assay was carried out to determine the DNA damage occurrence in Hela cells induced by goniothalamin. As shown in Figure 6 the DNA damage in Hela cells treated with goniothalamin increased in a concentration dependent manner. The percentage of total DNA damage induced by GN was 88% and 100% at IC₁₀ and IC₂₅ concentrations, respectively (Figure 7 and 8). As shown in Figure 6 and 7 untreated cells showed 100% cells with intact DNA (score 0). However, there was a significant decrease ($p<0.05$) in the percentage of cells with intact DNA (score 0) following treatment with IC₁₀ and IC₂₅ where the percentage was 12 and 0%, respectively. On the other hand, there was a significant increase in percentage of cells with damaged DNA following treatment with IC₁₀ GN, with 60, 24, 2 and 2% cells at scores 1, 2, 3 and 4, respectively. More cells with damaged DNA were observed in Hela cells treated with GN treatment at IC₂₅ where the percentage was 34, 48, 12 and 6% at scores 1, 2, 3 and 4, respectively. There was a significant difference between treated (IC₁₀ and IC₂₅) and untreated cells ($p<0.05$).

Activation of t the cellular level of caspase, 3/7, 8 and 9. Figure 8 shows the luminescence intensity values for caspase 3/7, 8 and 9. The chart shows a much higher luminescent intensity values of caspase 9 for Hela cells treated with goniothalamin (2.6×10^5) and (10×10^5) for 6h and 24h, respectively, compare with untreated cells (2.5×10^5). As shown in Figure 9 goniothalamin significantly ($p<0.05$) stimulated caspase 9 and there was no increase in caspase 3/7 and 8.

Quantification of Early apoptotic cells using fluorescent conjunction of 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. Table 2 showed the results of Annexin V/PI flow cytometry of HeLa cells after treatment with IC₅₀ value of goniothalamin. Untreated cell was found to be 6.4% of HeLa cells population while after 24h post-

treatment apoptotic cells increased to 26.45%.

Effects of goniothalamin on Cell Cycle Distribution. The state of HeLa cells was monitored by flow cytometry after propidium iodide staining nuclei. The treatment resulted in a loss of cells in G1 and G2/M phases of the cycle accompanied with a large increase in the sub-G1 and S phases. Goniothalamin induced time-dependent increase in the proportion of sub-G1 population. Apoptosis peak (sub-G1) was found in untreated cells with small percentage for about 2.44 and 6.62 for 24 and 48 hrs, respectively. The sub-G1 population in treated cells were 5.91 and 27.99 for 24 and 48 hrs, respectively. This GNT-induced cell cycle perturbation concurred with the results of the previous assay to suggest that HeLa cells undergo apoptosis more extensively With increasing in

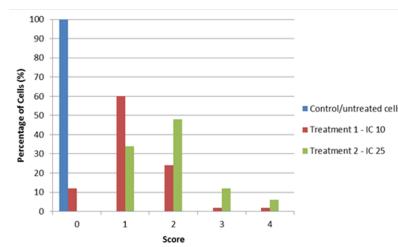


Figure 8. DNA Damage Scoring in Hela Cells after 2h Treatment with IC₁₀ and IC₂₅ of: Goniothalamin as Assessed by Alkaline Comet Assay. The results are the mean±SE. of three separate experiments. Each data are significantly different among group with $p<0.05$

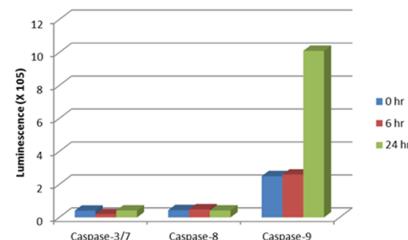


Figure 9. Luminescent Intensities Obtained after Adding Caspase 3/7, 8 and 9 for Hela Cells Treated with Goniothalamin after 0, 6 and 24 h. Goniothalamin has the ability to induce caspase 9 and there was no increase in caspase 3/7 and 8

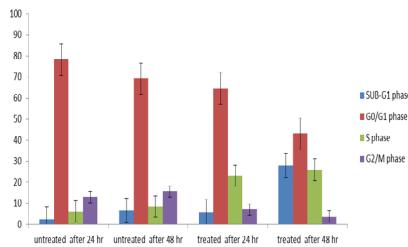


Figure 10. Effects of Goniothalamin on Cell Cycle Distribution. Goniothalamin induced time-dependent increase in the proportion of sub-G1 population

Table 2. Percentage of Early Apoptotic Cells in Hela Cell Line Population After Treated with Goniothalamin, Annexin V Positive and PI Negative

	Apoptotic cells %
Untreated Hela cells	6.40%
Hela cells after 24 h post-treatment	26.45%

time. On the other hand DNA histogram showed that goniothalamin increased the population of cells at S phase in a time-dependent manner (Figure 9). 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. DNA histogram showed that goniothalamin induce cell cycle arrest in S phase (Figure 10).

Discussion

Apoptosis is a regulated cell death process which leads to the complete cell destruction. These changes occur through characterized pathway including cell surface signalling, changes in morphology and caspase activation which lead to the DNA condensation and fragmentation. Most study had been carried out to determine the qualitative analysis of apoptosis in cervical cancer treated with anticancer compound. In this study we showed that goniothalamin decreases the human cervical cancer cells (Hela) viability via apoptosis. The cell viability decreased by goniothalamin was assessed by MTT Cytotoxicity Assay and Trypan Blue Exclusion Assay. Furthermore, the apoptosis induced by goniothalamin was confirmed by Propidium Iodide and Acridine Orange Double Staining, caspase- 9 activation, DNA fermentation.

In this study, goniothalamin isolated from Goniothalamus sp has indicated significant growth inhibition in Hela human cervical cancer cell line at low concentration of IC₅₀ values ($3.2 \pm 0.72 \mu\text{g/ml}$). 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; 2003; Chen et al., 2005; 2006; Nasir et al., 2004; Al-Qubaisi et al., 2011).

Based on the results of the trypan blue dye exclusion assay, Treatment of Hela cells with goniothalamin resulted in a dose- and time-dependent inhibition of cell growth rate. This observation was consistent with MTT results. It appears that Hela cells reacted differently when exposure to goniothalamin in the trypan blue dye exclusion assay. Cells count results indicated that viability of cells treated with goniothalamin was significantly inhibited while no obvious inhibition was detected in untreated cells. This may be contributed to by changes in the cell membrane triggered by the high goniothalamin concentrations, which may increase the uptake of trypan blue dye and the subsequent membrane alteration may have gradually resulted in cell death. Some evidence has indicated that goniothalamin exposure can alter the membrane properties (Inayat-Hussain et al., 2003).

Cell death can occur by two distinct processes: apoptosis and necrosis (Muneesh and Vishva, 1995). Apoptosis is natural phenomenon that occur in organism to eliminate a cell from organism without causing any inflammatory effect. The typical Morphological features of apoptosis in Hela cells after goniothalamin treatment observed through Phase Contrast Microscopy,

and characterized by the presence of shrunken cell with blebbing, nuclear condensation and fragmentation. Whereas the untreated Hela cells also showed a high confluence of monolayer cells compared to treated cells. This finding indicates that apoptosis had occurred on this method was previously done by Akira et al. (2003) on human colonic Adenocarcinoma cells and Dandan et al. (2006) on Hela cells (Murakami et al., 2003; Dandan et al., 2006).

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).

One of the most common methods used to confirm apoptosis occurred on cells is identification of DNA laddering. This is the biochemical hallmark of apoptosis with the fragmentation of the genomic DNA, an irreversible event that commits the cell to die but this method is qualitative rather than quantitative (Cohen et al., 1992). In this study we reported that goniothalamin stimulate DNA fragmentation characteristic of apoptosis in Hela cell line at 24, 48 and 72h post-treatment. DNA fragments reveal, upon agarose gel electrophoresis, a distinctive ladder pattern consisting of multiples of an approximately 180 base pairs subunit. DNA ladder formation is observed only when the extent of oligonucleosomal cleavage is prominent. Internucleosomal cleavage of DNA is likely to be in the later phase of apoptotic process (Cohen et al., 1992; Oberhammer et al., 1993; Gooch and Yee, 1999). In most cell types, the biochemical characteristics of apoptotic response include activation of endogenous calcium and magnesium dependent endonucleases, leading to fragmentation of the chromosomal DNA. Initially, the DNA fragments are large (50-300 kb) but are later digested to oligonucleosomal size (multimers of 180-200 bp). The formation of this distinct DNA ladder is considered to be a biochemical hallmark of apoptosis (Cohen, 1093).

Comet analysis of somatic cells electrophoresed under alkaline conditions measures single DNA strand breaks, whereas neutral conditions measure double strand breaks. However, many forms of structural damage can be converted to strand breaks during cell preparation and electrophoresis (Collins et al., 1997; Olive, 1999) . In this study, we report on the increased DNA strand breaks in Hela cells after exposure to goniothalamin. Comet assay confirms the observation that islet DNA is a target for cytotoxic levels of goniothalamin. In Untreated samples, the cell's nuclei remained unchanged but when exposed to goniothalamin a significant increase of DNA damage was observed. Low doses of goniothalamin (IC₁₀) caused

slightly lower extent of DNA damage than high dose (IC_{25}). These results conducted to conclusions of data show that GTN initially induces DNA damage which subsequently leads to cytotoxicity primarily via apoptosis in VSMCs (Chan et al., 2006). Furthermore, we indicate that GTN induced DNA damage in more than 90% cells after 2h in low dose IC_{10} and 100% in high dose (IC_{25}) however from MTT only about 25% of these cells died after 72 h. This is further supported by the proliferation assay which shows that the cells were still able to divide and proliferate in the same concentration of goniothalamin. This result was similar to a study where the anti-tumor quinone RH1 caused DNA damage in more than 80% of NQ16 breast cancer cells but only about 50% of the cells succumbed to cell death 48h later (Dehn et al., 2005) and similar to another study of the effect on GTN on VSMCs (Chan et al., 2006).

Different scores indicate different degrees of DNA damage was described (Collins, 1997). Collins et al. have described that at score 2, the proportion of DNA at the tail is between 25 and 75% and at score 3, more than 75% of DNA is at the tail. Other studies have shown that for the possibility for cells to undergo DNA repair or rejoining, the level of DNA damage should be below 25% of DNA at the tail (Chan et al., 2006). Although our result was similar to cahn et al. which indicated that more than 90% of the cells treated with IC_{25} of GTN had DNA damage, only a small proportion showed score 3. Therefore, the majority of DNA damaged cells would have the potential for rejoining of the break. Although DNA damage was determined after 2h, cells were left for 72h in the cytotoxicity experiments. This gives an opportunity for the cells to undergo rejoining of the breakage especially when the severity of DNA damage produced is not high. This is indicative of DNA repair mechanism and it is known that DNA repairing mechanisms which involve rejoining of single and double strands breaks can occur in cells. For example, strand-break rejoining has been observed in normal and cancer cell lines in vitro after ionizing radiation (Olive, 1999). In situation where the DNA strand breakages overwhelm the repair mechanisms, the cells will execute the apoptotic death program.

As stated by the researchers, apoptosis can be either caspase-dependent or caspase-independent (Zhao et al., 2004; Lee et al., 2006). However, the mechanism of caspase-independent apoptosis was still poorly understood until recently. Caspase plays important roles in execution of apoptosis through either extrinsic or intrinsic pathways (Chen et al., 2005). Thus, further investigation is needed to study the activation of caspase and apoptotic pathway in HeLa cells treated with GTN.

In this study, Caspase-3/7, -8, and -9 Assays were done to investigate the role of caspase-3/7, -8, and -9 and their activities in GTN-induced HeLa apoptosis. The executioner caspase-3/7 activity was increased in low level, less than one-fold at 6 hours and 24 hours of treatment with GTN at IC_{50} as compared to untreated cells. The activity of the caspase-3/7 should be in the equivalent level with the activity of initiator caspase-9. Caspase-3/7 is the executioner caspase that subsequently activated after the activation of initiator caspase-8 or -9. However, there

was no significant increase in the activity of caspase-3/7 after 24 hours of treatment ($p>0.05$).

In 2004, Tawa et al. studied reported that in contrast to pro-enzyme, caspase-3 is difficult to detect once activated in HeLa cells due to its rapid degradation. Previous study also showed similar result in which caspase-3 activity in GTN treated cells was increased up to 6 hours (1.68-folds) before a steady decline onwards (Chan et al., 2010). The short-lived increase of caspase-3 activity was further supported with detection by immunoblotting analysis or Western blotting at 4 and 6 hours (Chan et al., 2010). Caspase-3/7 activity may also be detected if the experiment time-interval is planned on 2, 4, 6, 12, and 24 hours.

However, GTN-induced HeLa apoptosis did not involved caspase-8 activation. Although there was a small significant increase of caspase-8 activity in HeLa cells at 6 and 24 hours of treatment ($p<0.05$). The significant increase of caspase-8 activity was probably due to the cell naturally undergoes apoptosis at senescence stage through extrinsic and intrinsic pathway with the activation of caspase-8 and -9 respectively. Therefore, the small increase of caspase-8 activity was detected in HeLa cells at 6 and 24 hours of treatment.

In comparison, caspase-9 activity was detected 4.5-folds and 16-folds higher than caspase-8 activity at 6 and 24 hours of treatment respectively. However, there was no significant increase of caspase-9 activity at 6 hours of treatment ($p>0.05$). This may due to high activity of caspase-9 was detected in untreated cells. Furthermore, there was a very high significant increase of caspase-9 activity (5.2-folds) at 24 hours after treatment of GTN ($p<0.05$). This showed that GTN was able to induce apoptosis in HeLa cells through the intrinsic pathway with the activation of caspase-9 as an initiator caspase. Previous study reported that the sequential activation of caspase-9 but not caspase-8 leading to the downstream caspase-3 cleavage was observed in GTN-treated CASMCs (Cononary Artery Smooth Muscle Cells) (Chan et al., 2010). It has also been reported that GTN induced apoptosis in HL-60 and Jurkat cells via mitochondrial pathway (Inayat-Hussain et al., 2003; 2010). Thus, this findings suggested that the insignificant role of caspase-8 as an initiator caspase. Caspase-8 is not essential in GTN-induced apoptosis in HeLa cells..

Overall, these data presented here reveal that the cytotoxic effect of Goniothalamin towards the cervical cancer cells via apoptosis.

Acknowledgements

This research was funded by Research Grant from Universiti Sultan Sultan Zainal Abidin (UniSZA), Malaysia.

References

- Adrain C, Creagh EM, Martin SJ (2002). Caspase cascades in apoptosis. caspases-their role in cell death and cell survival. Ed. marek los and henning walczak. *Molecular Biology Intelligence*, **24**, 41-51.

- Al-Qubaisi M, Rosita R, Yeap SK (2011). Selective cytotoxicity of goniothalamin against hepatoblastoma HepG2 cells. *Molecules*, **16**, 2944-59.
- Ali A, Mackeen MM, Hamid M, et al (1997). Cytotoxicity and electron microreactive oxygen speciescropy of cell death induced by goniothalamin, *Planta Med*, **63**, 81-3.
- Ali R, Alabsi AM, Ali AM, et al (2011). Cytolytic effects and apoptosis induction of newcastle disease virus strain AF2240 on anaplastic astrocytoma brain tumor cell line. *Neurochem Res*, **36**, 2051-62.
- Chan KM, Rajab NF, Ishak MHA, et al (2006). Goniothalamin induces apoptosis in vascular smooth muscle cells. *Chemico-Biological Interactions*, **159**, 129-40.
- Chan KM, Rajab NF, Siegel D, Din LB, Ross D Inayat-Hussain SH (2010). Goniothalamin induces coronary artery smooth muscle cells apoptosis: the p53-dependent caspase-2 activation pathway. *Toxicol Sci*, **116**, 533-48.
- Chen WY, Wu CC, Lan YH, et al (2005) Goniothalamin induces cell cycle-specific apoptosis by modulating the redox status in MDA-MB-231 cells. *European J Pharmacol*, **522**, 20-9.
- Cohen GM, Sun RT, Snowden D, Dinsdale DN (1992). Key morphological features of apoptosis may occur in the absence of internucleosomal DNA fragmentation. *Biochem J*, **286**, 331-4.
- Cohen JJ (1993). Apoptosis: the physiologic pathway of cell death. *Hosp Pract*, **28**, 35-43.
- Collins AR, Dobson VL, Duinsk'a M, Kennedy G, Ttina R (1997). The comet assay: what can it really tell us? *Mutat Res*, **375**, 183-93.
- Collins AR, Ai-guo M, Duthie SJ, Duthie (1995). The kinetics of repair of oxidative DNA damage strand breaks and oxidised pyrimidines in human cells. *Mutation Res*, **336**, 69-77.
- Dandan R, Guanghua P, Huang CH, Wang BH, Zhang S (2006). Effect of rhodoxanthin from potamogeton crispus L. on cell apoptosis in HeLa cells. *Toxicology in vitro*, **20**, 1411-8.
- De Fátima A, Modolo LV, Conegero LS, et al (2006). Styryl lactones and their derivatives: biological activities, mechanisms of action and potential leads for drug design, *Curr Medical Chemistry*, **13**, 3371-84.
- Dehn, DL, Inayat-Hussain SH, Ross D (2005). RH1 induces cellular damage in an NAD(P)H:quinone oxidoreductase 1-dependent manner: relationship between DNA cross-linking, cell cycle perturbations, and apoptosis. *J Pharmacol Exp Ther*, **313**, 771-9.
- Doonan F, Cotter XM (2008). Morphological assessment of apoptosis, *Methods*, 200-4.
- Gedik CM, Ewen SWB, Collins AR. (1992). Singlecellgel electrophoresis applied to the analysis of UV-C damage and its repair in human cells. *Int J Radiat Biol*, **62**, 313-20.
- Gooch JL, Yee D (1999). Strain-specific differences in formation of apoptotic DNA ladders in MCF-7 breast cancer cells. *Cancer Lett*, **144**, 31-7.
- HagueA, Paraskeva C (2004). Apoptosis and disease: a matter of cell fate. *Nature Cell Death and Differentiation*, **3**, 1-7.
- Inayat-Hussain SH, Chan KM, Rajab NF, et al (2010). Goniothalamin-induced oxidative stress, DNA damage and apoptosis via caspase-2 independent and Bcl-2 independent pathways in Jurkat T-cells. *Toxicol Lett*, **193**, 108-14.
- Inayat-Hussain, H, Annuar BO, Din LB, Ali AM, Ross D (2003). Loss of mitochondrial transmembrane potential and caspase-9 activation during apoptosis induced by the novel styryllactone goniothalamin in HL-60 leukemia cells. *Toxicol In vitro*, **17**, 433-9.
- Inayat-Hussain SH, Annuar O, Laily D, et al (1999). Caspases-3 and -7 are activated in goniothalamin-induced apoptosis in human Jurkat T-cells. *FEBS Letters*, **456**, 379-83.
- Khatijah H (2006). Anatomical Atlas of Malaysian Medicinal Plants. Malaysia, Universiti Kebangsaan Malaysia Bangi, Selangor (2006).
- Kuo K, Chen Y, Chen L, et al (2011). Involvement of phorbol-12-myristate-13-acetate-induced protein 1 in goniothalamin-induced TP53-dependent and -independent apoptosis in hepatocellular carcinoma-derived cells. *Toxicol and Applied Pharmacol*, **256**, 8-23.
- Lawen A (2003). Apoptosis-an introduction. *BioEssays*, **25**, 888-96.
- Lee WK, Abouhamed M, Thevenod F (2006). Caspase-dependent and -independent pathways for cadmium-induced apoptosis in cultured kidney proximal tubule cells. *Am J Physiol Renal Physiol*, **291**, 823-32.
- Lin TP, Pihie AHL (2003). Goniothalamin-induced apoptosis in human ovarian cancer cell line, Caov-3 through the regulation of Bcl-2 and Bax. *Borneo Sci*, **14**, 9-14.
- Madhuri S, Govind P (2009). Some Anticancer Medicinal Plants of Foreign Origin. *Current Science*, 1-5.
- Mishell BB, Shiiqi SM, Henry C (1980). Selected methods. In: Mishell BB, Shiiqi SM (eds) Cellular immunology. Freeman, San Francisco, 21-22.
- Muneesh T, Vishva MD (1995). Fas-and tumor necrosis factorinduced apoptosis is inhibited by the poxvirus crmA gene product. *Am Soc Biochem Mol Biol*, **270**, 3255-60.
- Murakami A, Hayashi R, Tanaka T, et al (2003) Suppression of dextran sodium sulfate-induced colitis in mice by zerumbone, a subtropical ginger sesquiterpene and nimesulide Separately and in combination, *Biochem Pharmacol*, **66**, 1253-61.
- Nasir UT, Mohamed Saifulaman MS, Rozita R, Laily BD, Leslie CL (2004). Genotoxicity of goniothalamin in CHO cell line. *Mutat Res*, **562**, 91-102 .
- Nasir UT, Saifulaman M, Rozita R, Laily BD, Leslie CL (2004). Genotoxicity of goniothalamin in CHO cell line. *Mutat Res*, **562**, 91-102 .
- Oberhammer F, Wilson JW, Dive C, et al (1993). Apoptotic death in epithelial cells: cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J*, **12**, 3679-84.
- Olive PL (1999). DNA damage and repair in individual cells: applications of the comet assay in radiobiology. *Int J Radiat Biol*, **75**, 395-405.
- Pihie AH, Stanslas J, Din, LB. (1998) Non-steroid receptor mediated antiproliferative activity ofstyrylpyrone derivative (SPD) in human breast cancer cell lines. *Anticancer Res*, **18**, 1739-44 .
- Rajab NF, Hamid ZA, Hassan H, et al (2005). Evaluation of the cytotoxicity and genotoxicity effects of goniothalamin in leukemic cell lines. *Environ Mutagen Res*, **27**, 161-4.
- Singh NP, McCoy MT, Tice RR, Schneider EL (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp Cell Res*, **175**, 184-91.
- Tawa P, Hell K, Giroux A, et al (2004). Catalytic activity of caspase-3 is required for its degradation: stabilization of the active complex by synthetic inhibitors. *Cell Death Differ*, **11**, 439-47.
- Vendramini-Costa D, de Castro I, Ruiz A, et al (2010). Effect of goniothalamin on the development of Ehrlich solid tumor in mice. *Bioorganic and Med Chem*, **18**, 6742-7.
- WHO (2011). Cervical Cancer. <http://www.who.int/reproductivehealth/topics/cancers/en/index.html>.
- Zhao S, Yang YN, Song JG (2004). Ceramide induces caspase-dependent and -independent apoptosis in A-431 cells. *J Cell Physiol*, **199**, 47-56.
- Zvavahera M C, Simbarashe R, Kirumbi L, et al (2001). Situation analysis for cervical cancer diagnosis and treatment in East, Central and Southern Africa Countries. *Bull World Health Organ*, **79**, 127-32.