ORIGINAL ARTICLE

Goniothalamin, a cytotoxic compound, isolated from *Goniothalamus macrophyllus* (Blume) Hook. f. & Thomson var. *macrophyllus*

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Abstract

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Goniothalamin, a cytotoxic compound, isolated from *Goniothalamus macrophyllus* (Blume) Hook. f. & Thomson var. *macrophyllus*

Songklanakarin J. Sci. Technol., 2005, 27(Suppl. 2) : 479-487

Bioassay guided fractionation by brine shrimp lethality test led to isolation of a cytotoxic compound, goniothalamin, from the root and stem of *Goniothalamus macrophyllus* (Blume) Hook. f. & Thomson var. *macrophyllus*. Goniothalamin showed a promising cytotoxicity (SRB assay) against colon cancer cell line (IC_{50} = 0.51±0.02 μg/ml), breast cancer cell lines (IC_{50}= 0.95±0.02 μg/ml) and lung carcinoma (IC_{50} = 3.51± 0.03 μg/ml). LDH assay of goniothalamin suggested that it had no toxicity on cell membrane. Cytotoxic evaluation of goniothalamin to normal cell revealed moderate toxicity against skin fibroblast (IC_{50} = 26.73± 1.92 μg/ml) and human fibroblast (IC_{50} = 11.99±0.15 μg/ml).

Key words : *Goniothalamus macrophyllus* var. macrophyllus, Goniothalamin, Cytotoxic, Brine shrimp lethality test, SRB assay

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Received, 3 October 2004 Accepted, 18 January 2005

บทคัดย่อ

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Goniothalamin สารที่มีฤทธิ์เป็นพิษต่อเซลล์แยกได้จากต้นชิงดอกเดียว (*Goniothalamus macrophyllus* (Blume) Hook. f. & Th var. *macrophyllus*)

ว.สงขลานครินทร์ วทท. 2548 27(ฉบับพิเศษ 2) : 479-487

การแยกสารจากลำต้นและรากของต้นชิงดอกเดียวโดยการทดสอบฤทธิ์ฆ่า brine shrimp ทำให้สามารถแยกสาร Goniothalamin ออกมาได้ เมื่อทดสอบฤทธิ์ความเป็นพิษต่อเซลล์มะเร็งของ Goniothalamin ด้วยวิธี SRB พบว่า Goniothalamin มีฤทธิ์ต้านเซลล์มะเร็งลำไส้ (IC_{50} = 0.51±0.02 μg/ml) เซลล์มะเร็งเต้านม (IC_{50} 0.95±0.02 μg/ml) และเซลล์มะเร็งปอด (IC_{50} = 3.51±0.03 μg/ml) ได้อย่างดี จากการวิเคราะห์หากลไกการออกฤทธิ์ของ Goniothalamin ด้วยวิธี LDH พบว่า Goniothalamin ไม่มีความเป็นพิษต่อเซลล์เมมเบรน และจากการวิเคราะห์ความเป็นพิษต่อเซลล์ปกติที่ได้จากหนู (skin fibroblast) และคน (human fibroblast) พบว่า Goniothalamin มีความเป็นพิษต่อเซลล์ปกติในระดับปานกลางด้วย IC_{50} = 26.73±1.92 μg/ml และ 11.99±0.15 μg/ml ตามลำดับ

ภาควิชาเภสัชเวทและเภสัชพฤกษศาสตร์ คณะเภสัชศาสตร์ มหาวิทยาลัยสงขลานครินทร์ อำเภอหาดใหญ่ จังหวัดสงขลา 90112

There are about 22 species of *Goniothalamus* found in Thailand, more than half of them in the southern part of Thailand (Chalermklin, 2001). *Goniothalamus macrophyllus* (Blume) Hook. f. & Thomson var. *macrophyllus* is a medicinal plant in the southern part of Thailand and known by the local name, "Ching Dok Diao" or "Rajchakru". Decoction of Ching Dok Diao was used by the ethnic group, Sakai, to nourish the blood and invigorate the body (Thonghom, 1993). Surprisingly *Goniothalamus* species and the isolated compounds from these plants have also been investigated for cytotoxic and antitumor properties (Ali *et al*., 1997; Cao *et al*., 1998; Hawariah and Stanslas, 1998; Bermejo *et al*., 1998) but there are very few reports for cytotoxicity of *Goniothalamus macrophyllus* against cancer cell lines especially lung, colon and breast cancer cells. Goniothalamin and goniothalamin oxide were isolated from the *Goniothalamus macrophyllus* as embryotoxic compounds (Sam *et al*., 1987).

This paper reports the isolation of cytotoxic compound, goniothalamin, from the methanolic extract of *Goniothalamus macrophyllus* var. macrophyllus roots and stems by using brine shrimp lethality test as a bioassay guide. The cytotoxicity of goniothalamin against colon cancer cell line, breast cancer cell line, lung carcinoma and normal cell lines was evaluated. Mode of action was conducted by LDH assay. The cytotoxicity of the leaf extract, stem extract and root extract was also evaluated against cancer cell lines and normal cell lines.

Materials and Methods

1. Plant Material

Roots, stems and leaves of *G. macrophyllus* var. *macrophyllus* were collected in 1996 from the area of Hat Yai district, Songkhla, Thailand. A specimen was identified by Mr.Charan Leeratiwong, Department of Biology, Faculty of Science, Prince of Songkla University, and the voucher specimen No. SKP 011-07-13-01 was deposited at the Herbarium of Pharmacognosy and Pharmaceutical Botany Department, Faculty of Pharmaceutical Sciences, Prince of Songkla University, Hat Yai, Songkhla, Thailand.

2. Extraction

Dried powdered roots (500 g), stems (1,400 g) and leaves (200 g) of *G. macrophyllus* var.

macrophyllus were extracted with methanol. The crude methanolic extracts of roots, stems and leaves gave 36.8 g, 27.0 g and 16.1 g, respectively.

3. Bioassay Guided Fractionation and Identification of Goniothalamin

The methanolic extracts of roots and stems were partitioned with dichloromethane and water. The brine shrimp lethality test guided fractionation indicated that the dichloromethane extracts of roots and stems showed a promising cytotoxicity (LD_{50} < 5.0 µg/ml). Further partition of dichloromethane extracts of both parts with hexane and 10 % methanol in water found that hexane extract of root and stem showed cytotoxicity in the brine shrimp lethality test with LD_{50} 20.73 and 57.77 µg/ml respectively whereas 10 % methanol of both parts showed cytotoxicity against brine shrimp lethality test with LD_{50} between 5 and 20 µg/ml. The cytotoxicity was lost after partition. However, 10 % methanol parts of both extracts were still more active than hexane extracts. The chromatographic separation of 10 % methanol part of both extracts using silica gel column was performed by eluting with hexane: ethyl acetate (9:1), leading to isolation of a white amorphous compound. The compound from the root (1.7848 g) and from the stem (0.4353 g) was identified as goniothalamin by comparing its spectral data and melting point with those of goniothalamin (Sam *et al*., 1987; Jewers *et al*., 1972).

4. Structural Analysis

NMR analyses were recorded in $CDCl_3$ on a Bruker Avance DPX-300 FT-NMR spectrometer, UV spectra were performed in methanol on HP 8452, Diode array spectrophotometer. IR spectra were obtained from a JASCO IR-180 Infrared spectrophotometer. Mass spectra were obtained from a MAT 95 XL Mass Spectrometer, Thermofinnigan and melting points were determined by Buchi Melting Point Apparatus SMP-20.

5. Brine Shrimp Lethality Test (Solis *et al*. 1990)

100 µl of artificial sea water containing 10-15 brine shrimp (Artemia salina) was pipetted into 96 microwell together with 100 µl of extract at concentrations of 1000, 100, 10 µg/ml (in artificial sea water with DMSO not more than 10% to dissolve the extract). The final concentration of each extract was 500, 50 and 5 µg/ml. Each concentration was run triplicate. A control was prepared by using 10% of DMSO in artificial sea water. The dead brine shrimp were counted within 24 hours using a microscope. Formalin was used to kill the rest of brine shrimp and the total number of brine shrimp was counted. LD_{50} was determined by using probit analysis.

6. *In vitro Assay* for Cytotoxic Activity

6.1 Cell Lines

Three types of cancer cell lines; large cell lung carcinoma COR-L23, human colon adenocarcinoma LS174T (ECACC No:86060401), and human breast adenocarcinoma MCF-7 (ECACC No:86012803) and two types of normal cells, fibroblast (ST3) of mouse skin and human fibroblast (HF) , were used. COR-L23 cells were established and kindly provided by Dr. P. Twentyman and Dr. P. Rabbitts of MRC Clinical Oncology & Radiotherapeutics Unit, Cambridge, UK, and cultured in RPMI 1640 medium supplemented with 10% heated-inactivated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin and 50 µg/ml streptomycin. LS-174T and MCF-7 cells obtained from the European Collection of Animal Cell Culture (PHLS Center for Applied Microbiology Research, Porton Down, Salisbury, UK) were cultured in Minimum Essential Media (MEM) with Earle's Salt (without glutamine medium supplement) with 10% heated-inactivated foetal bovine serum, 1% of 2 mM L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin and 1% non-essential amino acid, and maintained at 37ºC in a 5% CO_2 atmosphere with 95% humidity. (Keawpradub *et al*., 1997; 1999). ST3 was obtained from the Laboratory of Molecular Pharmacology and Pharmacotherapeutics, Graduate School of Pharmaceutical Sciences, Chiba University, Chiba, Japan. HF cell line was kindly provided by Dr. H. Navasaria, Department of Experimental Dermatology, St Bartholomews and Royal London School

of Medicine, Queen Mary and Westfield College, London UK. ST3 and HF were grown in an incubator with 10% CO_2 at 37ºC in Dulbecco's Modified Eagle Medium (DMEM) culture medium containing 10% foetal bovine serum, 1% of 10,000 U penicillin and 10 mg/ml streptomycin (Sampson *et al*., 2001). According to their growth profiles, the optimal plating densities of each cell line were determined ($1x10^3$, $1.5x10^3$, $3x10^3$ $4x10^3$ and $4x10^3$ cells/well for COR-L23, LS-174T, MCF-7, ST3 and HF) to ensure exponential growth throughout the experimental period and to ensure a linear relationship between absorbance at 492 nm and cell number when analysed by SRB assay.

6.2 Cytotoxicity Assay (Skehan *et al.*, 1990)

For the assay, cells were washed with magnesium- and calcium-free phosphate buffered saline (PBS) (Oxoid Ltd., UK). The PBS was decanted and the cells were detached with 0.025% trypsin-EDTA (Sigma). PBS was added to a volume of 50 ml. The cell pellet, obtained by centrifugation (1,000 g, 5 min), was resuspended in 10 ml of medium to make a single cell suspension. Viable cell density was counted by trypan blue exclusion in a haemocytometer and then diluted with medium to give concentrations of $1x10^4$, $1.5x10^4$, $3x10^4$, $4x10^4$ and $4x10^4$ cells/ml for COR-L23, LS-174T, MCF-7, ST3 and HF respectively. One hundred µl/well of these cell suspensions was seeded in 96-well microtiter plates and incubated at 37ºC to allow for cell attachment. After 24 h the cells were treated with the extracts or pure compounds. Each extract was initially dissolved in either dimethylsulfoxide, for ethanolic extracts, or sterile distilled water for water extracts. Vincristine sulphate (Sigma, Lot No.34H0447) was used as positive control. Each extract was diluted in medium to produce 8 concentrations of 0.1, 0.5, 1, 5, 10, 25, 50, 100 µg/ml. One hundred µl/well of each concentration was added to the plates in 6 replicates. Positive control was used at 0.05, 0.1, 0.5, 1,5, 10, 25, 50, 100 nM. The final dilution used for treating the cells contained not more than 1% of the initial solvent, this concentration being used in the solvent control wells. The plates were incubated for the selected exposure time of 72 hours. At the end of each exposure time, the medium was removed and the wells were washed with medium and 200 (l of fresh medium was then added. The plates were incubated at 37ºC for a recovery period of 6 days and cell growth was then analysed using the SRB assay. Three replicate plates were used to determine the cytotoxicity of each extract.

6.3 Sulphorhodamine B (SRB) assay

The antiproliferative SRB assay was performed to assess growth inhibition by a colorimetric assay, which estimates cell number indirectly by staining total cellular protein with the dye SRB (Skehan *et al*., 1990). In brief, cells were fixed by layering 100 µl of ice-cold 40% trichloroacetic acid (TCA, Aldrich Chemical) on top of the growth medium. Cells were incubated at 4ºC for 1 hour, after which plates were washed five times with cold water, the excess water was drained off and the plates were left to dry in air. SRB stain (50 µl; 0.4% in 1% acetic acid) (Sigma) was added to each well and left in contact with the cells for 30 minutes, after which they were washed with 1% acetic acid, rinsed 4 times until only dye adhering to the cells was left. The plates were dried and 100 µl of 10 mM Tris base pH 10.5 (Sigma) were added to each well to solubilise the dye. The plates were shaken gently for 20 minutes on a gyratory shaker and the absorbance (OD) of each well was read on a SLT 340 ATTC plate reader (SLT Lab instrument, Australia) at 492 nm. Cell survival was measured as the percentage absorbance compared to the control (non-treated cells). The IC_{50} values were calculated from the Anilisa program by plotting the percentage survival versus the concentrations, interpolated by cubic spine. According to National Cancer Institute guidelines (Boyd, 1997) extracts with IC_{50} values < 20 µg /ml were considered active.

6.4 Mode of Action of Cytotoxic Activity (LDH assay)

LDH or lactate dehydrogenase is a cytosolic enzyme which is not normally released from intact cells except under conditions involving cell death. LDH level is commonly used as an

indicator of cell viability when testing toxic compounds which affect the membrane and so cause cell death.

The principle of LDH assay is the presence of LDH in the supernatant which indicates cell damage. LDH activates the oxidation reaction of lactate to pyruvate with simultaneous reduction of nicotinamide adenine dinucleotide (NAD) as shown below:

$$\text{Lactate} + \text{NAD} \rightarrow \text{Pyruvate} + \text{NADH}$$

Formation of reduced nicotinamide adenine dinucleotide (NADH) results in an increase in absorbance at 340 nm. The rate of the increase in absorbance at 340 nm is directly proportional to LDH activity in the sample.

COR-L23 was treated with goniothalamin at different concentrations (50, 10, 1 and 0.1 µg/ml) and exposed for 72 hours. Then 10 µl of supernatant or medium was pipetted in duplicate into the 96-well plate, followed by 200 µl of Sigma Diagnostic® Lactate Dehydrogenase (LD-L) reagent, which was freshly prepared by reconstituting with Millipore water to give 50 mmol/L lactate, 7 mmol/L nicotinamide adenine dinucleotide (NAD), and buffer pH 8.9. The plate was immediately read at 340 nm at intervals of 9 sec over a period of 5 min in the Spectromax® 190 microplate spectrometer after initial shaking for 5 sec. The OD results were recorded at 5 min compared with Tritron X-100.

Results and Discussion

The percentage yields of the methanolic extract of root, stem and leaves were 7.36, 1.93, 8.05 respectively. Brine shrimp lethality test used as bioassay guided fractionation led to isolation of the cytotoxic compound from active fractions. It was found that the dichloromethane extracts of root and stem showed a promising cytotoxic activity against brine shrimp (LD_{50} < 5 µg/ml). After partition of dichloromethane extracts with hexane and 10% methanol in water each fraction was analysed with brine shrimp lethality test. The LD_{50} of hexane part of root and stem were 20.73 µg/ml and 57.77 µg /ml respectively. The LD_{50} of 10% methanol in water of both parts were between 5-20 µg/ml. The cytotoxicity was lost after partition. However 10 % methanol part of both extracts was still more active than the hexane extracts. The cytotoxic compound was isolated from 10% methanol of the root and stem of *Goniothalamus macrophyllus* var. *macrophyllus*. It was obtained as a white amorphous compound; EIMS found molecular ion peak at *m*/*z* 200, suggested a molecular formula as $C_{13}H_{12}O_2$; ^{1}H NMR (300 MHz, $CDCl_3$: δ 7.31 (m, 5H, aromatic protons), δ 6.94 (dt, 9.8 Hz, 4.3 Hz, 1H, H-4), δ 6.75 (d, 16.0 Hz, 1H, H-8), δ 6.29 (dd, 16.0 Hz, 6.3 Hz, 1H, H-7), δ 6.10 (dt, 9.8 Hz, 1.6 Hz, 1H, H-3), δ 5.12 (m, 1H, H-6), δ 2.57 (m, 2H, H-5); ^{13}C NMR (75 MHz, $CDCl_3$: 29.9 (C-5), 77.8 (C-6), 121.4 (C-3), 125.5 (C-7), 126.5 (C-10 & C-14), 128.1 (C-12), 128.4 (C-11 & C-13), 132.8 (C-8), 135.5 (C-9), 144.4 (C-4), 163.5 (C-2); strong uv absorption at wave length 254 nm and IR absorption at wave number 1720 cm^{-1}, 1658 cm^{-1}, 1244 cm^{-1} and 758 cm^{-1}; melting point 80-82ºC. It was identified as goniothalamin by comparing the spectrum data and melting point of this compound with those of the published papers by Sam *et al*., 1987 and Jewers *et al*., 1972. The chemical structure of goniothalamin is shown in Figure 1.

In previous research goniothalamin was isolated from several species of *Goniothalamus* (Jewers *et al*., 1972). Goniothalamin is known as an embryotoxic compound (Sam *et al*., 1987).

In cytotoxicity tests of each extract and goniothalamin against three human cancer cell lines by comparison with vincristine sulphate, a positive control, root and stem extracts showed the greatest cytotoxicity against three cancer cell lines but leaf extract showed the lowest cytotoxic activity

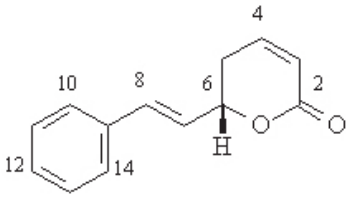

Figure 1. Goniothalamin.

Table 1. IC_{50} (µg/ml)±SEM of goniothalamin and the methanolic extracts of each part of *Goniothalamus macrophyllus* var. *macrophyllus* against cancer cell lines (N=3).

Sample/positive control	COR-L23	LS-174T	MCF-7	ST3	HF
Vincristine sulphate (µM)	1.38±0.16	1.66±0.13	2.67±0.14	>100	>100
Stem extract (µg/ml)	3.36±0.03	3.80±0.00	4.66±0.04	15.77±5.09	NT
Root extract (µg/ml)	3.16±0.14	4.54±0.35	5.04±0.03	4.83±0.33	NT
Leaf extract (µg/ml)	17.39±0.09	22.84±0.14	36.12±0.00	>100.0	NT
Goniothalamin (µg/ml)	3.51±0.03	0.51±0.02	0.95±0.02	26.73±1.92	11.99±0.15
Goniothalamin (µM)	17.55±0.18	2.57±0.09	4.75±0.09	133.63±0.19	59.95±0.11

NT = no test
N = number of independent experiments

(Table 1). Goniothalamin was a good cytotoxic compound against colon cancer cell line (LS-174T), breast cancer cell line (MCF-7) and lung carcinoma (COR-L23). Goniothalamin showed specific activity against colon and breast cancer cell line and less toxicity against lung cancer cells. Comparison of its activity against colon and against lung cancer cell lines indicated significance different by t-test from the Prism program ($P < 0.001$) (Figure 2). This compound exhibited less toxicity against a normal cell, especially ST3. The comparison between IC_{50} ratio of normal cell/cancer cell showed that the ratio of ST3/LS174T or fibroblast of mouse skin/colon cancer cells of goniothalamin showed the highest value (51.99) and P-value was extremely significant ($p<0.0001$). The ratio of HF/LS174T was also high (23.33) with $P<0.001$ (Table 2). This is the principal advantage of goniothalamin, because it showed great cytotoxic activity against colon and breast cancer cells and was less toxic against normal cells. Secondly, it showed specific activity when compared with vincristine sulphate as an anti-cancer drug. From Table 1, vincristine sulphate showed a ratio of cytotoxic activity against each cancer cell line less than 2 but goniothalamin showed ratios between lung cancer /colon cancer and lung cancer /breast cancer of 6.83 and 3.69 respectively. Both compounds showed less cytotoxicity against normal cells.

The amount of LDH released from each cell line was obtained by comparing the optical density (OD) of supernatant from cells treated by goniothalamin and each extract with those obtained for

Table 2. The ratio of IC_{50} (µg/ml) normal cells/IC_{50} (µg/ml) cancer cells of goniothalamin and each extract.

Extract/Compound	CORL-23		LS174T		MCF-7	
	ST3	HF	ST3	HF	ST3	HF
GMS	4.71	NT	4.16	NT	3.38	NT
GMR	1.53	NT	1.06	NT	0.96	NT
GML	>5.75	NT	>4.38	NT	2.76	NT
goniothalamin	7.61	3.41	51.99	23.33	28.13	12.62

Note: GMS, GMR and GML are the methanolic extract of *Goniothalamus macrophyllus* var. *macrophyllus* stems, roots and leaves respectively, NT = no test

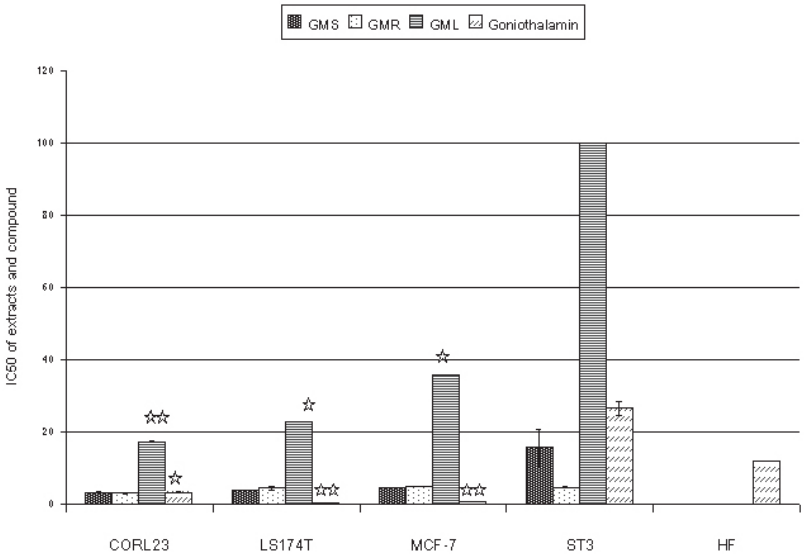

Figure 2. **IC_{50} (μg/ml) of goniothalamin and methanolic crude extracts on cell lines (N=3) with exposure time 72 h. Student t-test from the Prism program was used to determine the significant of differences between normal cell (ST3) and each cancer cell (COR-23, LS-174T and MCF-7) for * P<0.05 and for ** P<0.0001**
GMS = methanolic extract from *G. macrophyllus* var. *macrophyllus* stem
GMR = methanolic extract from *G. macrophyllus* var. *macrophyllus* root
GML = methanolic extract from *G. macrophyllus* var. *macrophyllus* leaf

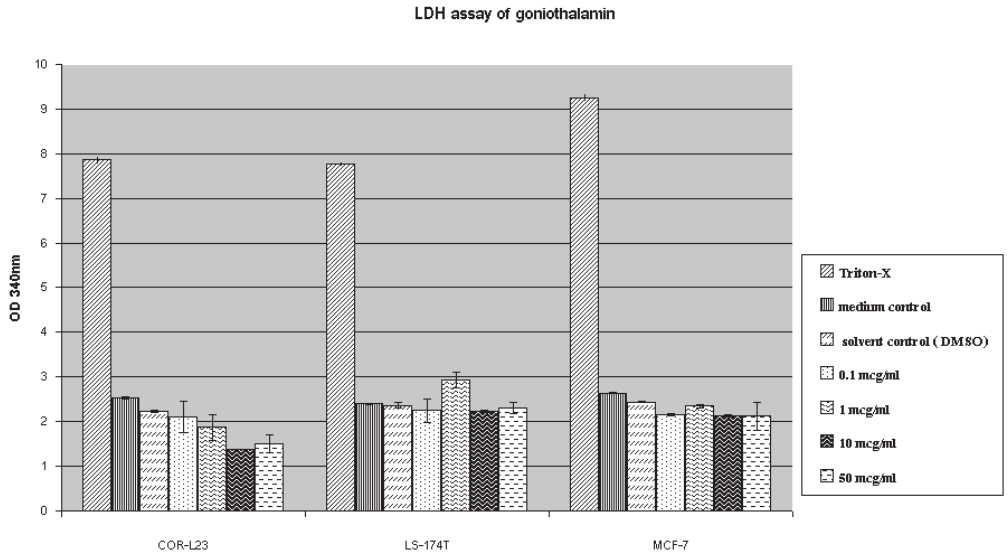

Figure 3. **LDH assay of goniothalamin. The histogram compares the OD values of medium control with OD values of samples treated with goniothalamin (μg/ml) against with lung (COR-L23), colon (LS174T) and breast (MCF-7) cell lines and also compared with Triton-X as positive control.**

cells treated with the detergent Triton X-100. Each cell line treated with goniothalamin was also compared with a control in which the cells were not treated. The LDH levels in each cell line (CORL-23, LS174-T and MCF-7) treated with the toxic compound, goniothalamin, are shown in Figure 3.

Goniothalamin showed no increase in OD values when compared with control but a significant difference ($p<0.0001$) when compared with Triton X-100 (Figure 3). It can be concluded that goniothalamin, which showed cytotoxic activity against cancer cell lines, had no toxicity against cell membrane. It was reported that goniothalamin-induced cytotoxicity revealed a necrotic mode of cell death towards MCF-7 (Ali *et al*., 1997).

In a study on HL-60 leukemia cell and human Jurget cell it was shown that goniothalamin induced apoptosis via the mitochondrial pathway in a caspase dependent manner (Inayat-Hussain 1999 & 2003). From these results, goniothalamin should be further studied for mode of action of cell death especially against colon cancer cells which it showed the highest cytotoxic activity.

Conclusion

This work suggests that goniothalamin is a promising antitumor agent against colon cancer cell line (LS-174T), breast cancer cell line (MCF-7), and large cell lung carcinoma (COR-L23), even though it showed more selective activity against colon cancer cell line and breast cancer cell line than against large cell lung carcinoma, and is a major component from the root and stem of *Goniothalamus macrophyllus* var. *macrophyllus*. However, the genotoxicity of goniothalamin may be the limiting factor in its use as an anticancer agent (Umar-Tsafe, 2004). The structure-activity relationship of goiothalamin and related styryl-lactones have to be studied to obtain more potent and less toxic lead compounds for anticancer drugs. The mode of action of goniothalamin suggest no toxic effect on cell membrane. Further study of the mode of action of goniothamin against different types of cancer cell lines should be carried out and *Goniothalamus macrophyllus* var. *macrophyllus* could be a source of goniothalamin.

Acknowledgements

The authors would like to express their gratitude to Dr. Khanit Suwanborirux, Department of Pharmacognosy, Faculty of Pharmaceutical Sciences, Chulalongkorn University, for NMR data, Mr. Charan Leeratiwong and Dr. Prakart Sawangchote, Department of Biology, Faculty of Science, Prince of Songkla University, for plant identification and plant collection, respectively.

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