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In vitro Response of Cancer Cells to the Growth-Inhibitory Effects of Dichloromethane Extract of *Goniothalamus umbrosus*

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Abstract: This present study aims to investigate whether *Goniothalamus umbrosus* extract has anticancer effects on several human cancer cells namely, the MCF-7 breast cancer cell line, HT-29 colon cancer cell line and CEM-ss leukemia cell line. Morphological changes and probable mode of cancer cell death induced by these *G. umbrosus* extract was examined. The dichloromethane plant extract of *G. umbrosus* was investigated for its cytotoxic activities on the human cancer cells using a 3 days MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The MTT assay results revealed that the extract has anticancer effects on human breast cancer cells (MCF-7). Half maximal inhibitory concentration (IC_{50}) of the hexane extract was found to be 19.5±0.338 mg mL^{-1} and this concentration was further used in investigations towards MCF-7 human breast cancer cells. Both inverted and fluorescence microscopic studies demonstrated that treated MCF-7 breast cancer cells using IC_{50} of the extract displayed a number of typical morphological changes. Appearance of membrane blebs, DNA condensation and fragmentation are significant signs of apoptosis. The above findings suggested, that the hexane extract of *Goniothalamus umbrosus* has potential therapeutic effect towards human breast cancer cells that requires further investigations in future.

Key words: *Goniothalamus umbrosus*, breast cancer, apoptosis, MCF-7, HT-29

INTRODUCTION

Cancer is an aberrant net accumulation of atypical cells, which can arise from an excess of proliferation, an insufficiency of apoptosis, or a combination of two (Hetts, 1998). Worldwide, >1 million women are diagnosed with breast cancer every year, accounting for one-tenth of all new cancers and 23% of all female cancer cases. It affects one in every ten women in Western Europe and the USA (Chang, 1998) and it is the second leading cause of cancer-related deaths (Parker, 1997). To date, >3000 plant species have been reported to be used in the treatment of cancer (Gordon and David, 2005; Aa and Mh, 2008). More than 60% of anti-cancer agents currently used are derived from natural sources, which include the plant kingdom and marine organisms (Siddiq and Dembitsky, 2008) and also micro-organisms (Gordon and David, 2005).

Regardless of the presence of cytotoxic acetogenins and styryl-lactones in the genus Goniothalamus, only 22 species in the genus Goniothalamus (Family: Annonaceae), out of 160 species (13.7%) have so far been investigated (Wiart, 2007). This genus is known to possess versatile biological activities such as immunosuppressive and anti-inflammatory (Tanaka *et al*., 2001), anti-malarial (Najila *et al*., 2002; Ichino *et al*., 2006; Noor-Rain *et al*., 2007), anti-cancer (Hawariah and Stanslas, 1998; Inayat-Hussain *et al*., 2003; Lee *et al*., 2003; Umar-Tsafe *et al*., 2004; De-Fatima *et al*., 2005; Zhong *et al*., 2005; Tian *et al*., 2006), antioxidant (Likhitwitayawuid *et al*., 2006), larvicidal activity (Kabir *et al*., 2003) and inhibitory effects on platelet-activating factor properties (Jantan *et al*., 2005).

To the best of our knowledge, there is only one scientific report published regarding only one biological property of *G. umbrosus* by Umar-Tsafe *et al*. (2004), which studied the genotoxicity of goniothalamin in CHO cell line. This present study aims to investigate whether *Goniothalamus umbrosus* plant extracts has anticancer effects on several human cancer cells namely, the MCF-7 breast cancer cell line, HT-29 colon cancer cell line and CEMss leukemia cell line.

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MATERIALS AND METHODS

Plant material and extraction procedure: Fresh leaves of *G. umbrosus* were collected from Selangor State, Malaysia in 2007. The plant was identified by Mr. Tajuddin Abd Manap, Assistant Agriculture Officer, Unit of Biodiversity, Institute of Bioscience, Universiti Putra Malaysia, Malaysia. The leaves were dried and grinded into powder before cold maceration as an extraction method. Before extraction with DCM, the powdered leaves (300 g) were extracted using hexane, the remaining powdered leaves were extracted with DCM. The extraction done for 7 days with occasional shaking and the process repeated for three times. The combined DCM extracts were filtered through Whatman® No. 41 filter paper (pore size 20-25 μm) and dried under vacuum using a rotary evaporator and kept at 4°C until required. The extracts were stored in the refrigerator.

Preparation of extract: To screen the DCM extract of *G. umbrosus*, dried extract of the plant were dissolved in 1 mL of DMSO to give stock solution of extract (10 mg mL^{-1}). Extract was stored in 4°C till the end of experiments. During the experiment, stock solutions were diluted with the complete media (RPIM1640) to obtain original concentrations of 0.469, 0.938, 1.875, 3.75, 7.5, 15 and 30 μg mL^{-1}. The MCF-7 human breast cancer cell lines and HT-29 human colon carcinoma cells were purchased from American Type Culture Collection (ATCC), USA. Human T4-lymphoblastoid cell line CEM-ss were obtained from NIH AIDS Reagent and Reference Reagent Program, USA and used in this study. The cell lines were grown at 37°C at humidified CO_2 incubator with 5% CO_2 in RPMI-1640 media (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp., Auckland, New Zealand).

Cell growth inhibition assay (MTT test): The cell suspension (0.1 million cells mL^{-1}) was plated out into 96-well microplates. Plant extracts were dissolved with Dimethylsulfoxide (DMSO) and the final concentration of DMSO was 0.1% (v v^{-1}). Different concentrations of the sample were prepared with serial dilution. Dimethylsulfoxide (0.1%) was used as a control. The toxicity profiles of the extracts were assessed using the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide (MTT) microculture tetrazolium viability assay as described by Mosmann (1983). Thereafter, the various concentrations of plant samples were plated out in triplicate. Each plate included untreated cell controls and a blank cell-free control. After 68 h of incubation, MTT (5 μg mL^{-1}) was added to each well and the plates incubated for a further 4 h and the media removed. DMSO was later added into each well to solubilize the formazan crystals. The absorbance was read at 595 nm using a microplate reader (Labsystems iEMS Reader MF).The percentage cellular viability was calculated with the appropriate controls taken into account. The concentration, which inhibited 50% of cellular growth (IC_{50} value) was determined. The inhibitory rate of cell proliferation was calculated by the following formula:

$$\text{Growth inhibition} = \frac{OD_{control} - OD_{treated}}{OD_{control}} \times 100$$

where:
OD = Optical Density

The cytotoxicity of sample on cancer cells was expressed as IC_{50} values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Morphological studies: The treatment of the MCF-7 human breast cancer cells with *G. umbrosus* extract was done in 6 flat-bottom well tissue culture plate. First, all the medium were discarded from the plate. Three mL of new medium were added into each wells of plate by followed with appropriate amount of extracts into treated wells. Finally, the plate was incubated at 37°C in 95% air and 5% CO_2 for 72 h (Tong-Ju *et al*., 2007).

Inverted light microscopy: After 72 h, the plate was taken out from incubator and checked to confirm that there was no contamination detected. The plate was observed under inverted light microscope. Cells were identified as apoptotic if they display condensed nuclear, fragmented nuclei and/or blebing.

Fluorescent microscopy of AO/PI stained cancer cells: The entire medium were removed and kept in two different tubes for treated and control cells. Five hundred microliter of PBS were added into each well for washing medium. About 500 μL of trypsin were added into each well for 5 min until the cells were detached from the plate. All the cells were transferred into sterile tubes and centrifuged (1000 rpm for 5 min). Then the supernatants were discarded and 1 mL PBS was added into tubes and followed by re-centrifugation. After the supernatant were discarded, 5 μL of Propidium Iodide (PI) and 5 μL of Acridine Orange (AO) were mixed with the cells. The 10 μL of mixture were placed on a glass slide and covered by cover slip and viewed under fluorescent microscopic study.

Cells were identified as apoptotic if they display condensed nuclear, fragmented nuclei and/or blebing. The percentages of viable cells (green intact), apoptotic cells (green shrinking cells with condensed or fragmented nucleus) and necrotic cells (red) were determined from 100 cells for the data to be statistically significant.

Statistical analysis: Data was expressed as mean±SEM. SPSS statistical package was used to analyze the results and 0.05 was set as a level of significance.

RESULTS AND DISCUSSION

In this study, the anti-cancer effects of DCM extract of *G. umbrosus* have been investigated using standard MTT cytotoxicity test. These anticancer effects were further studied using morphological assessment of cacner cells using inverted and fluorescent microscope after staining with AO/PI dyes.

Cell viability assay (MTT) is an appropriate method for fast screening of new substances for cytotoxicity on cancer cells. The MTT reduction as a cell viability measurement is now widely chosen as the most advantageous endpoint (Alley, 1988). The principle of this assay is to distinguish between viable and unviable cells. From the dose-response curves, the Inhibition Concentration (IC_{50}) for DCM extract of *G. umbrosus* against each cell line was determined. From the results obtained, only IC_{50} of DCM extract has shown an anti-tumor effect towards MCF-7 cancer cells, which could be determined as 19.5 ± 0.339 μg mL^{-1} (Table 1). On the other hand, the extract did not cause any cytotoxic effect against HT-29 cells and CEMss cells. This had been shown that DCM extract of *G. umbrosus* were conferred non-effective in inducing cell death towards these two cell lines according to the guidelines from American National Cancer Institute, which considered that the IC_{50} for potential plant should <30 μg mL^{-1} (Alenka *et al*., 2000). These findings will support the previous reports about the possessing of Goniothalamus genus to anticancer properties (Hawariah and Stanslas, 1998; Inayat-Hussain *et al*., 2003; Lee *et al*., 2003; Umar-Tsafe *et al*., 2004; De-Fatima *et al*., 2005; Zhong *et al*., 2005; Tian *et al*., 2006). However, MTT results have been further supported with a morphological study using inverted and fluorescent microscopy.

Morphological assessment of DCM extract of *G. umbrosus* treated-MCF-7 using inverted microscope demonstrated that apoptosis might occurred due to exhibition of typical morphological features of apoptosis (Fig. 1). Apoptosis, a type of programmed cell death, is an active process and a way of eliminating a cell from an organism without eliciting a major host inflammatory and/or immune response. Morphological changes associated with apoptotic cell death induced by DCM extract of *G. umbrosus* were characterized by the presence of shrunken cells with surface blebbing, nuclear condensation and fragmentation (Schwartzman and Cidlowski, 1993). This method was previously applied to human colonic Adenocarcinoma cells (Akira *et al*., 2003) and HeLa cells (Dandan *et al*., 2006).

Table 1: IC_{50} values (μg mL^{-1}) of crude extracts of *G. umbrosus* in MCF-7, HT-29 and CEMss cells

	IC_{50} (μg mL^{-1})		
Cell type	MCF-7	HT-29	CEMss
Treatment of extract	19.5±0.338	>30	>30

All the values above are mean of 3 different determinations and errors represent standard error of mean

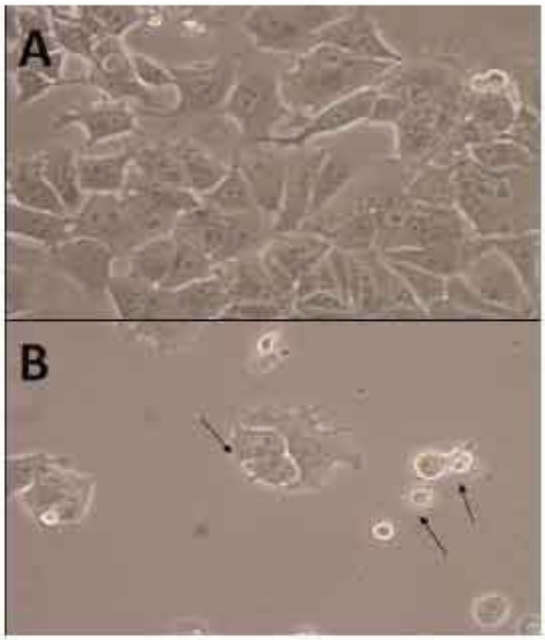

Fig. 1: A: MCF-7 control cells without any treatment with DCM extract of G. umbosus. B: Breast cancer cells (MCF-7) treated with IC_{50} of DCM extract of *G. umbrosus*. Appearance of membrane blebs and decreased number of cells signified that cell death had occurred (× 400)

Further study, was done to confirm the mode of cell death induced using fluorescent microscopy. Acridine Orange (AO) and Propidium Iodide (PI) staining was used. AO is a membrane-permeable, cationic dye that binds to nucleic acids of viable cells and that at low concentrations causes a green fluorescence (Fig. 2). PI is impermeable to intact membranes but readily penetrates the membranes of nonviable cells and binds to DNA or RNA, causing orange fluorescence (Mascotti *et al*., 2000). MCF-7 cells displayed green fluorescence with appearance of membrane blebs, nuclear condensation and fragmentation (Schwartzman *et al*., 1993). Based on morphological characterization, the untreated MCF-7 cells showed high viability with percentage of 97% and only 3% of apoptotic cells detected after 72 h incubation. Apoptotic cells found in untreated cells are due to natural cell death. This might be caused by nutrient depletion in growth media or contact inhibition. Besides the morphological changes of

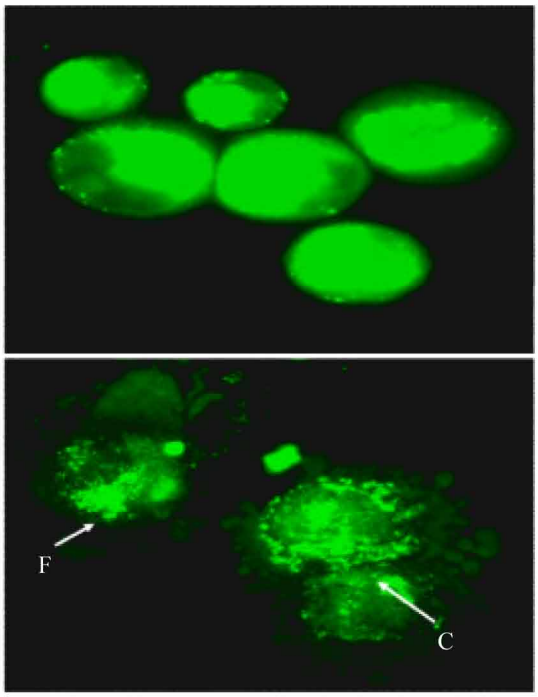

Fig. 2: Upper photo: Untreated cells at 72 h post inoculation after AO/PI staining. The cells were stained bright green fluorescence with intact cell membrane and nucleus. Lower photos: Cells treated at IC_{50} concentration (19.5 μg mL^{-1}) of dichloromethane extracts of *Goniothalamus umbrosus* at 72 h post treatment. Cell membrane blebbing (arrow) and nuclear fragmentation (F) were observed. Chromatin condenation (C) was highly bright green with AO stain. Picture taken at 40×10 magnifications

MCF-7 cells, the number of normal, apoptotic and necrotic cells were identified and counted after the treatment with 19.5 μg mL^{-1} (IC_{50}) DCM extract of *G. umbrosus*. Whereas, for the treated cells, the distribution of normal and apoptotic cells to be 51 and 49%, respectively (Fig. 3). Chi-Square test was carried out to evaluate statistical differences for the number of apoptotic cells in untreated and treated MCF-7 cells. Based on Chi-Square test ($p<0.05$), the number of apoptotic cell in the treatment is significantly ($p<0.005$) higher than those in untreated group (49% vs. 3%). Although, results of MTT and microscopic investigations are preliminary but it is encouraging to discover that this plant has anticancer effects towards breast cancer cells.

One of the most commonly used techniques for confirmation of apoptosis is identification of DNA ladders. Even though apoptotic features of the extract treated MCF-7 breast cancer cells could be seen in

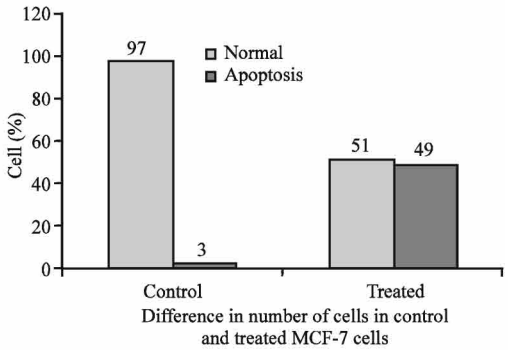

Fig. 3: The frequencies of normal and apoptotic cells in control and treated MCF-7 cells. Based on Chi-square test ($p<0.001$), in control, the frequencies of normal cells are significantly ($\chi^2 = 148.0$, $p<0.001$) high compared to normal cells in treated MCF-7 (97% vs 51%). While, frequencies of apoptosis are significantly ($\chi^2 = 52.0$, $p<0.001$) high in treated cells as compared to control (49 vs 3%)

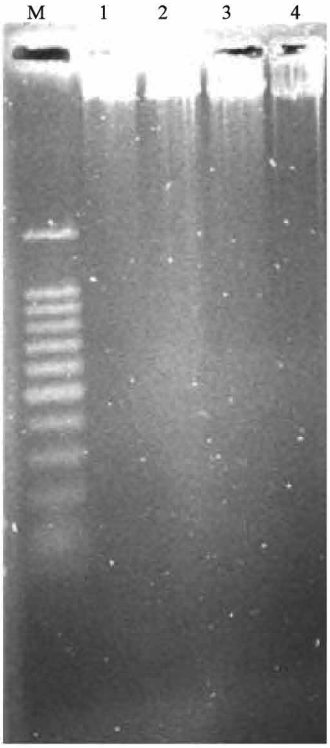

Fig. 4: Lane M is 100bp DNA marker, Lane 1-3 is DNA of control MCF-7 cells, Lane 4 is DCM extracts of *Goniothalamus umbrosus*-induced DNA of MCF-7 cells. The DNA of treated MCF-7 cells in Lane 4 does not fragment into characteristic laddered appearance. The DNA of MCF-7 cells were seen as smear at the top of the gel

inverted and fluorescent microscopic studies, no DNA ladder obtained in DNA fragmentation analysis (Fig. 4). The failure in obtaining DNA ladder was also reported by Hongmin *et al.* (1998) although they managed to find changes in nuclear structure consistent with apoptosis in MCF-7 breast cancer cells. It is also suggested that, DNA laddering represents a late event in the progression through apoptotic cell death and is not related to the ability of MCF-7 cells to undergo apoptosis, or to the amount of induction of apoptosis or the ability of MCF-7 cells to undergo DNA fragmentation appears to be an event, which has been either acquired or lost as a result of cell culture in some MCF-7 strains (Gooch and Yee, 1999). Thus, DNA fragmentation appeared to be a poor indicator of apoptosis in this study due to detection of DNA fragments failed to show ladder-like pattern, the hallmark of apoptosis. Nevertheless, the above findings suggested that the hexane extract of *Goniothalamus umbrosus* has potential therapeutic effect towards MCF-7 human breast cancer cells that requires further investigations in future.

CONCLUSION

In this investigation, the DCM extract of *Goniothalamus umbrosus* leaves showed potential anti-cancer activities towards breast cancer cells (MCF-7). The results obtained have suggested strongly that apoptosis has occur following the treatment by the plant extract. Such encouraging findings are a cornerstone to exploit this plant as a source for new anticancer drug for women breast cancer.

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