

Oncolysis of Breast, Liver and Leukemia Cancer Cells Using Ethyl Acetate and Methanol Extracts of *Goniothalamus umbrosus*

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Abstract: This current study, aims to investigate the ethyl acetate and methanol extracts of *Goniothalamus umbrosus* for their anticancer effects on several human cancer cells namely, the MCF-7 breast cancer, HT-29 colon cancer and CEM-ss leukemia cell lines using a 3 days MTT (3-(4 and 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide) assay. Morphological changes and probable mode of cancer cell death induced by bioactive *G. umbrosus* extract were examined. DNA laddering assay was performed to assess endonucleosomal fragmentation. The MTT assay results revealed that only the ethyl acetate extract has anticancer effects on human breast cancer cells (MCF-7). Half maximal Inhibitory Concentration (IC₅₀) of the ethyl acetate extract was found to be 24.5±0.12 µg mL⁻¹. Both inverted and fluorescence microscopic studies demonstrated that treated MCF-7 breast cancer cells using IC₅₀ of the extract displayed a number of typical morphological changes. Appearance of membrane blebs, DNA condensation and fragmentation are significant signs of apoptosis, were observed. The above findings suggested that the ethyl acetate extract of *Goniothalamus umbrosus* has potential therapeutic effect towards human breast cancer cells that requires further investigations in future.

Key words: *Goniothalamus umbrosus*, cancer, apoptosis, MCF-7, CEM-ss, HT29

INTRODUCTION

Human tumors are abnormal net accumulation of uncharacteristic cells, which can arise from an excess of proliferation, deficiency of apoptosis, or a combination of two (Hetts, 1998). Globally, >1 million patients 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 *et al.*, 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 (Wiar, 2007). This genus is known to

possess versatile biological activities such as immunosuppressive and anti-inflammatory (Tanaka *et al.*, 2001), anti-malarial (Siti 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-Fátima *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). In this respect, with absence of scientific report on *G. umbrosus*, the present study, was designed to investigate the oncolytic effects of ethyl acetate and methanol extracts of *Goniothalamus umbrosus* on several human cancer cells namely, the MCF-7 breast cancer cell line, HT-29 colon cancer cell line and CEMss leukemia cell line.

MATERIALS AND METHODS

Plant material and extraction procedure: The plant, *G. umbrosus* was collected from local area of Puchong, Malaysia. The plant was identified by the Assistant Agriculture Officer of the Unit of Biodiversity, Institute of

Bioscience, Universiti Putra Malaysia, Malaysia. Plant's leaves were dried and grinded into powder before cold maceration as an extraction method. Before extraction with ethyl acetate and methanol, the powdered leaves (0.3 Kg) were extracted using hexane and dichloromethane; the remaining powdered leaves were extracted with EA and MeTH. The extraction done for 7 days with occasional shaking and the process repeated for three times. The extracts were filtered using filter paper (pore size 20-25 µm) and dried and stored for further biological testing.

In vitro oncolytic assay of plant extracts: To screen the extracts 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 (RPIM 1640) 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 CO₂ incubator with 5% CO₂ in RPMI-1640 media (Sigma, MO, USA) supplemented with 10% fetal bovine serum (Invitrogen Corp., Auckland, New Zealand).

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 and 5-dimethylthiazol-2-yl)-2 and 5-diphenyltetrazolium bromide (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 (IC₅₀ value) was determined. The inhibitory rate of cell proliferation was calculated by the following formula:

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

OD = Optical Density

The cytotoxicity of sample on cancer cells was expressed as IC₅₀ values (the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells).

Morphological features of cancer cell: The treatment of the MCF-7 human breast cancer cells with ethyl acetate extract of *G. umbrosus* extract was done in 6 flat-bottom well tissue culture plate. First, all the medium were discarded from the plate. Total 3 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% CO₂ for 72 h (Tong-ju *et al.*, 2007).

Inverted photomicroscopy: 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 blebbing.

Fluorescent microscopy: The entire medium were removed and kept in 2 different tubes for treated and control cells. About 500 µL 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 blebbing. 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.

DNA fragmentation assay: MCF-7 cells, after treatment, were detached from culturing flask and collected in test tube using phosphate buffer saline. Apoptotic DNA ladder detection kit (Chemicon, USA) was used. Agarose gel electrophoresis was then carried out at 34 V for 3 h. The agarose gel was then stained with ethidium bromide for 15 min. Finally, the DNA was visualized by UV illumination.

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

RESULTS AND DISCUSSION

The current investigation was designed to explore the ethyl acetate and methanol extracts *G. umbrosus* for their oncolytic properties using standard MTT assay. These anticancer effects were further studied using morphological assessment of cancer cells using inverted and fluorescent microscope after staining with AO/PI dyes.

Proliferation assays, such as MTT test, are suitable method for screening of potential substances for their anti-proliferative against tumor cells. The MTT reduction as a cell viability measurement is now widely chosen as the most advantageous endpoint (Alley *et al.*, 1988). The principle of this assay is to distinguish between live and dead cells. From the dose-response curves, the Inhibition Concentration (IC_{50}) for both extracts of *G. umbrosus* against each cell line was determined. From the results obtained, only IC_{50} of ethyl extract has shown an anti-tumor effect towards MCF-7 cancer cells, which could be determined as $24.5\pm 0.12 \mu\text{g mL}^{-1}$ (Fig. 1). On the other hand, the extract did not cause any cytotoxic effect against HT-29 cells and CEMss cells. Methanol extract of this plant did not demonstrate any anti-tumor effects against all cancer cells used in this study (Table 1). This had been shown that extracts of *G. umbrosus* were conferred non-effective in inducing cell death towards these 2 cell lines according to the guidelines from American National Cancer Institute, which considered that the IC_{50} for potential plant should $<30 \mu\text{g mL}^{-1}$ (Alenka *et al.*, 2000). The current research findings are in consistence with previously published data, which indicates the possessing of *Goniiothalamus* genus to anti-proliferative activities (Hawariah and Stanslas, 1998; Inayat-Hussain *et al.*, 2003; Lee *et al.*, 2003; Umar-Tsafe *et al.*, 2004; De-Fátima *et al.*, 2005; Zhong *et al.*, 2005; Tian *et al.*, 2006).

Cytotoxicity of ethyl acetate extract was further confirmed using morphological assessment under inverted microscope, on MCF-7 cells. This assessment demonstrated that suspected cell death had occurred due to exhibition of morphological features changes (Fig. 2). 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 ethyl acetate extract of *G. umbrosus* were characterized by the presence of

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

Cell type	IC_{50} ($\mu\text{g mL}^{-1}$)		
	MCF-7	HT-29	CEMss
Ethyl acetate	$24.5\pm 0.12 \mu\text{g mL}^{-1}$	-	-
Methanol	-	-	-

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

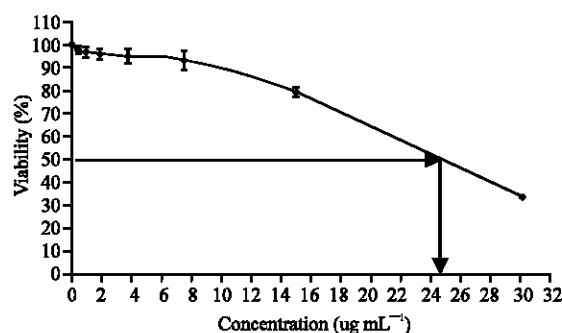


Fig. 1: A representative of dose response curve of MCF-7 cell lines treated with ethyl acetate extract of *G. umbrosus* at concentration of 0.469, 0.938, 1.875, 3.75, 7.5, 15 and $30 \mu\text{g mL}^{-1}$. The IC_{50} value for extract was determined to be $24.5\pm 0.12 \mu\text{g mL}^{-1}$

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).

Acridine Orange (AO) is a membrane-permeable, cationic dye that binds to nucleic acids of viable cells and that at low concentrations causes a green fluorescence. Propidium Iodide (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). AO and PI staining was used to study and confirm the mode of induced cell death using fluorescent microscopy. MCF-7 cells (Fig. 3) displayed green fluorescence with appearance of membrane blebs, nuclear condensation and fragmentation (Schwartzman and Cidlowski, 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 MCF-7 cells, the number of normal, apoptotic and necrotic cells were identified and counted after the treatment with $24.5 \mu\text{g mL}^{-1}$ (IC_{50}) ethyl acetate extract of *G. umbrosus*. The fluorescence

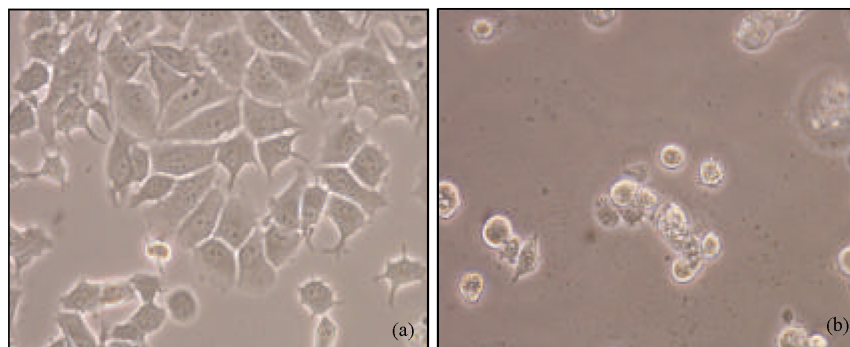


Fig. 2: A): MCF-7 control cells without any treatment with ethyl acetate extract of *G. umbosus*. B): Breast cancer cells (MCF-7) treated with IC_{50} of ethyl acetate extract of *G. umbosus*. Appearance of membrane blebs and decreased number of cells signified that apoptosis had occurred ($\times 400$)

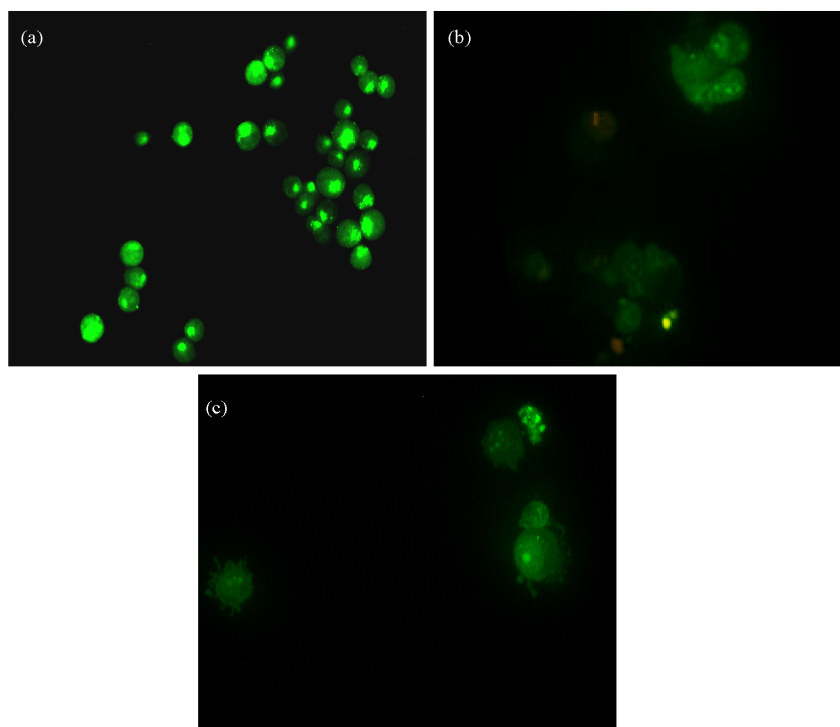


Fig. 3: Upper photo: MCF-7 control cells without any treatment with ethyl acetate extract of *Goniothalamus umbosus*. Middle and lower photos: breast cancer cells (MCF-7) treated with IC_{50} of ethyl acetate extract of *G. umbosus*. Morphological characterizations of cells undergo apoptotic death by acridine orange and propidium iodide staining. Appearance of membrane blebs (yellow arrows) and DNA fragmentation (white arrows) are signs of apoptosis of cells

microscopy analysis showed the distribution of normal, apoptotic and necrotic cells to be 96, 3 and 1%, respectively for the control. Whereas, for the treated cells, the distribution of normal, apoptotic and necrotic cells to be 42, 54 and 4%, 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 ($\chi^2 = 66.732$, $p < 0.05$), the number of apoptotic cell in the treatment is significantly ($p < 0.001$) higher than those in untreated group (54%

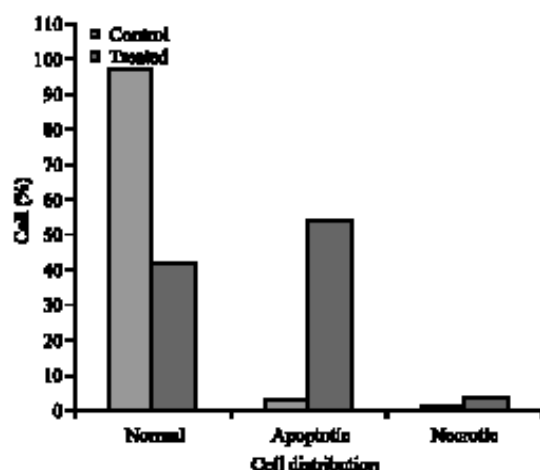


Fig. 4: Percentages of viable and apoptotic of MCF-7 cells before and after treatment with ethyl acetate extract of *G. umbrosus* at IC_{50} was determined with AO/PI staining. Based on Chi-Square test (High Chi-Squared value), the number of apoptosis in treatment is significantly ($p < 0.001$) higher than those in untreated group

vs. 3%) (Fig. 4). 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 (Fig. 2).

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 inverted and fluorescent microscopic studies, no DNA ladder obtained in DNA fragmentation analysis (Fig. 5). 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 ethyl acetate extract of *Goniothalamus umbrosus* has potential therapeutic effect towards MCF-7 human breast cancer cells that requires further investigations in future.

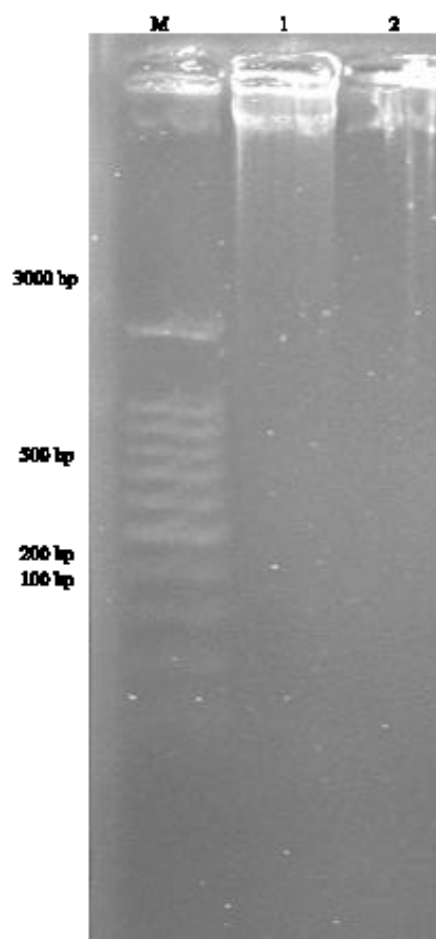


Fig. 5: DNA fragmentation of MCF-7 cells treated with $24.5 \mu\text{g mL}^{-1}$ of *G. umbrosus* extract for 72 h. Lane M: 100 bp ladder marker (Promega). Lane 1: Control (Untreated cells). Lane 2: MCF-7 cells were treated with extract at $24.5 \mu\text{g mL}^{-1}$

CONCLUSION

Only ethyl acetate extract of leaves of *Goniothalamus umbrosus* showed potential anti-cancer activities towards breast cancer cells (MCF-7). Both of the extract did not reveal any cytotoxicity towards liver and leukemia cancer cells. The obtained findings are suggesting that a type of cell death has occurred following the treatment by the plant extract. Such findings could be a basis to develop this plant as a source for new anticancer treatment for breast.

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