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Research Article

ANTIPROLIFERATIVE EFFECT OF THE METHANOL EXTRACT OF *GONIOTHALAMUS UMBROSUS* LEAVES ON HELA CELLS

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ABSTRACT

Use of natural products as an alternative medicine to treat cancer is increasing among people in Malaysia. Extracts of the herb *Goniothalamus umbrosus* (Kenerak) reportedly have cytotoxic and antiproliferative effects on breast, colon, kidney, leukemia, and pancreatic carcinoma cells, but scientific data are lacking. The goals of this study were to evaluate the phytochemical properties of the methanol extract of *G. umbrosus* leaves (GU-ML) to determine its antiproliferative effect on cervical cancer (HeLa) cells. Gas chromatography-mass spectrometry identified ten major phytochemical compounds present in the extract. The antiproliferative activities (IC₅₀ values) of the extracts were 45.31 µg/ml and 48.44 µg/ml as determined by the alamarBlue® and MTS assays, respectively. Morphological changes in cells treated with the extract included cell shrinkage, rounding of cells, vacuolization, and cell elongation, all of which indicate apoptosis. Overall, these data illustrate the antiproliferative effects of the GU-ML on cervical cancer.

Keywords: *Goniothalamus umbrosus*; GC-MS; MTS; alamarBlue®; antiproliferative; HeLa

INTRODUCTION

Cancer has become the second most common cause of death after cardiovascular disease¹ and it is the worst public health problem in the world. Omar and Tamin (2011) reported that cervical cancer is the third most common cancer among females². Development of cervical cancer can be triggered by several factors, including genetic and environmental aspects. Cancer is usually treated with chemotherapy, surgery, immunotherapy, radiation therapy, or monoclonal antibody therapy. However, these treatment options differ depending on the type of cancer and its location, cancer stage, the general health status of the patient³, cost of treatment, and possible side effects. The National Institutes of Health in the USA estimated that \$209.9 billion was invested throughout the world in 2005 for cancer treatment, thus it shown is a big economic challenge in cancer prevention development.

The use of natural products as an alternative medicine to treat cancer has been on the rise in Malaysia. The phytochemical contents of 22 (13.7%) out of 160 species in the genus *Goniothalamus*, including *Goniothalamus umbrosus*, have been investigated. Although the medicinal properties of *G. umbrosus* have not been verified via strict pharmacological experimentation, its chemical compounds have been studied. According to Abdelwahab et. al., 2009, in genus *Goniothalamus* and family Annonaceae the presence of lyphophilic secondary metabolic (acetogenins and stryl-lactone) has potential of cytotoxic⁴. Yang and Yu (2000) tested stryl-lactone and acetogenins for their chemical cytotoxicity activity and found that they were toxic to several human tumours cell lines⁵. Nasir

et al. (2004) reported that the bioactive stryl-lactone caused apoptosis of various human tumour cell and animal cell lines⁶.

Goniothalamus is the second largest genus of Annonaceae, and in Asia *Goniothalamus* has been widely used in traditional medicine⁶. *G. umbrosus* (known as 'kenerak' and the synonym name of *Goniothalamus tapis* Miq⁷ is a natural product found on the east coast of Peninsular Malaysia. It has been used for a long time in Asia as a traditional medicine to induce abortion and treat fever⁸. The goals of this study were to identify compounds in the volatile methanol extract of *G. umbrosus* leaves (GU-ML) and to determine the extract's antiproliferative effects on human cervical cancer (HeLa) cells. Ultimately, extracts from this plant might prove to be a source of new anticancer agents.

MATERIALS AND METHODS

Plant material

G. umbrosus leaves were obtained from Machang, Kelantan, Malaysia. Voucher specimens of this plant were deposited in the Herbarium Unit, School of Biology, Universiti Sains Malaysia (voucher number 11728).

Preparation of methanol extracts

Clean fresh leaves were extracted by grinding them with methanol and distilled water in a ratio of 4:1. The mixture was incubated on a shaker for 24 hours at 250 rpm. The mixture then was filtered through 125 mm filter paper, to isolate the supernatant. After that the methanol and distilled water in a ratio of 4:1 were added to the supernatant. The process was

repeated three times in 3 days about 72 hours. The filtered extracts were then pooled and dried in a freeze dryer for 1 week. The crude extracts (GU-ML) were stored at -20°C until used for analysis.

Gas chromatography-mass spectrometry (GC-MS)

The crude extracts was diluted with methanol, and a 1 μL aliquot was placed in a vial for analysis using an Agilent7890A GC-MS, (Agilent technologies, USA). An HP-5MS column (0.25 mm x 30 mm x 0.25 μm) was used. The column temperature was kept at 70°C for 2 minutes then programmed to reach 280°C at a rate of $20^{\circ}\text{C}/\text{min}$; it then was kept constant at 325°C for 0.5 minutes. The flow rate of helium (carrier gas) was 1 mL/min. The crude extract was analysed twice. The chemical components of GU-ML were identified by matching their mass spectra to mass spectral libraries (NIST).

Antiproliferative effect of the GU-ML

HeLa cell line

HeLa cells were obtained commercially (ATCC, Manassas, VA, USA). HeLa cells are cervix epithelial adenocarcinoma cells (i.e., attached cells). The cells were cultured in Dulbecco's Modified Eagle's Medium containing 10% fetal bovine serum and 1% penicillin-streptomycin. The cultures were incubated at 37°C with 5% CO_2 in a 25 cm^2 flask. The medium was replaced every 72 hours, and the cells were cultured until they reached 80–90% confluence. Only cell lines from passage 4 to passage 6 were used throughout the experiment.

Cell proliferation assay

HeLa cells were seeded at 5×10^3 cells per well of 96-well plates after the cells reached confluence and were subcultured with phosphate-buffered saline and trypsin to detach the cells. The cells were incubated at 37°C with 5% CO_2 . After 24 hours, cells were treated with 31.25, 62.5, 125, 250, 500, and 1000 $\mu\text{g}/\text{ml}$ of the GU-ML in triplicate. The negative control was complete medium without the GU-ML. The cells were incubated for 72 hours. The cells then were incubated with MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) and alamarBlue® to determine cell viability. The absorbance of cells was measured using a 96-well microplate reader (BioTek, US) Instruments at 460 nm.

In addition, morphological changes in the cells were observed and photographs of the cell cultures were taken at 40x and 100x magnification using an Olympus Xcam_Alpha camera attached to the phase contrast microscope (Olympus America Inc.).

Statistical analysis

The IC_{50} (50% inhibitory concentration) of the GU-ML was obtained by plotting the percentage cell survival against respective concentrations of extracts used in the assay. Values for data collected were from three independent experiments.

RESULTS

Extraction yield

Extraction (4:1 methanol: water) of *G. umbrosus* leaves (88.2 g fresh leaves) yielded 3.94 g of powder, for an extraction yield of 4.45%. (Table 1)

Volatile compounds in the GU-ML

Table 2 shows the ten major compounds in the GU-ML that were identified by GC-MS.

Antiproliferative effect of the GU-ML on HeLa cells

Percentage proliferation of HeLa cells treated with the GU-ML after 24 hours of exposure to alamarBlue® shows in Figure 1a whereas the percentage proliferation exposure by MTS shows in Figure 1b.

Figures 2 shows the effects of HeLa cells treated with the GU-ML at 31.25 to 1000 $\mu\text{g}/\text{ml}$ after 72 hours. However in Figure 3 shows morphological changes in HeLa treated with GU-ML observation under an inverted microscope.

DISCUSSION

In a previous study of *G. umbrosus* leaves collected from Machang, Kelantan, Malaysia, the aqueous methanol and aqueous ethanol extracts had the highest total phenolic content among the extracts tested⁹. This may be because phenolic compounds are often extracted in higher amounts in more polar solvents such as aqueous methanol or ethanol compared with absolute methanol or ethanol^{10,11,12}. Chatha et al. (2006) reported that the use of aqueous methanol solvent yielded the maximum extract yield (g/100 g) from rice bran¹³.

Table 1 lists the volatile compounds present in the GU-ML. All of the major volatile compounds appeared between 3.84 and 9.01 seconds. GC-MS is a functional device that extracts a polar solvent such as methanol extract and produced volatile oils with bioactive compounds. Nursyazana et. al. (2011) previously reported that methanol extracts of tualang honey had antiproliferative effects on human keloid fibroblasts due to the presence of fatty acids such as oleic acid, linoleic acid, octadecanoic acid, palmitic acid and stearic acid¹⁴. In *Jatropha curcas*, methanol extracts had cytotoxicity against MCF-7, HeLa, and Chang Liver cell lines¹⁵.

The major compounds detected in the GU-ML had biological potential for antiproliferative effects in HeLa cells due to the presence of 4H-Pyran-4-one, 2, 3-dihydro-3, 5-dihydroxy-6-methyl, 1, 2-benzenediol and phenol, 2, 6-dimethoxy-4-(2-propenyl) also were present. The presence of phenolics, flavonoids, and saponins in the extract provide antibacterial, antioxidant, and cytotoxicity activities¹⁵. In an animal study using propolis, which contains a lot of flavonoids, showed inhibited the development of several types of human cancer, including lung, oral, skin, esophagus, stomach, colorectal, prostate, and breast cancers¹⁶. Thus, the major compounds found in the GU-ML may have human health benefits.

The effects of the GU-ML on HeLa cells were evaluated using a range of concentrations. Antiproliferative activity of the GU-ML was determined by the MTS and Alamar Blue assays. The extracts had an inhibitory effect on HeLa cells, and the IC_{50} values obtained for the two methods were 45.31 $\mu\text{g}/\text{ml}$ (Figure 1a) and 48.44 $\mu\text{g}/\text{ml}$ (Figure 1b). Many types of assays based on different reagents are available to investigate cell proliferation^{17,18}. The MTS assay is a more advanced assay than the MTT (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide) method, as MTS reagents are reduced more efficiently compared to MTT in the cell. MTS is also less cytotoxic than the insoluble formazan used in the MTT assay. In this study, both methods showed significantly decreased cell viability relative to the control when the concentration of GU-ML was between 31.25 and 62.5 $\mu\text{g}/\text{ml}$. However, the percentage of

viable cells measured by both methods decreased with HeLa cells in a dose-dependent manner. increasing dosage of the extract. Thus, the GU-ML affected

Table 1: *G. umbrosus* leaves mass. 88.2g fresh leaves yielded, 3.94g powder. Methanol (4:1) water ratio phytochemical extraction yield was 4.45%

Items	Leaves (g)	Methanol : water (ml) (4:1)
Before extraction (fresh leaves)	88.22	400: 100
After extraction (concentrated & powdered)	3.93	-
% yield extraction	4.45	-

Table 2: GC-MS results for the GU-ML. Phytochemicals were identified based on the NIST Mass Spectrometry Data Centre reference library

No.	Name of compound	Rt ^a	Quality ^b
1	1,2-Cyclopentanedione	3.84	90
2	2-Cyclopenten-1-one, 2-hydroxy-	3.84	86
3	Benzaldehyde	4.11	94
4	4H-Pyran-4-one,2,3-dihydro-3,5-dihydroxy-6-methyl	5.83	90
5	1,2-Benzenediol	6.22	94
6	1,2-Benzenediol	6.29	87
7	2-Furancarboxaldehyde,5-(hydroxymethyl)	6.45	96
8	Ethanone, 2-hydroxy-1-phenyl-	6.61	87
9	Benzoic acid, methyl ester	6.61	83
10	Phenol, 2,6-dimethoxy-4-(2-propenyl)	9.01	97

^aRt = Retention time expressed in minutes

^bQuality = Compound similarities expressed in percentage (%)

Figure 1a: Percentage of proliferation on HeLa cell lines treated by *G.umbrosus* methanol leaves extracts after 24 h exposure by Alamar Blue.

Figure 1b: Percentage of proliferation on HeLa cell lines treated by *G.umbrosus* methanol leaves extracts after 24 h exposure by MTS.

Figure 2: Observation the effect of HeLa cells treated with *G.umbrosus* methanol leaves extracts at 72hours in 31.25ug/ml to 1000ug/ml concentration by inverted microscope (4 x magnification).

Figure 3: Morphological changes in HeLa cells treated with the GU-ML: (A) rounding of cells; (B) cell elongation; (C) cell shrinkage; (D) vacuolisation. Pictures were taken at 100x magnification.

Previous studies noted the morphological occurrence of cell shrinkage in dying cells, which has become a hallmark of apoptosis^{19,20}. Initial studies of cell shrinkage and apoptosis proved that a critical loss of intracellular potassium was important for the programmed cell death process that was independent of cell type or stimulus used to cause death²¹⁻²⁶. A few apoptosis-related morphological changes were observed in this study, such as cell shrinkage, cell elongation, rounding of cells, and vacuolization (Figure 3). These results suggest that the cell death or growth inhibition of HeLa cells treated with the GU-ML occurred through the apoptosis process.

Vacuolisation in the cell is a morphological characteristic of autophagy. Autophagy is a major intercellular protein degradation mechanism related to stress that involves amino acid or growth factor starvation²⁷. Autophagous cell death can be differentiated from other mechanisms of cell death by the formation of double or multiple membrane cytoplasmic vesicles that engulf bulk cytoplasm and also involve cytoplasmic organelles such as mitochondria and the endoplasmic reticulum. The lysosomal system in the cell releases secretions that destroy the autophagy vesicles and their contents. Hence, autophagous activity is far more extensive than that related to normal cytoplasmic and organelle turnover that occurs in normal cells. Consequently, the cell kills itself from the inside²⁸.

Overall, GU-ML treatment has the potential to cause both apoptosis and autophagous cell death in HeLa cells. However, further analysis is required to determine the actual cell death mechanism and to determine whether the treatment induced the observed cell death.

CONCLUSION

Screening of the GU-ML revealed that it has potential antiproliferative effects on HeLa cells. The extract inhibited

proliferation of HeLa cells with IC₅₀ values of 45.31 and 48.44 µg/ml for the Alamar blue and MTS assays, respectively. Treated cells showed morphological changes such as cell shrinkage, cell elongation, cell rounding, and vacuolization.

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