Journal of Medicinal Plants Research Vol. 3(11), pp. 883-888, November, 2009
Available online at http://www.academicjournals.org/jmpr
ISSN 1996-0875© 2009 Academic Journals

Full Length Research Paper

Biological and phytochemical investigations of *Goniothalamus umbrosus* leaves hexane extract

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Accepted 31 August, 2009

Antibacterial, antioxidant, anticancer properties and chemical compositions of *Goniothalamus umbrosus* (GU) hexane extract was investigated using disc diffusion method, DPPH assay, MTT cytotoxicity test (MCF-7 breast cancer cell line, HT-29 colon cancer cell line and CEMss leukemia cell line) and GC-MS, respectively. Anti-tumor effect of GU was further confirmed morphologically under inverted and fluorescent microscopy. Anticancer properties were only observed on MCF-7 with an IC_{50} of 20 ± 4.469 µg/ml. Morphology of MCF-7, after exposure to the extract, has suggested strongly the incidence of a cell death that might resemble to apoptosis. Antioxidant activity was not comparable significantly to the commercial standard antioxidant butylated hydroxytoluene (BHT). The extract failed to exhibit any antibacterial activity towards two Gram-positive bacteria, Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* B29, and other two Gram-negative bacteria, *Pseudomonas aeruginosa* 60690 and *Salmonella choleraesuis*. Analyses of the extract by gas chromatography and GC-mass spectrometry (GC-MS) tentatively identified 68 compounds, including a group naphthalene derivatives (18.33%) and eudesma-4(14),7(11)-diene (5.97%). A further research is recommended to verify the mechanism of oncolytic action of the hexane extract of *G. umbrosus*.

Keywords: *Goniothalamus umbrosus,* antibacterial, anti-oxidant; anti-tumor, GC-MS.

INTRODUCTION

The employment of medicinal plants for the cure of many diseases is related to folk medicine from different parts of the world. Natural products from some plants, fungi, bacteria and other organisms continue to be used in pharmaceutical preparations either as pure compounds or as extracts. There is an enormous diversity of compounds that can be isolated and elucidated from plants. One good example is the harmaline, one of the indole alkaloids found in *Peganum harmala* used in the treatment of dermatosis (Iwu et al., 1994). Another substance that can be found in plants is the morphine from the opium poppy, which has highly analgesic action and is still used. Its molecule is used as a model for design to reach new drugs (Phillipson, 1994). The isolation of these biological active compounds showed the real importance

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to investigate plants that can be sources of new compounds with clinical activities (Araujo and Leon, 2001).

Goniothalamus is the second largest genus of Annonaceae and widely used in traditional medicinal practices in Asia. *Goniothalamus umbrosus*, or known locally as kenerak, is perhaps one of the most interesting medicinal plants of the East coast of the Malay Peninsula, grown by generations of traditional practitioners mostly for healthcare. According to previous studies, the styrylpyrone goniothalamin, found within the family Annonaceae, is one of the bioactive styryl-lactones which appear to be mainly restricted to the genus *Goniothalamus*. Goniothalamin is the first styryllactone. It was crystallized from the light petroleum extract of *Goniothalamus species* bark. It has been shown to possess medicinal properties against various diseases and anti-cancer and apoptosis-inducing properties against various human tumour and animal cell lines (Jewers et al., 1972; Nasir et al., 2004). This compound can serve as an antimicrobial (Mosaddik and Haque, 2003), insecticidal (Kabir et al., 2003) and anti-fertility agent in rats. In particular, it was reported to exert cytotoxic properties in a variety of tumor cell lines including MCF-7, HeLa cells (Ali et al., 1997). In HL-60 cancer cells, it is demonstrated that goniothalamin induced apoptosis occurs via mitochondrial pathway (Inayat-Hussain et al., 2003). This compound has also been reported to be a potent mosquito larvicide in lymphatic filariasis (Kabir et al., 2003) and as an anti-fertility agent in rats without mating or behavioural side effects (Knowles, 2004). However, scientific investigation in order to determine the therapeutic potential of *G. umbrosus* is limited to its bioactive compound goniothalamin which isolated from the petroleum extract of this plant bark. Therefore, the aim of this study is to evaluate phytochemical and biological properties of hexane extract of *G. umbrosus* leaves.

MATERIALS AND METHODS

Plant material and extraction procedure

G. umbrosus was collected from Puchong, Selangor, Malaysia in October, 2007. The plant was identified by Mr. Tajuddin Abd Manap, Assistant Agriculture Officer, Unit of Biodiversity, Institute of Bioscience, University Putra Malaysia, Malaysia. Powdered leaves (300 gm) were extracted using hexane and cold maceration (3 x 500 ml). The combined 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.

Anti-cancer Activity of *G. umbrosus*

Human breast (MCF-7) and colon (HT-29) cancer cell lines were purchased from American Type Culture Collection (ATCC), USA. Human T4-lymphoblastoid cell line CEM-ss was obtained from Reagent and Reference Reagent Program, NIH-AIDS, USA. ATCC protocol recommended the use of RPMI 1640 (PAA, Germany) as a media for culturing cells. Cells were plated in 96-well plate. After an overnight incubation to allow cells attachment, medium were changed and 0.2 ml of new supplemented medium were added in each well. Cells were then treated, in a dose and time dependent-manner, with extract (0.469, 0.938, 1.875, 3.75, 7.5, 15 and 30 µg/ml), and were incubated at 37°C, 5% CO_2 for 72 h. Each concentration of the compounds was assayed in triplicates. MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay (Mosmann, 1983) reading was performed using ELISA plate reader (TECAN, SunriseTM, Männedorf, Switzerland). The cytotoxicity of sample on cancer cells was expressed as IC_{50} values which are known to be the drug concentration reducing the absorbance of treated cells by 50% with respect to untreated cells.

Morphological assessment using inverted microscopy

The treatment of the MCF-7 human breast cancer cells with *G. umbrosus* hexane extract was done in 6 flat-bottom well tissue culture plate. The plate was observed under inverted microscope. Cells were identified as apoptotic if they display condensed nuclear, fragmented nuclei and/or blebing.

Quantification of apoptosis using propidium iodide and acridine orange double staining

Extract-induced cell death on MCF-7 was quantified using Propidium Iodide (PI) and Acridine-Orange (AO) double staining and fluorescence microscope (Lieca attached with Q-Floro Software). Briefly, treatment was carried out in a 25-ml culture flask. Cells were plated at concentration of 1 x 10^6 cell/ml and treated with IC_{50}. The cells were then spun down at 1000 rpm for 10 min and washed twice using phosphate buffer saline. Ten microliters of fluorescent dyes containing AO (10 µg/mL) and PI (10 µg/mL) was added into the cellular pellet at equal volumes of each. Freshly stained cell suspension was dropped into a glass slides and covered using cover slip. Slides were observed under microscope within 30 min before the fluorescence color starts to fade. The percentages of viable and apoptotic cells were determined.

AO and PI are intercalating nucleic acid specific fluorochromes which emit a green and orange fluorescence, respectively, when they are bound to DNA. Of the two, only AO can cross the plasma membrane of viable and early apoptotic cells. Viewed by fluorescence microscopy, viable cells appear to have green nucleus with intact structure while apoptotic cells exhibit a bright green nucleus showing condensation of chromatin as dense green areas. Late apoptotic cells and necrotic cells will stain with both AO and PI. Comparatively, PI produces the highest intensity emission. Hence, late apoptotic cells exhibited an orange nucleus showing condensation of chromatin whilst necrotic cells display an orange nucleus with intact structure (Abdelwahab et al., 2009). This assay provides a useful quantitative evaluation and was done three times (n = 3).

Antibacterial activity

Gram-positive bacteria, Methicillin Resistant *Staphylococcus aureus* (MRSA) and *Bacillus subtilis* B29, and other two Gram-negative bacteria, *Pseudomonas aeruginosa* 60690 and *Salmonella choleraesuis* were obtained from Laboratory of Molecular Biomedicine, Institute of Bioscience, Universiti Putra Malaysia, Serdang, Malaysia. The screening of the extract antibacterial effect was carried out by determining the zone of inhibition using paper disc (6 mm) diffusion method (Sahoo et al., 2006; Rath et al., 1999). Bacteria were inoculated in a Petri-dish containing nutrient broth at 37°C for 24 h. The extract was dissolved in dimethyl sulphoxide which was previously tested for antibacterial activity against all test bacteria and found to have no antibacterial activity. The extract were diluted to concentration of 100 mg/ml and finally sterilized by filtration using 0.45 µm Millipore filters. The sterile discs were in-

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

Treatment of extract	IC_{50} (ug/ml)		
	MCF-7	HT-29	CEMss
Hexane	20 ± 4.469 µg/ml	-	-

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

pregnated with extract solution (0.005 ml from 100 mg/ml extract) to achieve desired concentration and placed in inoculated agar. Streptomycin (10 µg/disc) was used as standards. The controls were prepared using the same solvents without extract. The inoculated plates contain the test and standard discs were incubated at 37°C for 24 h.

DPPH radical scavenging assay

Radical scavenging activity of GU against stable DPPH (2, 2-diphenyl-2-picrylhydrazyl hydrate; Sigma-Aldrich, Steinheim, Germany) was determined spectrophotometrically (517 nm) (Changwei et al., 2008). When DPPH reacts with an antioxidant compound, which can donate hydrogen, it is reduced. The working solution (500 µg/ml) was prepared using methanol. The solution of DPPH in methanol (2.5 mg/ml) was prepared daily, before UV measurements (Labsystems iEMS Reader MF). Five µl of this solution were mixed with 100 µl extract solution 96-well plate. The samples were kept in the dark for 30 min at ambient temperature and then the decrease in absorption was measured. Absorption of blank sample containing the same amount of methanol and DPPH solution was prepared and measured daily. The experiment was carried out in triplicate. Radical scavenging activity was calculated by the following formula:

$$\% \text{ Inhibition} = [(A_B - A_A)/A_B] \times 100$$

Where: A_B: absorption of blank sample (t = 0 min); A_A: absorption of tested extract solution (t = 30 min). Commercial standard antioxidant butylated hydroxytoluene (BHT) was also tested against DPPH and used as a reference.

Gas chromatography–mass spectrometry (GC-MS) and mass spectrometer analysis

The GC-MS analysis was performed using a Shimadzu GS-MS – QP5050 spectrophotometer equipped with Shimadzu GC-17A, HP5MS (5% phenyl methylsilane) capillary column (30 x 250 µm x 0.25 µm) and helium as gas carrier. The constituents of oils were identified using GCMS technique, by comparing their mass spectral data with those from the Wiley mass spectral database.

Statistical analysis

Data was expressed as Mean ± SD. Percentages of viable and apoptotic cells before and after treatment (n = 3) was analyzed using Chi-Square test at 0.05 level of significance.

RESULTS AND DISCUSSION

In this study, some of the biological activities of hexane extract of *G. umbrosus* have been investigated. The anti-bacterial, anti-oxidant, anti-cancer properties and chemical composition was studied using disc diffusion method, DPPH assay, MTT assay and GC-MS, respectively. Anti-tumor effect of GU was further confirmed morphologically under inverted and fluorescent microscopy.

The microtitration assay (MTT) is appropriate method for rapid screening of large numbers of test substances for cytotoxicity. From the results obtained, the extract has shown only cytotoxic effect against MCF-7 (20 ± 4.469 µg/ml) (Table 1). This had been shown that hexane extract of *G. umbrosus* were conferred non-effective in inducing cell death towards these two cell lines (HT-29 and CEM-ss) according to the guidelines from American National Cancer Institute (Suffness and Pezzuto, 1990). Table 2

Morphological assessment of MCF-7 using inverted microscope demonstrated that suspected apoptosis had occurred due to exhibition of morphological features changes (Figure 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 (Amer, 2002). Morphological changes associated with apoptotic cell death induced by hexane extract of *G. umbrosus* were characterized by the presence of shrunken cells with surface blebbing, nuclear condensation and fragmentation.

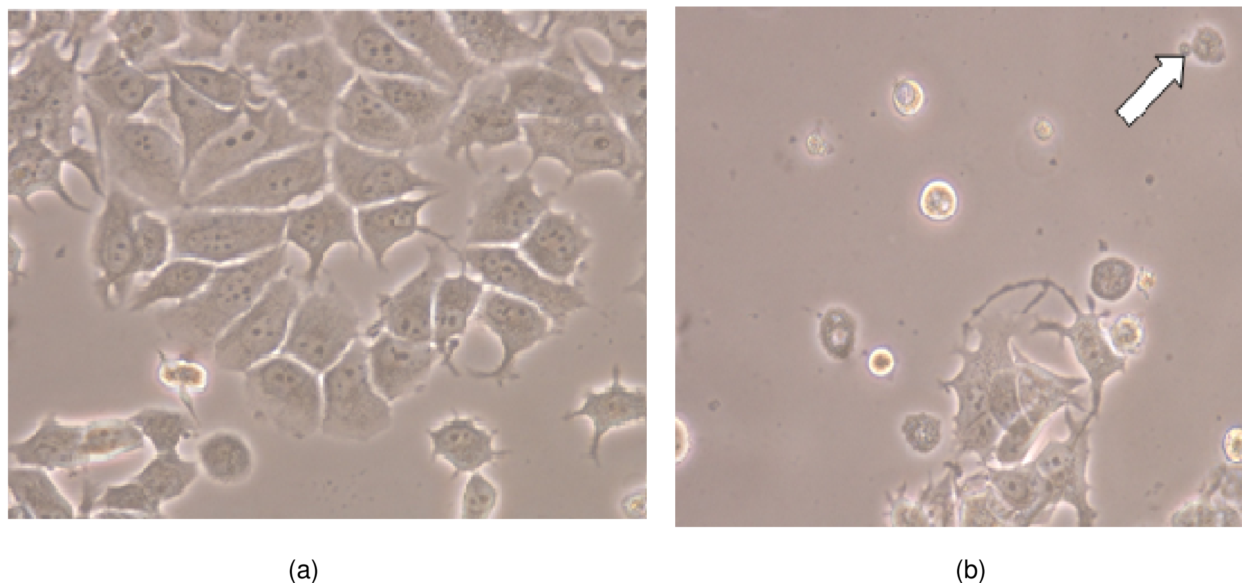
Further study was done to confirm the mode of cell death induced using fluorescent microscopy. Acridine Orange (AO) and Propidium Iodide (PI) staining was used. AO is a membrane-permeable, cationic dye that binds to nucleic acids of viable cells and that at low concentrations causes a green fluorescence (Figure 2). PI is impermeable to intact membranes but readily penetrates the membranes of nonviable cells and binds to DNA or RNA, causing orange fluorescence (Abdelwahab et al., 2009). MCF-7 cells displayed green fluorescence with appearance of membrane blebs, nuclear condensation and fragmentation. 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 treatment.

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. Whereby, the incidence of apoptosis at IC_{50} of hexane extract of *G. umbrosus* treated MCF-7 cells after 72 h increased to 51%.

Table 2. Composition of the hexane extract obtained by GC-MS.

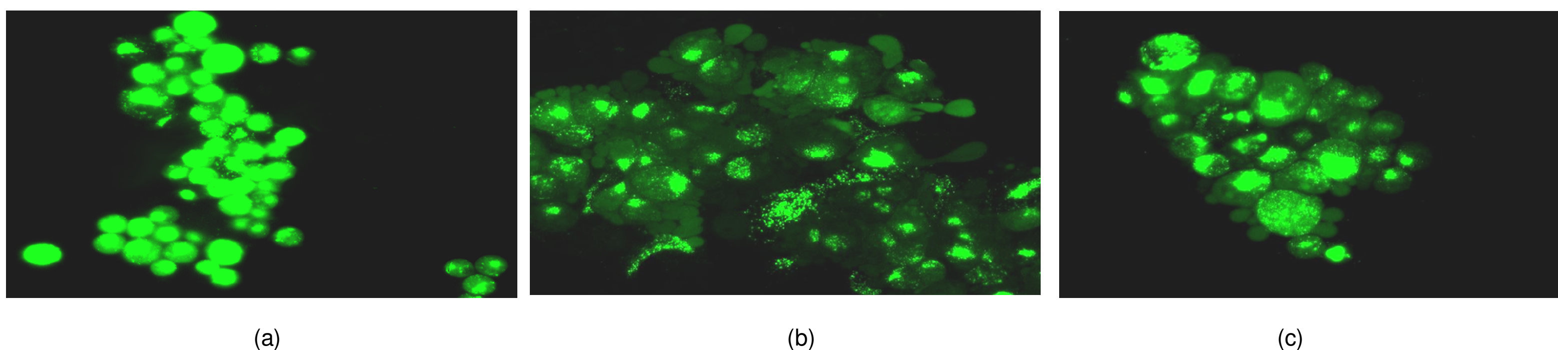
No	RT/min	Relative composition (%)	Compound	Homology %
1	13.292	3.22	Cyclohexane derivative	92
2	14.476	3.78	Napthalene derivative	94
3	14.742	5.97	eudesma-4(14),7(11)-diene	94
4	14.800	2.03	Napthalene derivative	92
5	14.817	5.15	Spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11 methylene-, (-)-	90
6	15.025	2.83	Napthalene derivative	92
7	15.886	3.03	Spathulanol	94
8	16.396	2.01	Globulol	84
9	16.833	2.80	Alpha cadenol	93
10	16.923	9.69	Napthalene derivative	89
11	17.693	2.92	trans-pinocarveol	82

RT = Retention time; Homology to MS standard = match degree (%) of mass spectrum to the standard compounds; Relative content = % of total absorbance of all peaks.



(a) (b)

Figure 1. A: Untreated MCF-7 control cells B: Treated MCF-7 with IC_{50} of hexane extract of *G. umbrosus.* Appearance of membrane blebs (Arrow) and decreased number of cells signified that apoptosis had occurred. (x400).



(a) (b) (c)

Figure 2. A: Untreated MCF-7 control cells B: Treated MCF-7 with IC_{50} of hexane extract of *G. umbrosus.*. Morphological characterizations of cells undergo apoptotic death by AO and PI. Appearance of membrane blebs and DNA fragmentation are signs of apoptosis of cells.

Cell viability decreased as cell death increased with the incidence of apoptosis (Figure 3). The percentages of dead cells exceeded the viable cells was due to apoptotic cell death mode. This indicated that hexane extract of *G. umbrosus* are able to induce apoptosis at half maximal inhibition concentration 20 µg/ml, which causes cell death in MCF-7 breast cancer cells.

Studies on the antibacterial activities of medicinal plants have clearly become a progressive trend using different screening method. Disc diffusion method was the first method of choice, possibly due to its simplicity and capability to analyze a large number of test samples. Many earlier publications used this method as a means of determining activity (Van-Vuuren, 2008). The antibacterial activities of this extract were evaluated using Gram-positive and Gram-negative bacteria. The solvent used for control and hexane did not show any activity (the results not shown). It has been noticed that all bacteria have shown resistance to hexane extract of *G. umbrosus.*

The results of DPPH inhibition by different plant extracts are summarized in Figure 4. Hexane extract of *G. umbrosus* has shown scavenging activity towards DPPH free radical (37.55%: Low absorbance values). Although this activity was not comparable with BHT, further studies are recommended to elucidate antioxidant activities of this plant. We selected the DPPH radical-scavenging assay due to its straightforwardness, quickness, sensitivity and reproducibility (Sanja et al., 2008). This method is also very handy for the screening of large numbers of samples with different polarity. As also used in this study, the extraction was performed in increasing order using sequentially different solvent systems.

Analysis of the hexane extract of *G. umbrosus* by GC–MS revealed a total of 68 compounds and tentatively identified 11 compounds, including Cyclohexane, 1-ethenyl -1- methyl-2,4-bis(1-methylethenyl)-, [1S-(1.alpha., 2beta., 4.beta.)]; napthalene derivative; eudesma-4(14) 11-diene; spiro[5.5]undec-2-ene, 3,7,7-trimethyl-11-methylene-, (-)-; Spathulanol; Globulol; Alpha cadenol and Trans-longipinocarveol. Naphthalene derivatives collectively were present in large amounts (18.33%) in the extract, as was eudesma-4(14), 11-diene (5.97%). Previous reports mentioned that napthalene derivatives have demonstrated a strong growth inhibitory activity against cell lines (Robert et al., 1961; Wang et al., 1998). Therefore, these derivatives of naphthalene might responsible for the anticancer properties of the hexane extract of *G. umbrosus* against breast cancer cell line (MCF-7).

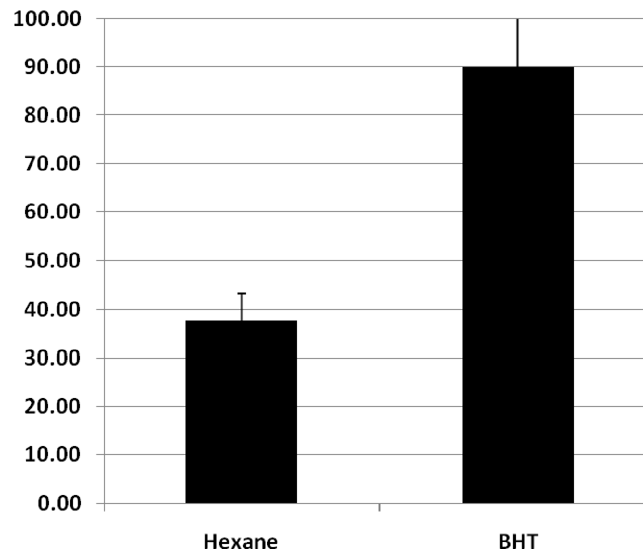


Figure 4. DPPH absorption inhibition (%) of *G. umbrosus* hexane extract (Hexane). BHT was used as a positive control.

Conclusion

However, scientific investigation in order to determine the therapeutic potential of *G. umbrosus* is limited to its bioactive compound goniothalamin which isolated from the petroleum extract of this plant. Although hexane extract of *G. umbrosus* did not show any potential antibacterial activities towards various tested microorganisms, it has shown potential activity in the profile of their anticancer and antioxidant activities. These activities could substantiated with the different chemical constituents existed in this hexane extract of *G. umbrosus* as revealed by GC-MS.

ACKNOWLEDGEMENT

The authors wish to thank gratefully University Putra Malaysia (RUGS 91143) and the Malaysian National Council for Cancer for financial support and The Laboratory of Molecular Biomedicine, Institute of Bioscience, University Putra Malaysia, Serdang, Malaysia for providing the bacterial strains.

REFERENCES

Abdelwahab SI, Abdul AB, Alzubairi AS, Elhassan MM, Mohan S (2009). *In Vitro* Ultramorphological Assessment of Apoptosis Induced by Zerumbone on (HeLa), J. Biomed. Biotechnol. doi:10.1155/2009/769568. 2009(760568): 10

Ali AM, Mackeen MM, Hamid M, Aun QB, Zauyah Y, Azimahtol HL, Kawazu K (1997). Cytotoxicity and electron micreactive oxygen speciescopy of cell death induced by goniothalamin. Planta. Med. 63: 81–83.

Amer AB (2002). Endogenous ligands of Toll-like receptors: implications for regulating inflammatory and immune responses. Trends Immunol. 23(11): 509-512.

Araujo CAC, Leon LL (2001). Biological activities of *Curcuma longa* L.. Mem. Inst. Oswaldo Cruz [online]. 96(5): 723-728.

Changwei A, Anping L, Abdelnaser AE, Tran DX, Shinkichi T (2008). Evaluation of antioxidant and antibacterial activities of *Ficus microcarpa* L. fil. Extract. Food Control 19(10): 940-948.

Inayat-Hussain SH, Annuar BO, Din LB, Ali AM, Ross D (2003). Loss of mitochondrial transmembrane potential and caspase-9 activation during apoptosis induced by the novel styryl-lactone goniothalamin in

HL-60 leukemia cells. Toxicol. *In Vitro,* 17: 433–439.
Iwu MM, Jackson JE, Schuster BG (1994). Medicinal plants in the fight against leishmaniasis. Parasitol. Today 10: 6568.
Jewers K, Davis JB, Dougan J, Machanda AH, Blunden G, Kyi A, Wetchaipan S (1972). Goniothalamin and its distribution in four *Goniothalamus* species. Phytochemistr 11: 2025–2030.
Kabir KE, Khan AR, Mosaddik MA (2003). Goniothalamin: a potent mosquito larvicide from *Bryonopsis laciniosa*. L. J. Appl. Ent. 127: 112–115.
Knowles M (2004). Report on Middle East and North Africa Regional Conference of Psychology. Int. Assoc. Appl. Psychol. Newslett. 16(2):10.
Mosaddik MA, Haque ME, (2003). Cytotoxicity and antimicrobial activity of goniothalamin isolated from Bryonopsis laciniosa. Phytother. Res. 17: 1155–1157.
Mosmann T (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J. Immunological Methods. 65: 55-63.
Nasir UT, Mohamed Saifulaman MS, Rozita R, Laily BD, Leslie CL (2004). Genotoxicity of goniothalamin in CHO cell line. Mutat. Res. 562: 91–102.
Phillipson JD (1994). Natural products as drugs. Trans. R. Soc. Trop. Med. Hyg. 88: 17-19.
Rath CC, Dash SK, Mishra RK, Charyulu JK (1999). *In vitro* evaluation of antimycotic activity of turmeric (*Curcuma longa* L.) essential oils against *Candida albicans* and *Cryptococcus neoformans*. J. Essent. Oil Bearing Plants 43(4): 172-178.
Robert CE, Mervyn I, Joseph H R, James AW (1961). Synthesis of Potential Anticancer Agents. X. Nitrogen Mustards Derived from 4-Quinoline- and 1-Naphthalenemethanols1,2. J. Org. Chem. 26(8): 2827–2831.
Sahoo S, Kar DM, Mohapatra S, Rout SP, Dash SK (2006). Antibacterial activity of *Hybanthus enneaspermus* against selected UTI pathogens. Indian J. Pharm. Sci. 68(5): 653-655.
Sanja C, Milka M, Marija EŠ, Anesa JM, Renata B (2008). Chemical composition and antioxidant and antimicrobial activity of two Satureja essential oils. Food Chem. (111): 648–653.
Suffness M, Pezzuto JM (1990). Assays related to cancer drug discovery, In: Hostettmann, K. (Ed). Methods Plant Biochemistry: Assays for Bioactivity. London: Academic Press 6: 71-133.
Van-Vuuren SF (2008). Antimicrobial activity of South African medicinal plants. J. Ethnopharmacol. (119): 462-472.
Wang TC, Lee KH, Chen YL, Liou SS, Tzeng CC (1998). Synthesis and anticancer evaluation of certain γ-aryloxymethyl-α-methylene-γ-phenyl-γ-butyrolactones. Bioorg. Med. Chem. lett. 8(19): 2773-2776.