BAHAGIAN PENYELIDIKAN DAN PEMBANGUNAN
CANSELORI
UNIVERSITI SAINS MALAYSIA

Laporan Komprehensif ProjekPenyelidikan Jangka Pendek USM
(April 2005 – May 2007)

Nama Penyelidik/Penyelaras : **Tan Mei Lan PhD**

Pusat Pengajian: **Institut Perubatan dan Pergigian Termaju, Universiti Sains Malaysia**

Tajuk Projek Penyelidikan: **Analysis of signal transduction pathways responsible for the *c-myc* mediated regulation of the herb-induced Type II (autophagic) Programmed Cell Death: Identification of new therapeutic intervention of cancer**

Jumlah Geran Diluluskan : **RM 19,960.00 (2 tahun)**

Geran Tambahan yang diluluskan : *Tiada*

Lanjutan tempoh yang diluluskan (*sekiranya berkaitan*) : *Tiada berkaitan*

(i) **2 bulan (15 Mac 2007-14 May 2007)**

(ii)

(iii)

Laporan Kemajuan dari segi kerja-kerja yang telah dijalankan:
(*Sila beri Laporan Kemajuan yang terperinci setakat mana yang boleh*)

1. Purchasing of reagents and consumables - ongoing

Reagents and consumables were brought from various companies starting from the month of May 2005.

2. Appointment of research assistant/postgraduate student - ongoing

1. Recruitment of student helper (with daily rate of RM25) was made starting from 9 June 2006 (Tay Liang Xin).
2. Training and co-supervisorship of PhD candidate Lamek Marpaung (School of Chemical Sciences, USM)

3. Progress in laboratory work

Completed and ongoing experiments

A. Stabilization of T-47D mammary carcinoma cell line

i) T-47D cells was established in the School of Biological Sciences Cell Culture Laboratory (USM) in the month of June 2005 and maintained till this date.

ii) T-47D cells were subcultured in T25 flasks and maintained in the CO2 incubator to be used for subsequent experiments

B. Preparation of extracts -completed

i) Raw plant materials of *Epipremnum pinnatum* (L.) Engl. was harvested and dried in the oven (School of Biological Sciences, USM)

ii) The dried plant material were subsequently weighed and exhaustively extracted with the hexane, followed by chloroform and methanol solvents by soxhlet extraction

iii) The hexane extract collected was dried (firstly by rotary evaporator and then air dried), weighed and reconstituted with DMSO for the preparation of stock solution (5mg/ml)

C. Analysis of signal transduction pathways of the herb-induced (*E. pinnatum* hexane extract) Type II Programmed Cell Death - completed

Brief experimental design

i) A panel of inhibitors with specific actions on components of known signal transduction pathway (s) was used in this study in order to identify intracellular signaling routes which may be potentially involved in the up-regulation of apoptotic-related gene expression as described previously. The most prominent

apoptotic genes induced by *Epipremnum pinnatum* hexane extract in T-47D cells were *c-myc* gene.

ii) The nature and mode of the inhibitors used were listed below. The range of concentration of each inhibitor used was based on previous published studies, which produced significant inhibition on the specific pathways: 30 µM each of H7 (Ahn *et al.*, 1996) and PD098059 (Lin *et al.*, 2002), 25 µM of H89 (Fujihara *et al.*, 1993), 1 µM of herbimycin A (Eason and Martin, 1995) and 90 µM of Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide (Glass, 1983).

iii) The concentration of *Epipremnum pinnatum* (L.) Engl. hexane extract used in the cellular incubation was at EC_{50} value at 72 hours. The incubation time corresponded to the time, which produced a maximal and significant mRNA expression of the *c-myc* gene was used as described in previous experiments (2.90 µg/ml, 30 minutes post incubation) (Tan *et al.*, 2005).

iv) Briefly, before the start of the experimental incubations, cells were maintained for 4 hours in RPMI 1640, containing a reduced concentration of FCS [0.5% (v/v)]. Following washing with PBS, the cells were pretreated with the inhibitor for 2 hours in fresh medium containing 0.5% (v/v) FBS (Tengku Muhammad *et al.*, 1999). The extracts were then added and the incubation continued for a specified incubation time (30 minutes). The concentration of hexane extract used was the concentration achieved 50% inhibition of cell growth after 72 hours treatment (EC_{50}, 2.90 µg/ml) as determined earlier. Total cellular RNA was isolated from untreated cells and treated cells after a specified duration of incubation time with the extract as described earlier and used, subsequently, for RT-PCR using the appropriate specific primer sets to amplify the *c-myc* and *β-actin* gene, using the optimized conditions as described in previous experiments.

v) RNA samples and PCR products was analyzed by electrophoresis and subsequently viewed using Spectroline Ultraviolet Transilluminator 312nm (Spectronics Corporation, USA) and photographed using the Syngene GeneGenius Bio Imaging System (Syngene, UK). Gene expression signals at each point of time were quantified by densitometric scanning, using the Gel Analysis Software Genetools (Syngene, UK). The signals of *c-myc* will be normalized to that of *β-actin*, assigning the ratio in unstimulated cells as 100%.

Table 1: Inhibitors used in the study of signal transduction in T-47D cells

Inhibitors	Chemical name	Actions
H7	1-(5-isoquinolinylsulfonyl)-2-methylpiperazine	Inhibitor of protein kinase C
H89	N-[12-(p-bromocinnamylamino)-ethyl]-5-isoquinolenesulfonamide	Selective inhibitor of cAMP-dependent protein kinase
PD098059	[2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]	Specific inhibitor of the activation of the MAPK cascade, by inhibiting the MAPK-activating enzyme, MAPK/ERK kinase (MEK)
Herbimycin A		Inhibitor of tyrosine kinases
Peptide Arg-Lys-Arg-Ala-Arg-Lys-Glu		Inhibitor of cGMP-dependent protein kinase

D. Analysis of signal transduction pathways responsible for the caspase 3-mediated regulation of the herb-induced (*E. pinnatum* chloroform extract) apoptotic cell death - completed

The materials and methods used are similar to the above except:

i) The concentration of *Epipremnum pinnatum* (L.) Engl. chloroform extract used in the cellular incubation was at EC_{50} value at 72 hours. The incubation time corresponded to the time, which produced a maximal and significant mRNA expression of the *caspase-3* gene was used as described in previous experiments (5.60 µg/ml, 3 h post incubation) (Tan *et al.*, 2005).

ii) Briefly, before the start of the experimental incubations, cells were maintained for 4 hours in RPMI 1640, containing a reduced concentration of FCS [0.5% (v/v)]. Following washing with PBS, the cells were pretreated with the inhibitor for 2 hours in fresh medium containing 0.5% (v/v) FBS (Tengku Muhammad *et al.*, 1999). The extracts were then added and the incubation continued for a specified incubation time (30 minutes). The concentration of chloroform extract used was the concentration achieved 50% inhibition of cell growth after 72 hours treatment (EC_{50}, 5.60 µg/ml) as determined earlier. Total cellular RNA was isolated from untreated cells and treated cells after a specified duration of incubation time with the extract as described earlier and used, subsequently, for RT-PCR using the appropriate specific primer sets to amplify the *caspase-3* and *β-actin* gene, using the optimized conditions as described in previous experiments.

Results and Progress:

a. The effects of inhibitors against components of known signal transduction pathways on the *c-myc* mRNA expression of T-47D resting cells

T-47D cells exhibited a very low mRNA expression of *c-myc* gene at rest or unstimulated condition. However, when pre-treated with the inhibitors of the signal transduction pathways, the mRNA expression of *c-myc* was noted to decrease at different levels. As shown in Figure 1, the resting mRNA expression of *c-myc* gene was generally reduced by all the inhibitors, most prominently by herbimycin A followed by Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide. Both of these inhibitors down-regulated the mRNA expression of *c-myc* in T-47D cells, with more than 50% reduction in expression, indicating that the constitutive mRNA expression in T-47D cells requires the major tyrosine kinase and cGMP-dependent protein kinase pathways (Figure 2).

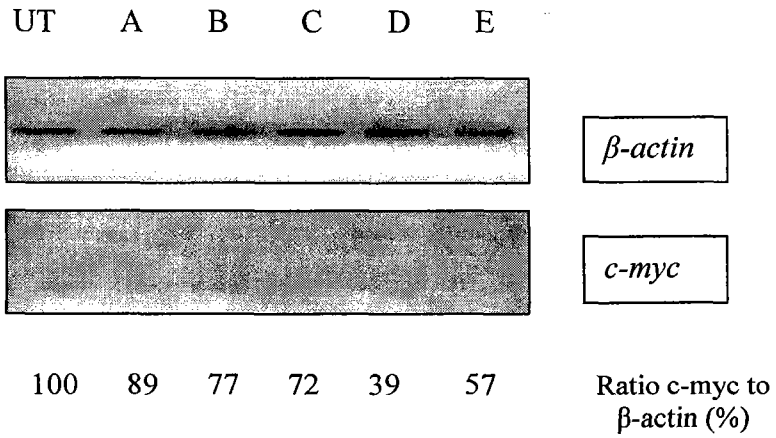

Figure 1: The effects of inhibitors against components of known signal transduction pathways on the *c-myc* mRNA expression of T-47D cells

T-47D cells were treated for 2 hours with the signal transduction inhibitors before RNA extraction and RT-PCR. The *c-myc* to β-actin ratio in untreated cells has been assigned as 100%, with the ratio for the remaining samples being represented relative to this control value. Data shown were representatives of two independent experiments.

UT	Untreated cells
A	Cells were treated with 30 μM H7
B	Cells were treated with 25 μM H89
C	Cells were treated with 30 μM PD098059
D	Cells were treated with 1 μM Herbimycin A
E	Cells were treated with 90 μM Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide

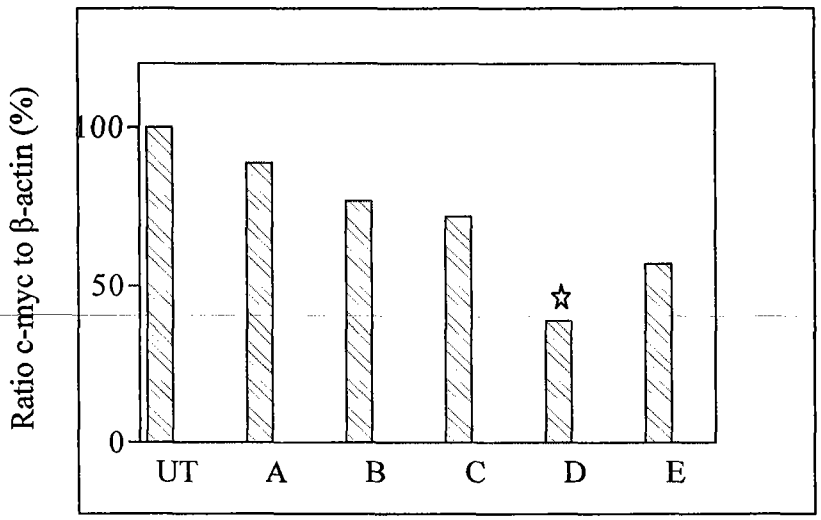

Figure 2: The effects of inhibitors against components of known signal transduction pathways on the *c-myc* mRNA expression of T-47D cells
T-47D cells were treated for 2 hours with the signal transduction inhibitors before RNA extraction and RT-PCR. The *c-myc* to β-actin ratio in untreated cells has been assigned as 100%, with the ratio for the remaining samples being represented relative to this control value. Data shown were representatives of two independent experiments.

☆	More than 50% reduction in expression
UT	Untreated cells
A	Cells were treated with 30 μM H7
B	Cells were treated with 25 μM H89
C	Cells were treated with 30 μM PD098059
D	Cells were treated with 1 μM Herbimycin A
E	Cells were treated with 90 μM Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide

Table 1: The effects of inhibitors against components of known signal transduction pathways on the *c-myc* and β-actin mRNA expression of T-47D resting cells (densitometry semi-quantification) and *c-myc*: β-actin ratio of two independent experiments

	Beta actin	*cmyc*	Ratio	Ratio to beta actin
Experiment 1				
Untreated	46916.79	13762.15	0.293331	1.000
H7	73037.73	12759.04	0.174691	0.596
H89	64115.71	11937.27	0.186183	0.635
PD098059	47530.74	11189.52	0.235416	0.803
HERBI A	76140.4	7660.43	0.100609	0.343
Peptide	70017.21	7664.68	0.109469	0.373
Experiment 2				
	Beta actin	*c-myc*	Ratio	Ratio to beta actin
Untreated	29777.64	17596.73	0.590938	1.000
H7	31115.22	16736.52	0.537889	0.910
H89	33552.8	14242.85	0.424491	0.718
PD098059	33395.34	13575.24	0.406501	0.688
HERBI A	43087.07	11274.67	0.261672	0.443
Peptide	28956.61	10686.42	0.369049	0.625

However, pre-treatment of T-47D cells with H7, H89 and PD098059 only reduced the expression of *c-myc* mRNA level by 11%, 23% and 28%, respectively, as compared to control (untreated sample) indicating the minor role of protein kinase C, cAMP-dependent protein kinase and MAP kinase in the constitutive expression of *c-myc* mRNA in T-47D cells.

b. The effects of inhibitors against components of known signal transduction pathways on the *c-myc* mRNA expression of T-47D cells treated with *Epipremnum pinnatum* hexane extract

A representative results from two independent experiments. Both experiments showed similarity in pattern of expression.

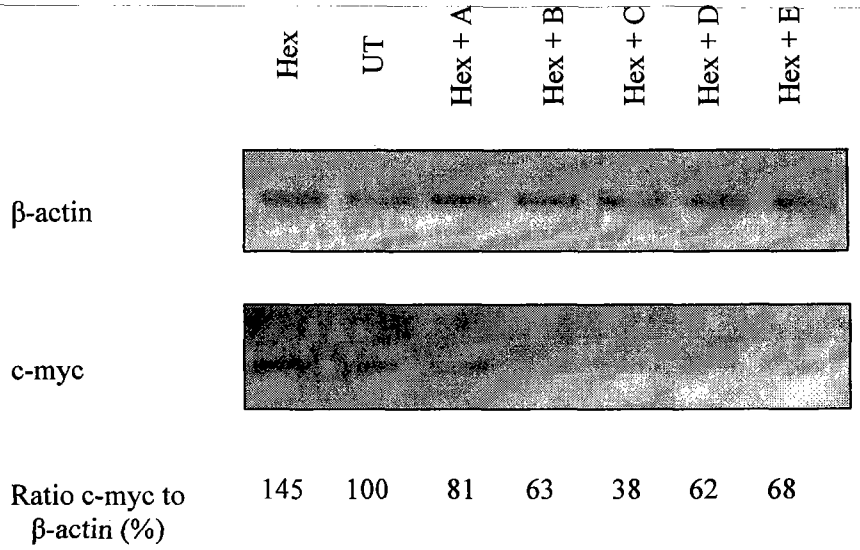

Figure 1 The effects of inhibitors against components of known signal transduction pathways on the *c-myc* mRNA expression of T-47D cells treated with *Epipremnum pinnatum* hexane extract

T-47D cells were either treated with hexane extract (Hex), untreated (UT) or treated with hexane extract in the presence of the inhibitors of the signal transduction pathways. The gene signals at each point were determined by densitometric scanning. The *c-myc* to β-actin ratio in untreated cells has been assigned as 100%, with the ratio for the remaining samples being represented relative to this control value. Data shown were representatives of two independent experiments.

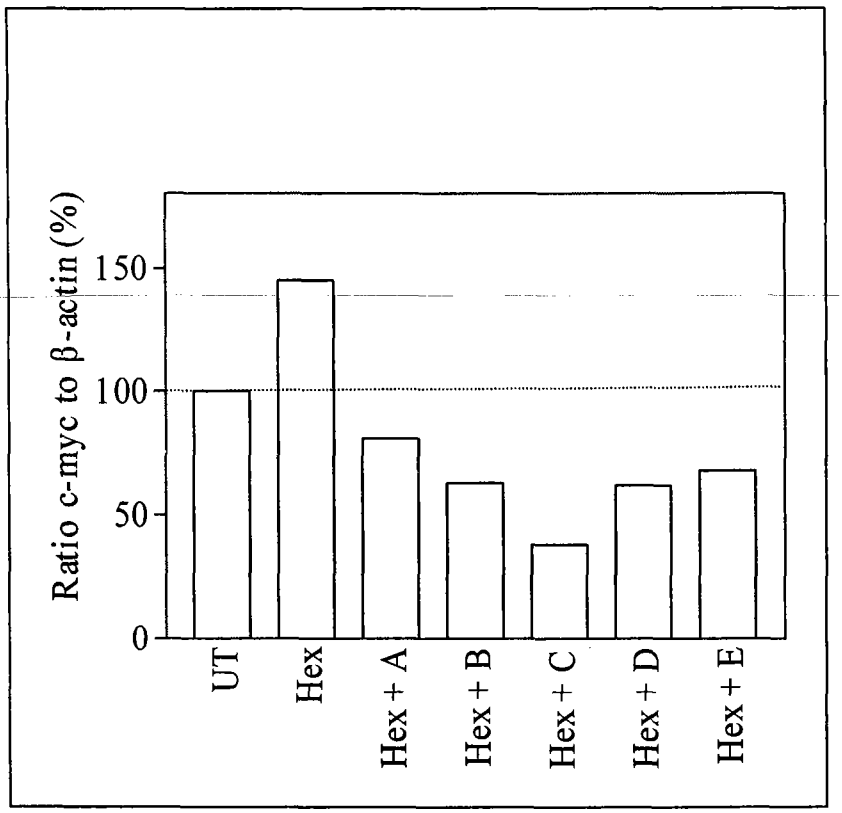

Figure 2 The effects of inhibitors against components of known signal transduction pathways on the *c-myc* mRNA expression of T-47D cells treated with *Epipremnum pinnatum* hexane extract

Hex	Cells treated with 2.90 µg/ml hexane extract
UT	Untreated cells
Hex + A	Hexane extract in the presence of 30 µM H7
Hex + B	Hexane extract in the presence of 25 µM H89
Hex + C	Hexane extract in the presence of 30 µM PD098059
Hex + D	Hexane extract in the presence of 1 µM Herbimycin A
Hex + E	Hexane extract in the presence of 90 µM Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide

As shown in Figures 1 and 2, it is interesting to note that all five inhibitors of the signal transduction pathways reduced the mRNA expression of *c-myc* to below baseline levels, as induced by the *Epipremnum pinnatum* hexane extract. The effect of PD098059 appeared to be the greatest (reduction to 38% of the ratio as compared to 145% in the hexane extract-treated cells), followed by herbimycin A (reduction to 62%), H89 (reduction to 63%), Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide (reduction to 68%) and H7 (reduction to 81%). This result suggested that a combination of various pathways (MAP kinase, tyrosine kinase, cAMP-dependent protein kinase, cGMP-dependent protein kinase and protein kinase C) may be involved with MAP kinase pathway playing a major role in the signal transduction pathway of hexane extract-induced up-regulation of mRNA expression of *c-myc* gene.

Several previous studies have used specific inhibitors against known signal transduction pathways in order to identify potential intracellular events (Tetsuka and Morrison, 1995; Tengku Muhammad, *et al.*, 1999). A panel of such inhibitors (Table 1) was, therefore, used in the present study to help elucidate the signaling pathways involved in the *Epipremnum pinnatum* extracts mediated induction of mRNA expression of *c-myc* in T-47D cells.

The breast carcinoma cell line, T-47D cells, has been shown to express constitutively low *c-myc* mRNA levels. The results demonstrated that the constitutive mRNA expression of *c-myc* was sensitive to H7, H89, PD098059, herbimycin A and Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide. This, therefore, suggested that the constitutive expression of *c-myc* required multiple signaling pathways, including protein kinase C, cAMP-dependent protein kinase, cGMP-dependent protein kinase, MAP kinase and tyrosine kinase pathways.

As expected, all signal transduction inhibitors used to treat the T-47D cells affected the mRNA expression of *c-myc* in T-47D cells induced by the *Epipremnum pinnatum* hexane extract. Multiple signal transduction pathways may be mediating the mRNA expression of *c-myc* induced by the hexane extract, with MAP kinase playing a major role as PD098059 exerted the most effect as compared to other inhibitors. Inhibition of the MAP kinase pathways have maximally down-regulated the mRNA expression of *c-myc* induced by the extract, indicating the programmed cell death exerted by the hexane extract was mainly mediated by up-regulation of *c-myc* gene via the major MAP kinase (and other signaling pathways) pathway.

Although the role of *c-myc* and MAP kinase in the non-apoptotic programmed cell death remained unclear, the role of *c-myc* and MAP kinase in apoptosis was well demonstrated in a number of studies, with *c-myc* and MAP kinase demonstrating dual roles, respectively. For example, NAMI-A, a novel ruthenium-containing experimental anti-metastatic agent, inhibited the MEK/ERK signaling pathway, which led to the down-regulation of *c-myc* gene expression and inhibited cell proliferation. NAMI-A and PD098059 down-

regulated *c-myc* gene expression to the same extent in serum-cultured endothelial cells and ultimately abolished the increased in *c-myc* gene expression elicited by PMA (phorbol 12-myristate 13-acetate) in serum-free cells (Pintus *et al.*, 2002). In another example, studies using rat cell lines revealed that the JNK MAP kinase pathway was involved in the promoting the apoptosis-inducing ability of c-myc (Noguchi *et al.*, 2000). In addition, the MKK6/p38 MAP kinase pathway affected the *myc*-mediated apoptosis and may play a role in the c-myc-dependent signaling upstream of caspase activation (Noguchi *et al.*, 2000).

However, in another study, treatment of keratinocytes with PD098059, inhibited the *c-myc* activation and autocrine keratinocyte proliferation, where the function of *c-myc* was to promote proliferation and down-regulation of *c-myc* was a prerequisite for the initiation of terminal differentiation, indicating that the relationship between MAP kinase and *c-myc* appeared to be anti-apoptotic or pro-survival (Praskova *et al.*, 2002). Although the p38 and JNK MAP kinase pathways were considered as pro-apoptotic whereas the ERK MAP kinase pathways were considered to be anti-apoptotic, the ultimate results were rather unpredictable.

However, there were some exceptions, for example, Taxol (paclitaxel), anticancer agent of natural origin, activated ERK 1/2 MAP kinases and p38 MAP kinases in human ovarian carcinoma cells, which coincided with inhibition of proliferation and the onset of apoptosis (Seidman *et al.*, 2001). In another study, inhibition of MEK/ERK (usually a pro-survival pathway) with a pharmacologic inhibitor, UO126, resulted in a dramatic enhancement of apoptosis (MacKeigan *et al.*, 2000). Thus, it was tempting to speculate that c-myc may induced the Type II non-apoptotic programmed cell death in hexane extract-treated T-47D cells via p38 and JNK MAP kinase pathways. Further functional analysis experiments are required to validate the claim.

c. The effects of inhibitors against components of known signal transduction pathways on the caspase-3 mRNA expression of T-47D cells treated with *Epipremnum pinnatum* chloroform extract

All five inhibitors of the signal transduction pathways partially reduced the chloroform extract-induced mRNA expression of caspase-3 in T-47D cells (Figures 1). H7 produced the most reduction (reduced to 170% of ratio as compared to 605% in chloroform-treated cells), followed by herbimycin A (reduced to 175% of ratio), PD098059 (reduced to 300%), Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide (reduced to 385%) and H89 (reduced to 399%), indicating all five signal transduction pathways may be partially mediating the chloroform extract-induced mRNA expression of caspase-3 in T-47D cells. Thus, protein kinase C and tyrosine kinases may play a major role in mediating the signal transduction pathway of chloroform extract-induced mRNA expression of caspase-3 in T-47D cells.

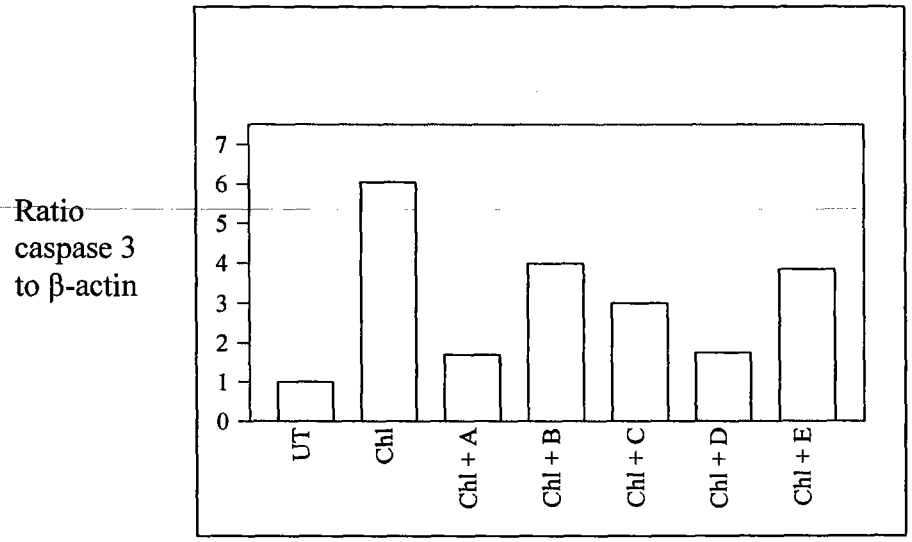

Figure 1: The effects of inhibitors against components of known signal transduction pathways on the caspase-3 mRNA expression levels in T-47D cells treated with the *Epipremnum pinnatum* chloroform extract

UT	Untreated cells
Chl	Cells treated with 5.60 µg/ml Epipremnum pinnatum chloroform extract for 3 h
Chl + A	Chloroform extract in the presence of 30 µM H7
Chl + B	Chloroform extract in the presence of 25 µM H89
Chl + C	Chloroform extract in the presence of 30 µM PD098059
Chl + D	Chloroform extract in the presence of 1 µM Herbimycin A
Chl + E	Chloroform extract in the presence of 90 µM Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide

Pls see attached manuscript submitted for publication (for this part, contributing to one small section in that manuscript)

However, the earlier part, on the MAP kinase mediated c-myc mRNA expression in mammary cells undergoing autophagic cell death, more work is needed, before it can be published.

d. This USM Short Term Grant was also used to partly support a project undertaken by my postgraduate student Lamek Marpaung (TML is co-supervisor since 13-4-05)

Title of Project:	Cytotoxicity of flavanones isolated from fruits of *Macaranga gigantean* M.A. (Euphorbiaceae) in hepatocarcinoma and mammary carcinoma cell lines
Status:	Experiments and thesis write-up completed
Publication title:	Cytotoxic activities of flavanone compounds from fruits of *Macaranga gigantea* M.A.
Authors:	Marpaung. L., Tan, M.L., Tengku Muhammad, T.S., Lim, B.P., Wong, K.C. (2007)

Submission of manuscript and poster presentation:

Proceedings of the 9th Malaysian Applied Biology Symposium, Penang, 30-31 May 2007

To acknowledge USM Short Term Grant in the extended abstract

Epipremnum pinnatum (L.) Engl. chloroform extract causes both apoptotic and non-apoptotic cell death in T-47D mammary carcinoma cells

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Abstract

Epipremnum pinnatum (L.) Engl. chloroform extract elicited both apoptotic and non-apoptotic cell death in T-47D mammary cells. T-47D cells treated with the plant extract at EC_{50} concentration (72 h) for 24 h demonstrated typical DNA fragmentation associated with apoptosis, as carried out using a DNA fragmentation detection assay. However, ultrastructural analysis using transmission electron microscope demonstrated distinct vacuolated cells, which indicated a Type II non-apoptotic cell death although the presence of cell and nuclear blebbing, apoptotic bodies and chromatin changes associated with apoptosis were also detected. T-47D cells exposed to the extract produced a significant up-regulation of c-myc and caspase-3 mRNA expression levels as compared to untreated cells. The up-regulation of caspase-3 mRNA expression appeared to be mediated mainly via both protein kinase C and tyrosine kinases pathways, respectively, which most likely contributed to the apoptotic cell deaths. The presence of both apoptotic and non-apoptotic programmed cell death was also suggested with annexin-V, propidium iodide and trypan blue staining. These findings suggested *Epipremnum pinnatum* (L.) Engl. chloroform extract produces both apoptotic and non-apoptotic cell deaths in the mammary carcinoma cells, indicating the potential usage of this plant in breast cancers.

Keywords: *Epipremnum pinnatum* (L.) Engl., Growth inhibition, T-47D, apoptotic and non-apoptotic cell death

Introduction

Epipremnum pinnatum (L.) Engl., commonly known as "Dragon Tail Plant" or "centipede togavine" is a large root-climber, belongs to the botanical family of Araceae. This terrestrial plant is widespread in monsoon forest, growing occasionally on rocks and in coastal forest. The *Epipremnum pinnatum* (L.) Engl. plant is widely known in Malaysia and Singapore and has had a reputation as a traditional anticancer preparation as well as a remedy for skin diseases (1)(2)(3). A decoction of the fresh leaves with meat or eggs or as tea was reported to be a common practice among the locals (2). A previous study has revealed that crude ethyl extract from the *Epipremnum pinnatum* (L.) Engl. indeed exhibited cytotoxic activities against murine as well as human cell lines such as Molt 4 (leukemic cells), KB (nasopharynx carcinoma cells) and SW 620 (colon adenocarcinoma cells) (1). However, its anti-proliferative effects and cell death mechanisms has not been clearly defined. Investigations into the more hydrophilic extracts may explain the way the indigenous medicine works as most of the time, the aqueous decoction were used therapeutically.

The new chemical entities (NCE) paradigm of the twentieth century attempts to treat complex disease with a "single golden molecular bullet". The first flaw in this paradigm appeared relatively recently when problems of resistance to antimicrobial and anticancer drugs became apparent (4). The multifactorial nature of most diseases especially cancer cannot be ascribed to a single genetic or environmental change but arise from a combination of genetic, environmental or behavioral factors (5). Unlike the western NCE paradigm, traditional medicinal systems of the East always believed that complex combinations of botanical and non-botanical remedies should be adjusted to the individual patient and stage of the disease. This approach, emphasized the mutually

potentiating effects of different components of complex medicinal mixtures, is articulated and developed in traditional medicinal systems. Interaction between different molecular components is generally required for an optimal therapeutic effect of plant extracts. Although the development of NCE from plants remained the major objective of most researchers, the importance of traditional and herbal preparation in crude form should not be overlooked. Therefore, the crude extract of *Epipremnum pinnatum* (L.) Engl. chloroform extract is being evaluated for its possible anticancer effects

Materials and Methods

Chemicals

The MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carbonxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay) and the Deadend™ Colometric Apoptosis Detection System were purchased from Promega, USA. Annexin-V-FLUOS kit was purchased from Roche Diagnostics, Germany. DMSO was obtained from Amresco, USA, propidium iodide and vincristine sulphate from Sigma Aldrich, USA. Etoposide was purchased from DBL, Australia. All culture media and additives were from Hyclone, USA. All other chemicals were reagents of molecular grade.

Preparation of extract

The *Epipremnum pinnatum* plant was collected from the Herb Garden, School of Biological Sciences, Universiti Sains Malaysia. The voucher specimen (No. USM-TML-002) was preserved and deposited in the School of Biological Sciences, Universiti Sains Malaysia. The leaves and stems were washed, dried and chopped finely using a

blender. The dried plant material was exhaustively extracted with hexane by soxhlet extraction, followed by chloroform. The chloroform extract were then filtered and concentrated using a rotary evaporator and then evaporated to dryness at room temperature. The dried chloroform extract is then weighed using a microbalance (Sartorious, Germany) and reconstituted with DMSO to prepare stock concentration of 5mg/ml and diluted serially to eight different working concentrations.

Cell lines and culture medium

T-47D (human breast carcinoma) cells were purchased from American Type Culture Collection (ATCC), USA and cultured in RPMI 1640, supplemented with 10% Fetal Bovine Serum (FBS), 100 U/ml Penicillin and 100 mg/ml Streptomycin solution, 2 mM L-glutamine, 0.01 mg/ml bovine insulin, 10 mM HEPES and 1 mM sodium pyruvate, as recommended by ATCC.

In vitro cytotoxicity assay

Cellular growth in the presence or absence of experimental agents was determined using MTS assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay, Promega, USA) as described by the manufacturer. Briefly, T-47D cells were plated onto 96 well plates at cell density of approximately 6000 cells/well and grown at 37^0C in a humidified incubator supplemented with 5% (v/v) CO_2 for 24 – 48 h. Cell viability was routinely determined using trypan blue exclusion test and in all cases, cell viability was always in excess of 95%. When the cells reached between 80–90% confluency, the medium was removed and replaced with medium containing only 0.5% (v/v) FBS. The cells were then incubated for a further 4 h. Subsequently, the cells were then treated with different concentrations of the chloroform extract (Fig. 1). Untreated control cells

were cultured in 0.5% (v/v) FBS-containing medium in the presence of 1% (v/v) DMSO (vehicle). DMSO was used to dissolve and dilute the extracts and the final concentration of DMSO present in each well was adjusted to 1% (v/v). The cells were subsequently incubated for 24, 48 and 72 h. Vincristine sulphate and etoposide were used as positive controls.

After 24, 48 and 72 h incubation, 20 µl/well of combined MTS/PMS solution was added and the plates were incubated for a further 1–4 h in the humidified 5% (v/v) CO_2 incubator at 37^0C. Absorbance was then read at 490 nm using Vmax Kinetic Microplate Reader (Molecular Devices, USA). Wells with complete medium and MTS/PMS solution but without cells were used as blanks. EC_{50} values were expressed as µg of compound/ml that caused a 50% growth inhibition as compared to controls. Experiments were carried out in triplicate in three independent experiments (n=9).

Determination of apoptotic marker gene expression

The mRNA expression levels of c-myc, p53 and caspase-3 were performed using reverse RT-PCR (6)(7). T-47D cells were cultured in T-25 flasks and starved under reduced FBS concentration [0.5% (v/v)] for 4 h. *Epipremnum pinnatum* chloroform extract at concentration of EC_{50} at 72 h (5.60 µg/ml) was used to stimulate the cells over a period of 24 h. Total RNA was then isolated using Tri Reagent LS according to manufacturer's protocol.

Subsequently, 1µg of isolated total cellular RNA was reverse-transcribed into cDNA and subjected to PCR amplication. The PCR conditions used were: denaturation at 94^0C for 45 s, annealing at 55^0C for 1 min and extension at 72^0C for 2 min for 32 cycles (c-myc and β-actin) and 34 cycles (caspase-3 and p53). The PCR conditions including the quantity of RNA and cDNA samples used to amplify c-myc, caspase-3, p53 and β-actin genes were in the exponential phase of amplification (data not shown)

indicating that the conditions were optimized for semi-quantitative studies (6)(7). The mRNA level of β-actin was used as an internal control for amount of template used. The oligonucleotide sequences of the PCR primers (Operon Technologies, USA) used herein were designed based on the human mRNA encoding the respective genes obtained from the Genbank (Table 1). Cloning and sequencing of the amplified PCR fragments were also carried out to determine and confirm the target genes identified. The PCR products were electrophoresed on a 1.2% (w/v) agarose gel and visualized with ethidium bromide staining. Gene expression signals at each point of time was determined by densitometric scanning using the Gel Analysis Software Genetools (Syngene,UK). The signals from c-myc, p53 and caspase 3 were normalized to that from β–actin and the ratio in unstimulated samples were assigned as 1.

Analysis of signal transduction pathways mediating the caspase-3 mRNA regulation of apoptotic cell death in T-47D cells

A panel of inhibitors with specific actions on components of known signal transduction pathway(s) were used in this study in order to identify intracellular signaling routes which may potentially be involved in the up-regulation of caspase-3 gene expression as described earlier. The nature and mode of the inhibitors used were listed in Table 2. The range of concentration of each inhibitor used was based on previous published studies, which produced significant inhibition on the specific pathways: 30 μM each of H7 (8) and PD098059 (9), 25 μM of H89 (10), 1 μM of herbimycin A (11) and 90 μM of Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide (12). The concentration of *Epipremnum pinnatum* chloroform extract used in the cellular incubation was 5.60 μg/ml (EC_{50}, 72 h). The incubation time corresponded to the time, which produced a maximal and significant mRNA expression of the caspase-3 gene was used as described earlier (3 h).

Briefly T-47D cells were prepared and maintained in medium containing a reduced concentration of 0.5% v/v FBS for 4 h. Following that, the cells were pretreated with the inhibitor for 2 h in fresh medium containing 0.5% (v/v) FBS (13). The chloroform extract were then added and the incubation continued for 3 h. Total cellular RNA was isolated from untreated cells and treated cells as described earlier and used, subsequently, for RT-PCR using the appropriate specific primer set (Table 1) to amplify caspase-3 and β-actin, using the optimized conditions as described previously. Gene expression signals at each point of time were quantified by densitometric scanning, using the Gel Analysis Software Genetools (Syngene, UK). The signals of caspase 3 were normalized to that of β-actin, assigning the ratio in unstimulated cells as 1. All data shown were representatives of two independent experiments.

Detection of DNA fragmentation (apoptosis) in T-47D cells

Briefly, near confluent cultures of T-47D cells were subcultured into Labtek® Chamber Slides (Nalge Nunc, Denmark) and then incubated for 24–48 h. When the cells reached between 80-90% confluency, the medium was replaced with fresh medium containing 0.5% (v/v) FBS, incubated for a further 4 h and then treated with the *Epipremnum pinnatum* chloroform extract at concentration of EC_{50} at 72 h (5.60 µg/ml). Untreated control cells were treated with similar concentration of DMSO. Positive control cells were treated with DNase I (1U/ml) and etoposide at EC_{50} concentration (72 h, 1.90 µg/ml). In all cases, the final concentration of DMSO in each control slide did not exceed 1% (v/v). The slides were subsequently incubated for 24 h, then washed with PBS twice and subsequently processed according to the Deadend™ Colometric Apoptosis Detection System (Promega, USA) protocol as described by the manufacturer's manual. The slides were observed using the light microscope (Olympus

BH2 Light microscope attached with 3CCD JVC KYF55B Colour Video Camera). In another set of experiments in order to determine cell viability, T-47D cells were plated onto 12-well plates at similar cell densities and then treated accordingly with the compound. After 24 and 72 h, the treated cells were stained with 0.4% (w/v) trypan blue and left for about 5 minutes. The cell samples were then viewed using an inverted light microscope. Experiments were carried out in duplicate.

Ultrastructural analysis of T-47D cells using transmission electron micrograph (TEM)

Cells were treated with *Epipremnum pinnatum* chloroform extract (EC_{50}, 72h) and incubated for 24 h. Subsequently, treated cells were washed, centrifuged and then resuspended in 2.5% (v/v) glutaraldehyde in 0.1 M phosphate buffer pH 7.3.at 4^0C for 24 h. Cells were then centrifuged and rinsed in 0.1M phosphate buffer for 10 min and repeated three times. Postfix was carried out with 1% (w/v) osmium tetroxide prepared in 0.1M phosphate buffer for 1-2 h at room temperature. Cells were washed using distilled water for 10 min and repeated. Dehydration were carried out at follows; cells were washed with 50% (v/v) ethanol for 15 min, followed by 75% (v/v) ethanol for 15 min, 95% (v/v) ethanol for 15 min and repeated, 100% (v/v) ethanol for 30 min and repeated and lastly 100% (v/v) acetone for 100 min and repeated. Infiltration was carried out using the mixture of acetone: Spurr's resin mixture (1:1) in a rotator for 2-3 days, with daily change of Spurr's mixture of each sample specimen. Finally the cells were embedded and cured at 60^0 C for 12-48 h. Sectioning of thin sections (<1μm) was carried out using Reichart Supernova Ultra Microtome. The thin sections were initially stained with uranyl acetate for 15 min and repeated using lead citrate. Subsequently the sections were rinsed, dried and examined under a Philips CM 12 Transmission Electron Microscope. Experiments were carried out in duplicate.

Detection of phosphatidylserine externalization (programmed cell death) in T-47D cells

T-47D cells were prepared and stimulated with *Epipremnum pinnatum* chloroform extract (EC_{50}, 72 h) as described earlier. After stimulation, medium, chambers and silicon borders of chamber slides were removed and the treated cells were incubated with the Annexin-V-FLUOS labeling solution (combination of annexin V and propidium iodide solution) (100 µl/chamber) for 10–15 min at 15–25^{0}C as described by the manufacturer's protocol. Subsequently, the slides were immediately analyzed using a fluorescence microscope using an excitation wavelength in the range of 450–500 nm and detection in the range of 515–565 nm (green) (Olympus BH2-RFCA Fluorescence Microscope with Olympus camera attachment). Annexin V and propidium iodide positive cells were stained in green and red, respectively.

Calculations and statistical analysis

EC_{50} values for growth inhibition was derived from a nonlinear regression model (curvefit) based on sigmoidal dose response curve (variable) and computed using GraphPadPrism (Graphpad, USA). Data were given as mean ± standard error mean (S.E.M.). The significant differences in growth inhibition (between test concentration and control) and the significant differences in the ratio to β-actin (between each time point and control) in gene expression studies were determined using one way analysis of variance (ANOVA) and Dunnett's Multiple Comparison Test for post comparison tests computed using GraphPadPrism software (Graphpad, USA). The significance level was determined at 0.05 (α).

Results

Effects of Epipremnum pinnatum chloroform extract on T-47D cell growth

Exposure of T-47D breast tumor cells to *Epipremnum pinnatum* chloroform extract produced a concentration dependent inhibition of cell growth at both 48 and 72 h (Fig. 1). Although concentrations above 3.125 μg/ml produced significant growth inhibition against T-47D cells as early as 24 h ($p<0.05$), EC_{50} could not be determined at the range of concentration used (Fig. 1). Concentrations 12.5 μg/ml and above produced significant growth inhibition ($p<0.05$) against T-47D cells after 48 and 72 h incubation with EC_{50} at 12.30 μg/ml and 5.60 μg/ml respectively (Fig. 1). The concentration needed to cause a 50% growth inhibition of T-47D cells reduced to almost half when the incubation time increased for 24 h, indicating there was consistent activity by the extract. Generally, the low EC_{50} values indicated that the chloroform extract exhibited a high cytotoxic activity against T-47D cell line at 48 and 72 h as judged by the criterion set by the National Cancer Institute, USA (Geran et al., 1972). Surprisingly, vincristine sulphate produced an EC_{50} of 21.70 μg/ml at 72 h, which appeared to indicate that the compound was less toxic against the T-47D cells as compared to the crude chloroform extract of *Epipremnum pinnatum* plant. However, etoposide produced a better cytotoxic activity with an EC_{50} of 1.90 μg/ml.

The effects of the Epipremnum pinnatum chloroform extract on the p53, caspase-3 and c-myc mRNA expression levels and the signal transduction pathways mediating the up-regulation of caspase-3 mRNA expression in T-47D cells

The RT-PCR analysis showed a differential expression profile for p53, caspase-3 and c-myc mRNA levels in T-47D cells treated with the *Epipremnum pinnatum* chloroform extract (EC_{50}, 5.60 μg/ml) over 24 h period. The expression levels of p53

were not significantly induced by the extract at all time points (data not shown). However, the mRNA expression of caspase-3 and c-myc was significantly increased at different time points.

The mRNA expression of caspase-3 was generally increased from 15 min onwards and reached a peak at 30 min (4.70-fold increase) and 3 h (5.20-fold increase) of stimulation (Fig. 2). Semi-quantitative analysis of the caspase-3 expression levels revealed a significant increase in the expression levels at 3 h ($p<0.05$) (Fig. 2). Although there was a steady decline in the caspase-3 mRNA levels after that, the expression levels remained high and relatively constant throughout the 24 h incubation period (more than 2 fold-increases). As for the c-myc gene, mRNA expression was up-regulated as early as 15 min following stimulation of the cells with the chloroform extract and reached the highest level at 1 h (2.50-fold increase) (Fig. 2). However, the mRNA expression of c-myc declined rapidly thereafter, gradually reaching the lowest level at 24 h. The up-regulation was statistically significant at 15 min, 30 min, 1 h and 2 h post stimulation ($p<0.05$) as compared to untreated control cells. Graphical representation of the expression profiles is shown in Fig. 2.

All five inhibitors of the signal transduction pathways partially reduced the chloroform extract-induced mRNA expression of caspase-3 in T-47D cells (Fig 3). H7 produced the most reduction (reduced to 1.7 of ratio as compared to 6.05 in chloroform-treated cells), followed by herbimycin A (reduced to 1.75), PD098059 (reduced to 3.00), Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide (reduced to 3.85) and H89 (reduced to 3.99), indicating all five signal transduction pathways may be partially mediating the chloroform extract-induced mRNA expression of caspase-3 in T-47D cells. However, both H7 and Herbimycin A produced more than 50% reduction of the caspase-3 mRNA level as compared to the original level of treated T-47D cells. Thus, protein kinase C

and tyrosine kinases may play a major role in mediating the signal transduction pathway of chloroform extract-induced mRNA expression of caspase-3 in T-47D cells.

Influence of Epipremnum pinnatum chloroform extract on DNA fragmentation and cell morphology of T-47D cells

Alterations in DNA integrity and ultrastructural morphology of T-47D cells were examined following 24 h exposure to 5.60 μg/ml *Epipremnum pinnatum* chloroform extract. The typical appearance of T-47D cells after 24 h exposure to the chloroform extract is shown in Fig. 4A as compared to the DNase I-treated T-47D cells (Fig. 4B), etoposide-treated T-47D cells (Fig. 4C) and negative control T-47D cells (Figure 4D). As shown in Fig. 4A, cells treated with the chloroform extract exhibited apparent DNA fragmentation, one of the criteria used to define apoptosis (the presence of brown nucleus indicate DNA fragmentation). A high proportional of T-47D cells produced distinct stained nucleus, suggests that apoptosis was a main mode of cell death elicited by the extract. Similarly for DNase I-treated and etoposide-treated T-47D cells, distinct stained cells were clearly visible. By contrast, the negative control cells produced no DNA fragmentation, indicating reliability of assay. The chloroform extract-treated cells did not show morphological evidence of necrotic cell death, as confirmed using the trypan blue exclusion assay. Fig. 4E and F showed only a few cells were stained with trypan blue at 24 and 72 h of incubation, indicating that most cells were viable and plasma membrane were intact at both time points. Thus, necrosis was ruled out as a probable cause of cell death in the chloroform extract-treated T-47D cells.

Ultrastructural analysis revealed that T-47D cells treated with the *Epipremnum pinnatum* chloroform extract demonstrated different stages of apoptotic and non-apoptotic type of cell death. There was clumping of nuclear chromatin (Fig. 5A, B and C), margination of chromatin against the nuclear membrane (Fig 5A and B), apoptotic

bodies (Fig. 5A and B), cell blebbing (Fig. 5C) and vacuolated type of morphology (Fig. 5D), identified as Type II non-apoptotic programmed cell death.

The presence of high intensity and weakly stained annexin V positive cells was evident (green fluorescence) (Figure 6A, B, C and D), indicating late and early phase of apoptosis or programmed cell death, respectively. It is interesting to note that a moderate percentage of cells produced homogeneous and high intensity staining of propidium iodide (red fluorescence), indicating necrosis death (Fig. 6A, C and D). However, based on the trypan blue exclusion assay, necrosis was ruled out as the possible mode of cell death. Thus these cells which were simultaneously stained with both annexin-V and propidium iodide, could indicate that programmed cell death, possibly Type II cell death has taken place and not necrosis. Thus, the results strongly suggest that *Epipremnum pinnatum* chloroform extract produces both apoptotic and non-apoptotic programmed cell death in the T-47D mammary carcinoma cells.

Discussion

The studies presented in this report revealed the potential anticancer properties of the *Epipremnum pinnnatum* plant as demonstrated by the low EC_{50} values at both 48 and 72 h (12.30 and 5.60 μg/ml, respectively). The chloroform extract was demonstrated to cause a significant increase in the mRNA expression of c-myc in T-47D cells as early 15 min before reaching its peak at 30 min and continued to be up-regulated at 2 h. Although it was generally known that exposure to cytotoxic agents involved down-regulation instead of up-regulation of proto-oncogenes, which resulted in programmed cell death in many different cell types (14), there were studies which reported that over-expression of c-myc can induce programmed cell death depending on circumstances. For example, droloxifene, a tamoxifen derivative induced apoptosis in

cultured luteal cells with significant increase of c-myc mRNA expression levels as treatment duration increased (15).

Similarly, an increased in the mRNA expression of caspase-3 was also observed to be significantly upregulated at 3 h. As expected, the sustained up-regulation of caspase-3 over a period of 24 h was associated with the morphological features of apoptosis. There was clear evidence of DNA fragmentation, as assayed using the Deadend^{TM} Colometric Apoptosis Detection System (Promega, USA), which explained further the role of caspase-3 expression in eliciting the morphological changes associated with apoptosis. Caspase-3 is a crucial component of the apoptotic machinery in most cell types. The activation of caspase-3 is a central event in the process of apoptosis (16)(17)(18). This cysteine protease, which is proteolytically activated by cleavage of pro-caspase-3 by caspase-8, cleaves several intracellular polypeptides, including major structural elements of the cytoplasm and nucleus, components of the DNA repair machinery and a number of protein kinases. Collectively, these scissions disrupted survival pathways and disassembled important architectural components of the cell, contributing to the stereotypic morphological and biochemical changes that characterized apoptotic cell death (19). Thus, the up-regulation of caspase-3 and c-myc mRNA expression has undoubtedly contributed to the apoptotic cell death in T-47D cells following exposure to the *Epipremnum pinnatum* chloroform extract.

The mRNA expression of caspase-3 in T-47D cells stimulated with *Epipremnum pinnatum* chloroform extract appeared to be mainly mediated via the protein kinase C (PKC) and tyrosine kinase pathways (although other pathways may be partially involved). This was not surprising as both pathways were inter-related. The atypical PKC isozymes were activated via the PI-3-K-dependent pathways, which were also the main signaling pathway utilized by the tyrosine kinases (20). The pro-apoptotic role of

caspase-3 gene mediating apoptosis via the protein kinase C pathways was well described in various studies. For example, the tumor necrosis factor α (TNFα) induced cytotoxicity and apoptosis in the rat epithelial cell line, IEC-18, via the activation caspase-3 enzyme and its effects on the activity were reduced by selective inhibition of novel PKCε and δ, indicating that the PKCs mediated the activation of caspase-3 activity (21). In another example, cDDP [cis-diamminedichloroplatinum (II), an anticancer agent] caused a time and concentration-dependent increased in the generation of the catalytic fragment of novel PKCδ and ε that was associated with activation of caspase-3 and 7 (22). In addition, one study demonstrated that phorbol 12-myristate 13 acetate (PMA) induced cell death in SNU-16 gastric adenocarcinoma cells via the caspase-3 activation and the activity was completely suppressed by Go6983, a specific inhibitor of PKC, indicating that the activation of caspase-3 was mediated via the PKC pathways (23). Similarly, PKCδ was found to increase the pro-apoptotic effects of caspase-3 in Jurkat-T leukemic cells exposed to ionizing radiation (24).

Ultrastructural analysis demonstrated the presence of apoptotic and non-apoptotic morphology in T-47D cells treated with chloroform extract. The presence of highly vacuolated cells (indicated as the Type II non-apoptotic programmed cell death) as demonstrated in the TEM analysis, clearly suggested that the extract caused cell death via both apoptotic and non-apoptotic pathway. The chloroform extract-treated cells produced positive results with the annexin V staining, indicated that the negative-charged phospholipid phosphotidylserine found on the interior surface of the plasma membrane of the cells translocated to the cell surface, which confirmed the presence of programmed cell death. Furthermore, the presence of the cells simultaneously stained with propidium iodide and annexin V, possibly suggests the presence of non-apoptotic cell death, since necrosis was ruled out earlier.

Recent studies revealed that active self-destruction of cells were not confined to apoptosis alone. Cells actually used different pathways to commit suicide, including the non-apoptotic or Type II PCD, known as vacuolar or autophagic cell death. Briefly, autophagic cell death is often associated with bulk degradation of proteins and organelles, a process essential for cellular maintenance and cell viability (25). It was shown to be essential; for differentiation, development as well as cellular maintenance (26). The most recent reviews have clearly indicated that malignant transformation was frequently associated with the suppression of autophagic cell death (27)(28)(29)(30)(31). Autophagic PCD was also significantly associated with other diseases such as neurodegenerative diseases, cardiomyopathies, bacterial and viral infections (25). During autophagy, a cup-shaped structure, the preautophagosome, engulfed cytosolic components like organelles to form the autophagosome, which subsequently fused with a lysosome, leading to the proteolytic degradation of the components (25). This process usually was caspase-independent, thus, no DNA fragmentation. The most prominent evidence that can be visible at the electron microscope level was the formation of autophagic vacuoles, which gradually degrade the cytoplasmic structures (32)(33). However, many relevant pathways regulating this Type II cell death and the role and function of this type of programmed cell death in cancer has yet to be ascertained.

In conclusion, the findings in this study indicated that the *Epipremnum pinnatum* chloroform extract exerted cytotoxicity in T-47D cells via both apoptotic and non-apoptotic programmed cell death.

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List of abbreviations

ATCC	American Type Culture Collection
cDNA	complementary deoxyribonucleic acid
CO_2	carbon dioxide
DMSO	dimethyl sulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
EC_{50}	effective concentration that causes a 50% growth inhibition as compared to controls
EGFR	epidermal growth factor receptor
FBS	fetal bovine serum
h	hour
min	minute
mRNA	messenger ribonucleic acid
MTS	3-(4,5-dimethythiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
PBS	phosphate buffer saline
PCR	polymerase chain reaction
PMS	phenazine methosulphate
RPMI	Rosewell Park Memorial Institute
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
s	second
S.E.M.	standard error mean
TEM	transmission electron microscopy
TNF-α	tumor necrosis factor alfa
TUNEL	Terminal deoxytransferase-mediated deoxyuridine nick end-labeling (assay)
USA	United States of America
µg	microgram
w/v	weight/volume
v/v	volume/volume

Figure 1 Growth inhibitory effects of different concentrations of chloroform extract of *Epipremnum pinnatum* plant on T-47D cell line at 24, 48 and 72 h. Each value represented the mean ± S.E.M. (n=9); * $p<0.05$.

Figure 2 Semi-quantitative analysis of the caspase-3 and c-myc mRNA level in T-47D cells treated with the *Epipremnum pinnatum* chloroform extract. The ratio of caspase-3, c-myc and β-actin signals of each point was determined by densitometric scanning of the signals. The caspase-3: β-actin and c-myc: β-actin ratio in unstimulated cells (control) has been assigned as 1, with the ratio for the remaining samples being represented relative to this control value. The data shown are the representative of three independent experimental series, each of which produced the same expression profile. Each value represented mean ± S.E.M. (n=3) * $p<0.05$ (as compared to control).

Figure 3 The effects of inhibitors against components of known signal transduction pathways on the caspase-3 mRNA expression levels in T-47D cells treated with the *Epipremnum pinnatum* chloroform extract.

Figure 4 The effects of (A) *Epipremnum pinnatum* chloroform extract (EC_{50} at 72 h, 5.60 μg/ml), (B) DNase I (1 U/ml), (C) etoposide (EC_{50} at 72 h, 1.90 μg/ml) and (D) DMSO 1% (v/v) on T-47D cells as assayed with Deadend™ Colometric Apoptosis Detection System (Promega, USA). Arrows show dark stained nuclei of T-47D cells indicating DNA fragmentation. Trypan blue exclusion assay of the T-47D cells treated with *Epipremnum pinnatum* chloroform extract (5.60 μg/ml) for (E) 24 h and (F) 72 h. Arrows indicating necrotic cells.

Figure 5 TEM showing the morphological features of T-47D cells treated with *Epipremnum pinnatum* chloroform extract (EC_{50} at 72 h, 5.60 μg/ml), for 24 h. (A) Chromatin clumping (thick arrow), chromatin margination (thin arrow) and apoptotic bodies (a) (B), chromatin clumping (thick arrow), cell blebbing (thin arrow) and apoptotic bodies (a) (C) cell blebbing and (D) vacuolated morphology (arrows), were observed. (Original magnification X 9250)

Figure 6 The effects of *Epipremnum pinnatum* chloroform extract (EC_{50} at 72 h, 5.60 μg/ml), on T-47D cell line as stained with Annexin-V-Fluos™ kit (Roche, Germany). The presence of high intensity and weakly scattered annexin V (green fluorescence, thin arrows) and propidium iodide stained cells (red fluorescence, thick arrows) were evident in most cells. Note (A) and (C), cells stained with annexin V and PI simultaneously, indicating programmed cell death, mostly Type II.

Table 1

Primer	Sequence (5' – 3')
c-myc forward	GAACAAGAAGATGAGGAAGA
c-myc reverse	AGTTTGTGTTTCAACTGTTC
p53 forward	TGTGGAGTATTTGGATGACA
p53 reverse	GAACATGAGTTTTTTATGGC
caspase-3 forward	TCACAGCAAAAGGAGCAGTTT
caspase-3 reverse	CGTCAAAGGAAAAGGACTCAA
β-actin forward	TCACCCTGAAGTACCCCATC
β-actin reverse	CCATCTCTTGCTCGAAGTCC

Table 2

Inhibitors	Actions
H7 (1-(5-isoquinolinylsulfonyl)-2-methylpiperazine)	Inhibitor of protein kinase C
H89 (N-[12-(p-bromocinnamylamino)-ethyl]-5-isoquinolenesulfonamide)	Selective inhibitor of cAMP-dependent protein kinase
PD098059 [2-(2'-amino-3'-methoxyphenyl)-oxanaphthalen-4-one]	Specific inhibitor of the MAPK-activating enzyme, MAPK/ERK kinase (MEK)
Herbimycin A	Inhibitor of tyrosine kinases
Peptide Arg-Lys-Arg-Ala-Arg-Lys-Glu	Inhibitor of cGMP-dependent protein kinase

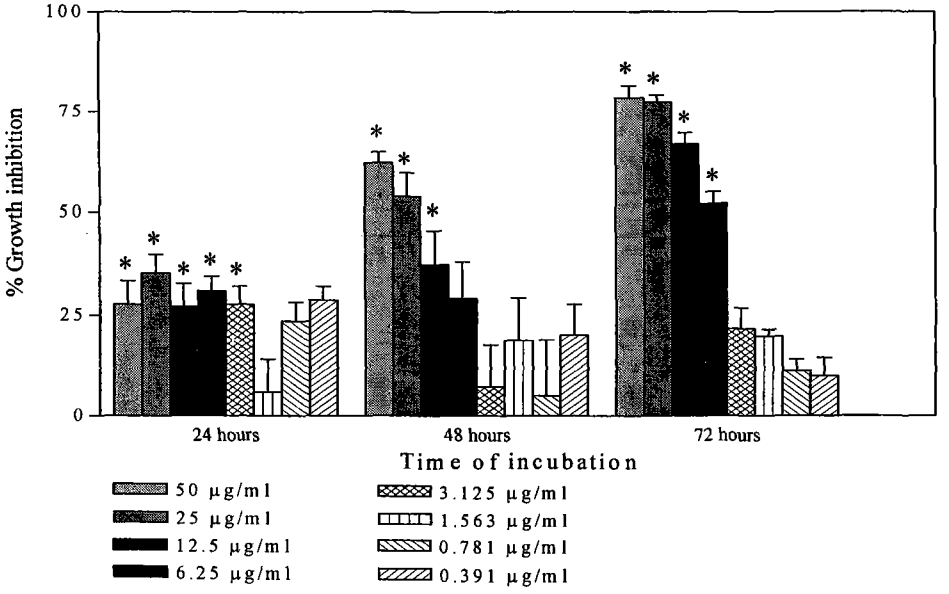
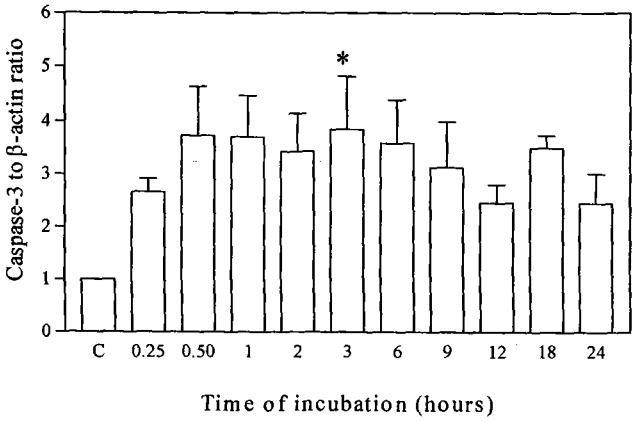

100
75
50
25
0
% Growth inhibition
24 hours
48 hours
72 hours
Time of incubation
50 µg/ml
25 µg/ml
12.5 µg/ml
6.25 µg/ml
3.125 µg/ml
1.563 µg/ml
0.781 µg/ml
0.391 µg/ml

Figure 1


Caspase-3 to β-actin ratio
0
1
2
3
4
5
6
*
C
0.25
0.50
1
2
3
6
9
12
18
24
Time of incubation (hours)

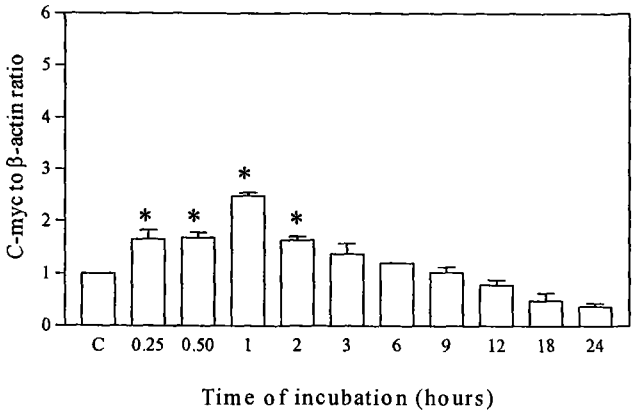

C-myc to β-actin ratio
0
1
2
3
4
5
6
*
*
*
*
C
0.25
0.50
1
2
3
6
9
12
18
24
Time of incubation (hours)

Figure 2

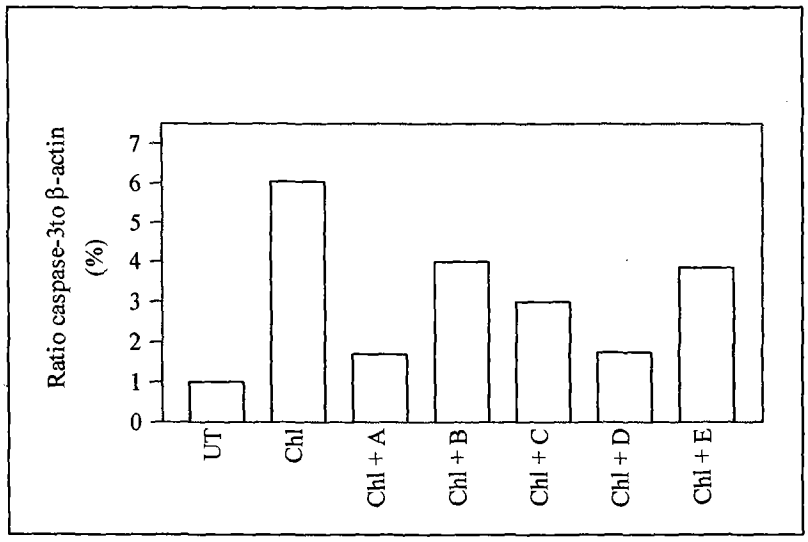


Figure 3

UT	Untreated cells
Chl	Cells treated with 5.60 μg/ml Epipremnum pinnatum chloroform extract for 3 h
Chl + A	Chloroform extract in the presence of 30 μM H7
Chl + B	Chloroform extract in the presence of 25 μM H89
Chl + C	Chloroform extract in the presence of 30 μM PD098059
Chl + D	Chloroform extract in the presence of 1 μM Herbimycin A
Chl + E	Chloroform extract in the presence of 90 μM Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide

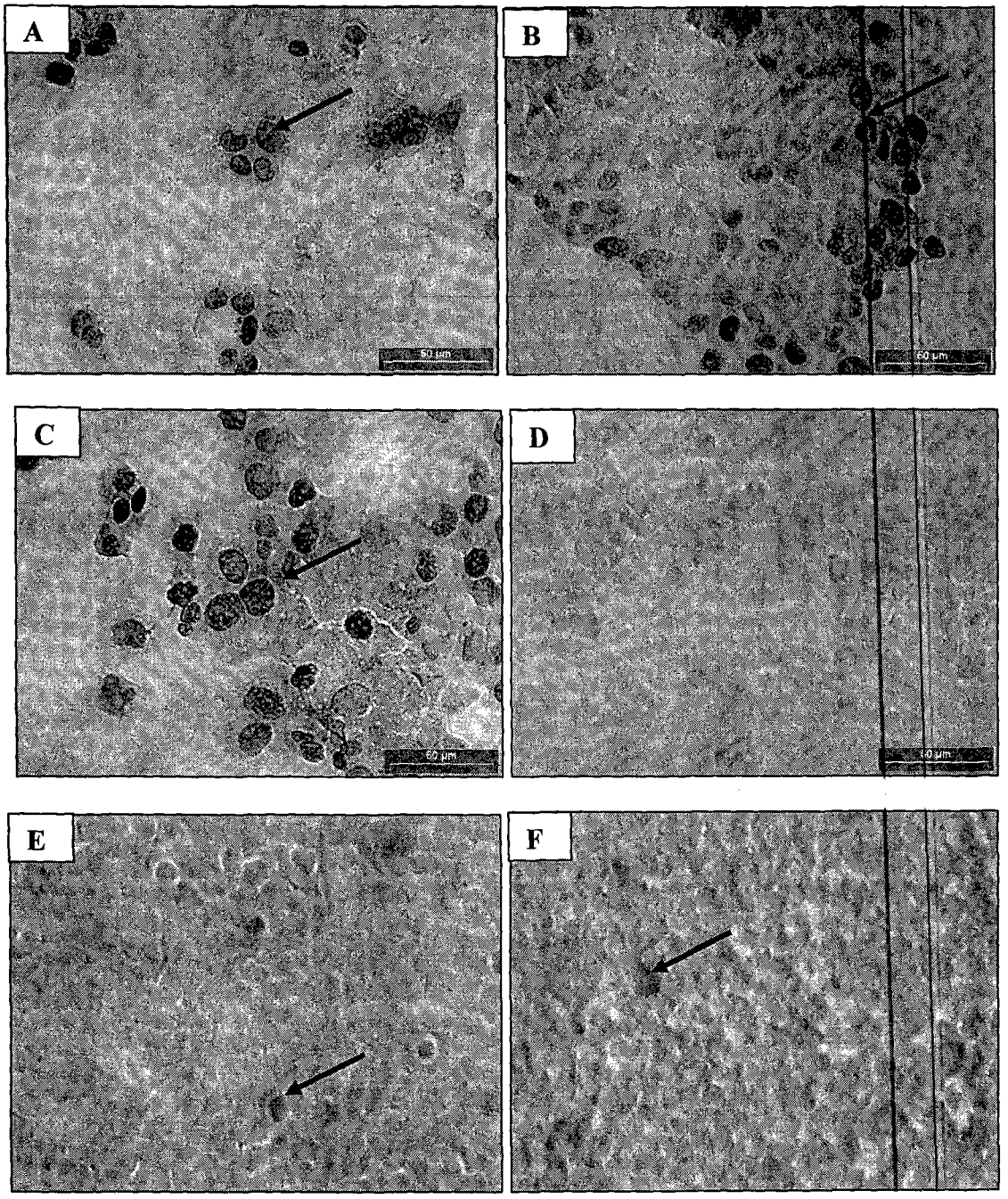


Figure 4

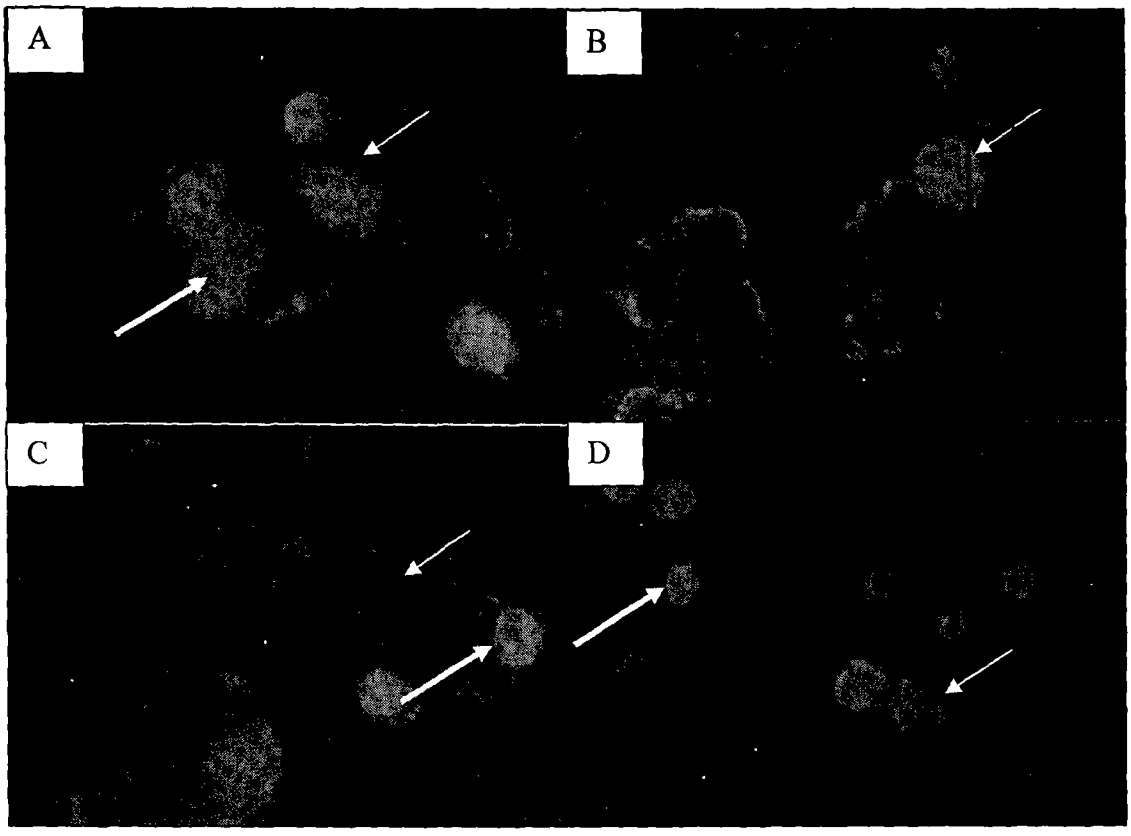


Figure 6

Cytotoxic activities of flavanone compounds from fruits of *Macaranga gigantea* M.A

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Introduction

The genus *Macaranga* (Euphorbiaceae) consists of approximately 250 species that are widely distributed in the Asia Pacific (Jang *et al.*, 2004). The genus is known for wide range of mutualistic association with ants, although the degree of interaction between plants and ants may vary from loosely facultative, non-specific myrmecophylic to obligate myrmecophytic associations (Fiala *et al.*, 1994). Many *Macaranga* species are characteristic of secondary forest in South Asia, where plants provide food and nesting space for specific ant partners (*Slik et al.*, 2003; Vogel *et al.*, 2003). The usages of *Macaranga* plant in various ailments are reported in some literature. For example the gum-powder from *M. peltata* are used in Indian Medicine for the treatment of venereal disease (Ramaiah *et al.*, 1979), the leaves of *M. triloba* are used to treat stomach ache and skin itches in Indonesia and Malaysia (Ahmed and Holdsworth, 1994; Grosvenor *et al.*, 1995). Another species, *M. kilimandsgcharica* Pax (leaves and stems) are traditionally used for afterpains (post-partum cramps) (Cos *et al.*, 2002). The decoction of the *M. griffithiana* and *M. hullettii* king root is taken orally for fever and stomach discomfort, respectively (Burkill, 1935). *M. hypoleuca*, M.A decoction is reportedly used as febrifuge, expectorant, and anti-spasmodic (Burkill, 1935).

Another genus, *M. populifolia*, M.A. is administered after childbirth (Burkill, 1935). The decoction of the *M. gigantea* root-bark is taken by the Malay community for diarrhea and dysentery (Burkill, 1935; Mat-Salleh, 1997). Various compounds are isolated from some *Macaranga* species and investigated for their biological activities. For example, cytotoxic geranyl stillbene are isolated from leaves of *Macaranga schweinfurthii* (Beutler *et al.*, 1998). Mappain, a cytotoxic prenylated stillbene is isolated from leaves *M. mappa* (van der Kaaden *et al.*, 2001). Activity-guided fractionation of the leaves of *M. triloba*, resulted in the isolation of a rotenoid and other compounds capable of inhibiting the cyclooxygenase-2, which is an important mechanism for chemoprevention (Jang *et al.*, 2004). Prenylated flavonoids with inhibiting activity against cyclooxygenase-2 are isolated from *M. conifera* (Jang *et al.*, 2002). Vitexin, a flavonoid is also isolated from *M. triloba* (Vinh *et al.*, 2002). Active antibacterial compounds such as prenylated flavanones, Macarangaflavanones A and B, euchrestaflavanone A, bonannione A are isolated from *M. pleiostemona* (Schutz *et al.*, 1995).

Although some chemical composition of leaf volatiles from *M. gigantea* such as fatty acids, benzenoids, monoterpenoids and sequiterpenoids were reported, there was no biological assessment of their activities (Jurgens *et al.*, 2006). In this study, various compounds were isolated from the fruits of *M. gigantea* and their

biological activities, specifically cytotoxic activities were evaluated using *in-vitro* cell lines.

Materials and Methods

Chemicals

The fruits (raw material) of *M. gigantea* were collected fresh from the Universiti Sains Malaysia campus, Pulau Pinang, Malaysia. Identification of plant was carried out by morphological examination assisted by staff of the School of Biological Sciences Herbarium, Universiti Sains Malaysia. The voucher specimen (No.10833) was registered and given a serial number according to the classification system of the order, family, genus and species. The voucher specimen was deposited in the School of Biological Sciences Herbarium, Universiti Sains Malaysia. The six compounds used in the experiment were isolated and characterized previously (Table 1). The MTS [3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay (CellTiter 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay) were purchased from Promega, USA. Dimethyl sulfoxide (DMSO) was obtained from Amresco, USA. Doxorubicin HCl was purchased from DBL, Australia. All culture media and additives were obtained from Hyclone, USA.

Cell lines and culture medium

Two human tumor cell lines were used, namely T-47D (human breast carcinoma) and Hep G2 (human hepatocellular carcinoma) were purchased from American Type Culture Collection (ATCC), USA. T-47D cells were cultured in RPMI 1640 and Hep G2 in MEM/EBSS (Eagle's Minimal Essential Medium/Earle's Balanced Salt Solution). All media was supplemented with 10 % (v/v) Fetal Bovine Serum (FBS), 100 U/ml and 100 mg/ml HyQ penicillin-streptomycin solution and 2 mM HyQ L-Glutamine. Additional additives such as 0.01 mg/ml bovine insulin were added to T-47D and 0.1 mM non-essential amino acids to Hep G2 as recommended by ATCC.

In vitro cytotoxicity assay

Near confluent cultures of cell were harvested with 0.05 % (w/v) Trypsin-EDTA The cells were then centrifuged, pellet re-suspended with complete medium with 10 % (v/v) FCS and plated onto 96 well plates (Costar, USA) at cell density of approximately 6000 cells/well. Cell viability was routinely determined using trypan blue exclusion to make sure cell viability was always in excess of 95%. The cells were then allowed to attach and incubated at 37^0C in the CO_2 incubator for a further 24-48 h. When the cells reached 80-90 % confluence, the medium was removed and replaced with medium containing only 0.5 % (v/v) FCS. The cells were then incubated for a further 4 h. The cells were then treated with different concentration extract and isolated pure compounds. Control cells were cultured in 0.5 % (v/v) FCS -containing medium alone. The pure compounds were dissolved and diluted in DMSO 99.9 % (v/v) and the final concentration of DMSO in every test well was not more than 1 % (v/v) .The cells were then incubated for 72 h. Doxorubicin HCl was used as a positive control. Cytotoxicity assays were carried out using CellTiter 96® Aqueous Non Radioactive Cell Proliferation Assay (Promega, USA) as described by the manufacturer.

Calculations

Experiments were carried out in triplicate using CellTiter 96®A_{Queous} Non-Radioactive Cell Proliferation Assay. The mean and standard error mean (SEM) of measured values were calculated using Graph Pad Prism software (Graphpad, USA). EC_{50} values for growth inhibition were derived from 50% interpolation on fit spline point to point plots. The significance of differences in growth inhibition between each test concentration and control were determined using one way analysis of variance (ANOVA), and Dunnett's Multiple Comparison Tests, for post-comparison test, and computed using Graphpad Prism software (Graphpad, USA). The criterion of cytotoxic or non-cytotoxic activities was adapted from the guidelines set by the National Cancer Institute, United States of America (Geran *et al.*, 1972). The screening protocol (Protocol 1.600) has indicated that pure compounds with $EC_{50} < 4$ µg/ml were considered to be cytotoxic and non-cytotoxic if otherwise (Geran *et al.*, 1972). In this experiment, the highest and lowest concentration used was 100 µg/ml and 1 µg/ml, respectively. The highest and lowest concentration used for Doxorubicin was 2 µg/ml and 1 x 10^{-6} µg/ml, respectively. The PG (Percentage Growth) is calculated by using the formula as published by Valeriote, 1990.

Table 1: Pure compounds isolated and screen for cytotoxic activities

Compounds	Name
(1)	5,7,3',5'-tetrahydroxy -6 -geranylflavanone
(2)	5,7,3',4'-tetrahydroxy-2'-geranylflavanone (Nymphaeol- B)
(3)	5,7,3',4'-tetrahydroxy-6-prenyl-2'-geranylflavanone (Nymphaeol-C)
(4)	5,7,4'-trihydroxy-6-geranylflavanone Bonannione A
(5)	5,7,3'-trihydroxy - 4'- geranylflavanone
(6)	5,7,3',5'-tetrahydroxy - 2'- geranylflavanone
Positive control	Doxorubicin HCl

Results

M. gigantea methanol extract appeared to be non-cytotoxic against both Hep G2 and T-47D cell line, as judged by the criterion by the National Cancer Institute (Geran *et al.*, 1972). The EC_{50} for the methanol extract cannot be determined within the range of concentration used (1-100 µg/ml), although the methanol extract at concentration 100 µg/ml showed some marginal cytotoxic activities against T-47D cell lines (Fig. 1 and Fig.2). By contrast, the pure compounds isolated from fruits of M. gigantean appeared to have prominent cytotoxic activities against both the cell lines (Fig. 1). Compound (**1**), (**3**), (**5**), (**6**) appeared to have similar cytotoxic activities against HepG2 with EC_{50} values of 2.50 µg/ml, 2.50 µg/ml, 2.60 µg/ml, 2.60 µg/ml, respectively (Fig 1, Table 2). However, (**2**) and (**4**) compounds appeared to be non-cytotoxic with EC_{50} values of 6.70 µg/ml and 4.20 µg/ml, respectively, ($EC_{50} > 4$ µg/ml) (Table 2).

All compounds, (1), (2), (3), (4), (5), (6), appeared to have cytotoxic activities against T-47D with EC_{50} less than 4 μg/ml, respectively (Fig. 2, Table 2). The EC_{50} for (1), (2), (3), (4), (5) and (6) were 2.50 μg/ml , 2.30 μg/ml, 3.50 μg/ml, 2.40 μg/ml , 3.50 μg/ml and 2.20 μg/ml, respectively. In general, (1), (2), (3), (4), (5) and (6) exhibited cell killing activities at concentration 10 μg/ml above and percentage of cytotoxic activities at each concentration appeared to be similar across the compounds. All compounds except (1) exhibited cytostatic (growth inhibitory) at concentration as low as 1μg/ml, strongly indicating that T-47D cell were sensitive to all these compounds (Fig. 2). However, concentration of compound (1) at 1 μg/ml appeared to produce positive growth as compared to control cells indicating possible growth stimulation effects (percentage growth >100 %). However, this needs to be verified further.

The overall results showed that all pure compounds isolated from *M. gigantea* fruits have growth inhibitory activities at lower concentration and cell killing activities at higher concentration against T-47D and HepG2 carcinoma cell lines respectively. These compounds may be potentially developed as anticancer agents, although further tests are warranted.

Table 2 Cytotoxicity (E_{50} values) of various compounds on Hep G2 and T- 47D cell lines

Compound	**Hep G2 cell line** EC_{50} (μg/ml)	**T- 47D cell line** EC_{50}(μg/ml)
(1)	2.50	2.50
(2)	6.70	2.30
(3)	2.50	3.50
(4)	4.20	2.40
(5)	2.60	3.50
(6)	2.60	2.20
Methanol extract	(ND) > 100	(ND) > 100
Doxorubicin HCl	0.0082	0.0052

(1): 5-,7-,3'-,5'-tetrahydroxy - 6 - geranylflavanone

(2): Nymphaeol-B = 5,7,3',4'-tetrahydroxy -2'-geranylflavanone

(3): Nymphaeol-C = 5,7,3',4'-tetrahydroxy-6-prenyl -2'-geranylflavanone

(4): Bonannione A = 5, 7,4'-trihydroxy - 6 - geranylflavanone

(5): 5, 7, 3'-trihydroxy - 4'- geranylflavanone

(6): 5, 7, 3', 5'-tetrahydroxy -2'- geranylflavanone

ND: Not determined

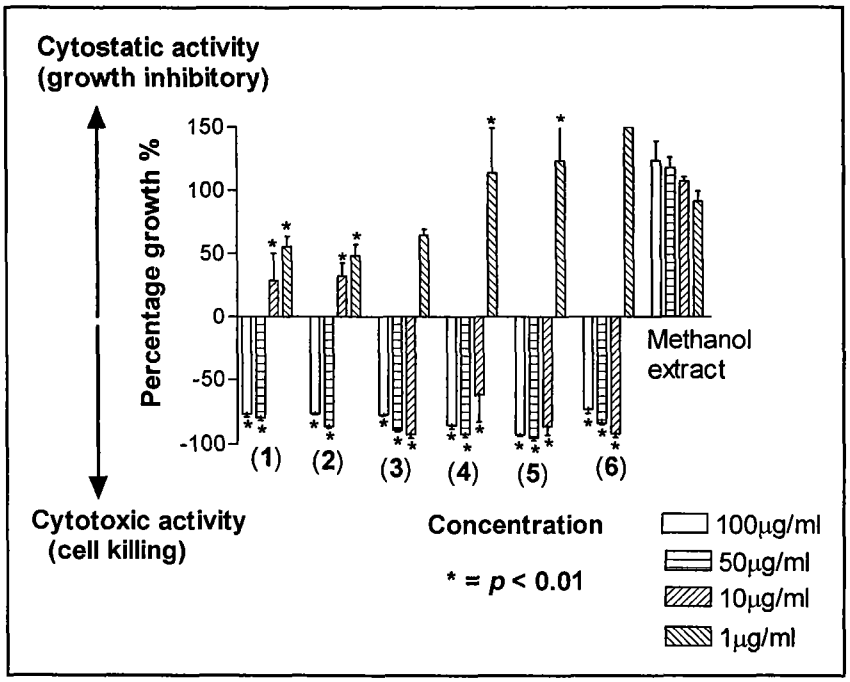

Fig.1 Growth inhibitory effect of different concentration of pure compounds and methanol extract on Hep G2 cell line. Each value represented the mean ± S.E.M. of nine replicates (three independent, $p< 0.01$).The 100% growth corresponds 0% growth inhibition compared with controls and -100% growth corresponds to total lethality compared with number of cells initially plated

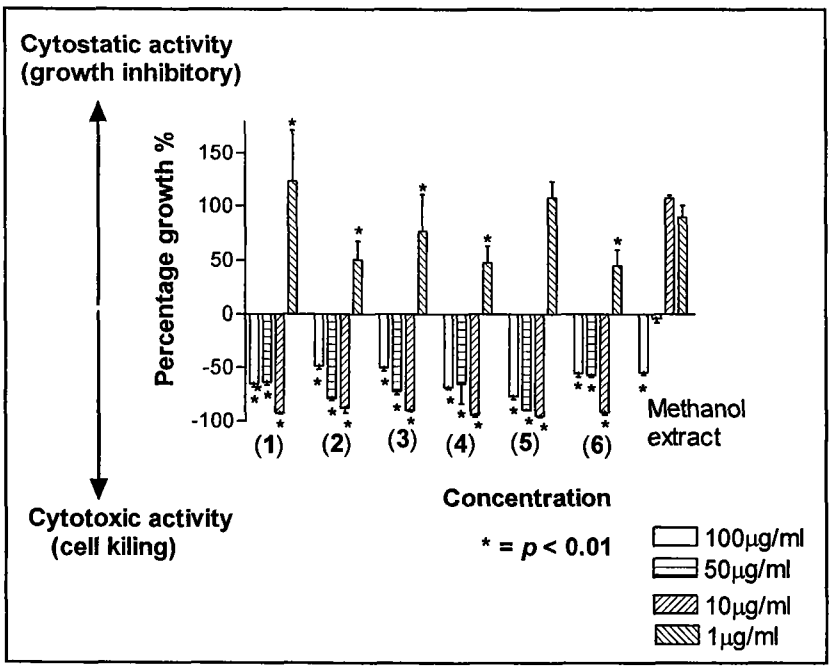

Fig. 2 Growth inhibitory effect of different concentration of pure compounds and methanol extract on T-47D cell line. Each value represented the mean ± S.E.M. of nine replicates (three independent experiments, $p < 0.01$). The 100 % growth corresponds to 0 % growth inhibition compared with controls and -100 % correspond to total lethality compared of cells initially plated.

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