ANTICANCER MEDICINAL PLANT, *Epipremnum pinnatum* (L.) Engl. CHLOROFORM EXTRACTS ELICITED BOTH APOPTOTIC AND NON-APOPTOTIC CELL DEATHS IN T-47D MAMMARY CARCINOMA CELLS

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ABSTRACT

***Epipremnum pinnatum* (L.)** Engl. chloroform extract produced significant growth inhibition against T-47D breast carcinoma cells and analysis of cell death mechanisms indicated that the extract elicited both apoptotic and non-apoptotic programmed cell deaths. 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. T-47D cells exposed to the 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. The presence of non-apoptotic programmed cell death was also detected with annexin-V and propidium iodide staining. These findings suggested that up-regulation of caspase-3 and c-myc mRNA expression may have contributed to both apoptotic and non-apoptotic programmed cell death, respectively in the ***Epipremnum pinnatum* (**L.) Engl. chloroform extract-treated T-47D cells.

KEYWORDS: Medicinal Plant, *Epipremnum pinnatum* (L.) Engl., apoptotic, non-apoptotic

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1. 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 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 [1-4]. 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. 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. Although the hexane extract of the plant is found to induce the non-apoptotic programmed cell death in breast carcinoma cells, the bioactive compounds in the hydrophobic extract may not be present in the aqueous solution prepared traditionally [5]. Investigations into the more hydrophilic extracts may explain the way the indigenous medicine works as most of the time, the aqueous decoction is used in practice.

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 [6]. The multifactorial nature of most diseases especially cancer is unlikely as a result of a single genetic or environmental change but arise from a combination of genetic, environmental or behavioral factors [7]. 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 developed in traditional medicinal systems. Interactions 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.

Human cancers development is often mainly a consequence of deregulated cell cycle control and/or suppressed apoptosis [8-9]. Impairment of apoptosis is related to cell immortality and carcinogenesis, thus, the induction of apoptosis in neoplastic cells is therefore, important in cancer treatment [10]. The word "apoptosis" is used to describe a common series of morphological changes involving the nucleus, cytoplasm and plasma membrane that accompanied the death of cells from a variety of tissue sources [11]. The earliest recognized morphological changes are compaction and segregation of nuclear chromatin, chromatin margination, convolution of nuclear and cell outlines and followed by breaking of the nucleus into discrete fragments by budding or blebbing of the cell to produce membrane-bounded apoptotic bodies [12]. All these morphological characteristics could be observed using transmission electron microscope. The other important characteristics are internucleosomal DNA fragmentation and phosphatidylserine translocation from the inner to the outer monolayer of plasma membrane as an early event of programmed cell death, which could be determined using several conventional markers of apoptosis such as TUNEL based assays and annexin V staining methods [13-19].

A range of proto-oncogenes and tumor suppressor genes has been identified that regulates apoptosis in mammals. For example, over-expression of the tumor suppressor gene, p53 leads to growth arrest and apoptosis [20-23]. The increased in the expression of c-myc proto-oncogene is also reported to lead cells to the apoptotic route as shown in several cell lines such as CHO (Chinese hamster ovary cells) [24], fibroblasts [25-26] and myeloid 32D cells [27]. Another apoptotic gene, the caspase-3 is required to trigger apoptosis has been demonstrated in caspase knockout mice [28], in strategies for inducing apoptosis, including Fas activation or exposure to ionizing radiation [29] and as demonstrated in MCF-7 breast cells transfected with caspase-3 gene [30]. Thus, in this experiment these three genes were used as markers in the investigation of cell

death mechanisms elicited by this medicinal plant. The signal transduction pathways mediating the caspase-3 mRNA expression in T-47D cells were also determined.

2. MATERIALS AND METHODS

2.1 Chemicals

The CellTiter^{TM} 96 AQ_{ueous} Non-Radioactive Cell Proliferation Assay and the Deadend^{TM} 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.

2.2 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 prepared 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.

2.3 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% (v/v) 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.

2.4 *In vitro* cytotoxicity assay

Cellular growth in the presence or absence of experimental agents was determined using CellTiter^{TM} 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 (Figure 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).

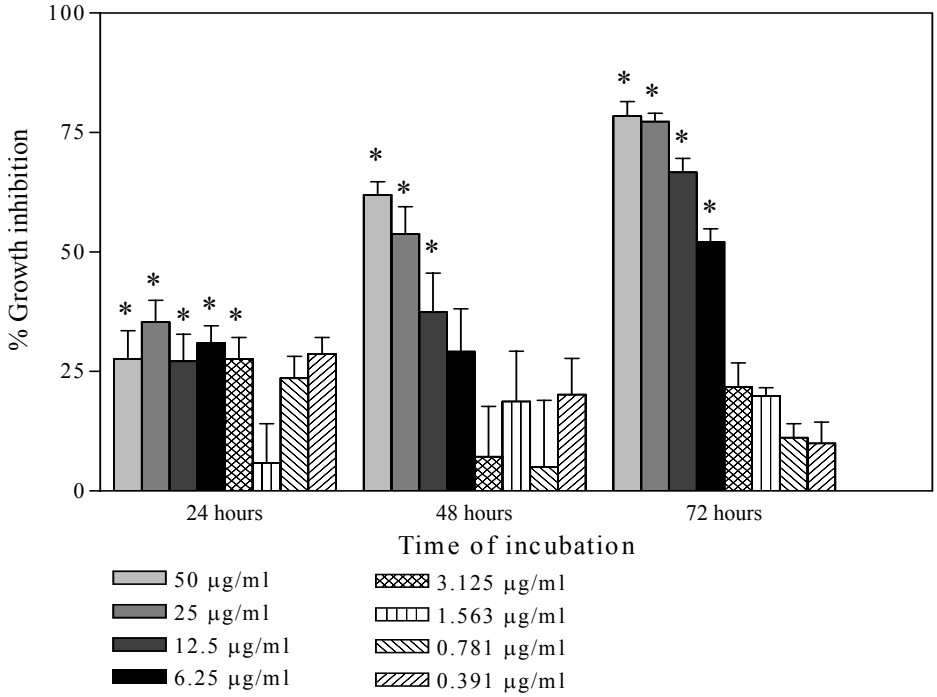

Figure 1 Growth inhibitory effects of different concentrations of chloroform extract of *Epipremnum pinnatum* plant on T-47D cell line at 24, 48 and 72 h. Each value represented the mean ± S.E.M. (n=9); * $p<0.05$.

2.5 Determination of apoptotic marker gene expression

The mRNA expression levels of c-myc, p53 and caspase-3 were performed using reverse RT-PCR [31-32]. 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 [31-32]. 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 was assigned as 1.

Table 1 The sequence of primers used in PCR

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

2.6 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 [33] and PD098059 [34], 25 μM of H89 [35], 1 μM of herbimycin A [36] and 90 μM of Arg-Lys-Arg-Ala-Arg-Lys-Glu peptide [37]. 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).

Table 2 Inhibitors used in the study of signal transduction in T-47D cells

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

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 [38]. 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.

2.7 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.

2.8 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, pelleted 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 pelleted 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. Subsequently the samples were resin-embedded and 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.

2.9 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 15–25^0C 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.

2.10 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 $\pm$ 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).

3. RESULTS

3.1 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 (Figure 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 (Figure 1). 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 [39]. Surprisingly, vincristine sulphate produced an EC_{50} value 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 higher cytotoxic activity with an EC_{50} of 1.90 μg/ml.

3.2 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). Semi-quantitative analysis of the caspase-3 expression levels revealed a significant increase in the expression levels at 3 h ($p<0.05$) (Figure 2). As for the c-myc gene, mRNA expression was up-regulated significantly 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 Figure 2.

All five inhibitors of the signal transduction pathways partially reduced the chloroform extract-induced mRNA expression of caspase-3 in T-47D cells (Figure 3). 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.

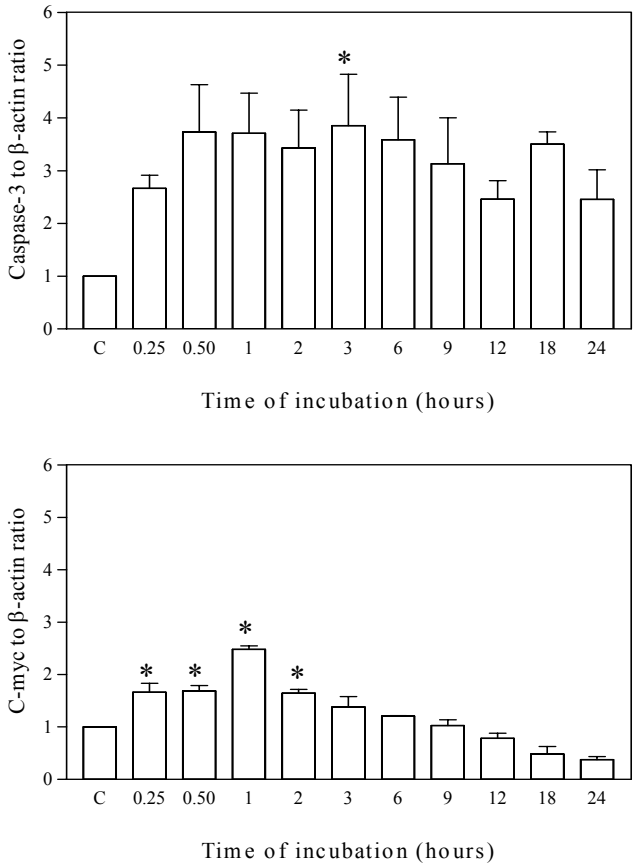

Figure 2 Semi-quantitative analysis of the caspase-3 and c-myc mRNA level in T-47D cells treated with the *Epipremnum pinnatum* chloroform extract. The ratio of caspase-3, c-myc and β-actin signals of each point was determined by densitometric scanning of the signals. The caspase-3: β-actin and c-myc: 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 $\pm$ S.E.M. (n=3) * $p<0.05$ (as compared to control).

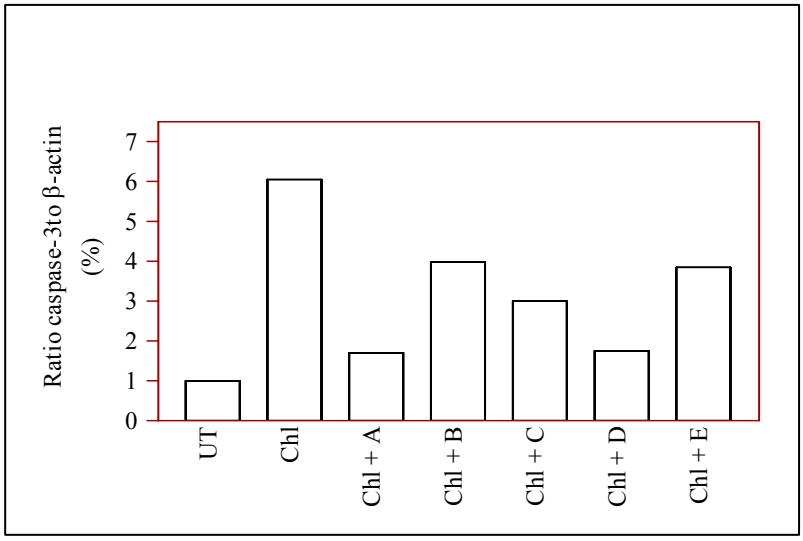

Figure 3 The effects of inhibitors against components of known signal transduction pathways on the caspase-3 mRNA expression levels in T-47D cells treated with the *Epipremnum pinnatum* chloroform extract.

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

3.3Influence 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 Figure 4A as compared to the DNase I-treated T-47D cells (Figure 4B), etoposide-treated T-47D cells (Figure 4C) and negative control T-47D cells (Figure 4D). As shown in Figure 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 apoptosis cell death. 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. Figure 4E and F showed only a few cells were stained with trypan blue at 24 and 72 h of incubation, indicating that 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 (Figure 5A, B and C), margination of chromatin against the nuclear membrane (Figure 5A and B), apoptotic bodies (Figure 5A and B), cell blebbing (Figure 5C) and vacuolated type of morphology (Figure 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 either necrosis or Type II programmed cell death (Figure 6A, C and D). However, based on the trypan blue exclusion assay, necrosis was ruled out as the possible mode of cell death. Thus, the results strongly suggest that programmed cell death was evident in the *Epipremnum pinnatum* chloroform extract-treated T-47D cells and both apoptotic and non-apoptotic programmed cell death (Type II cell death) was equally involved.

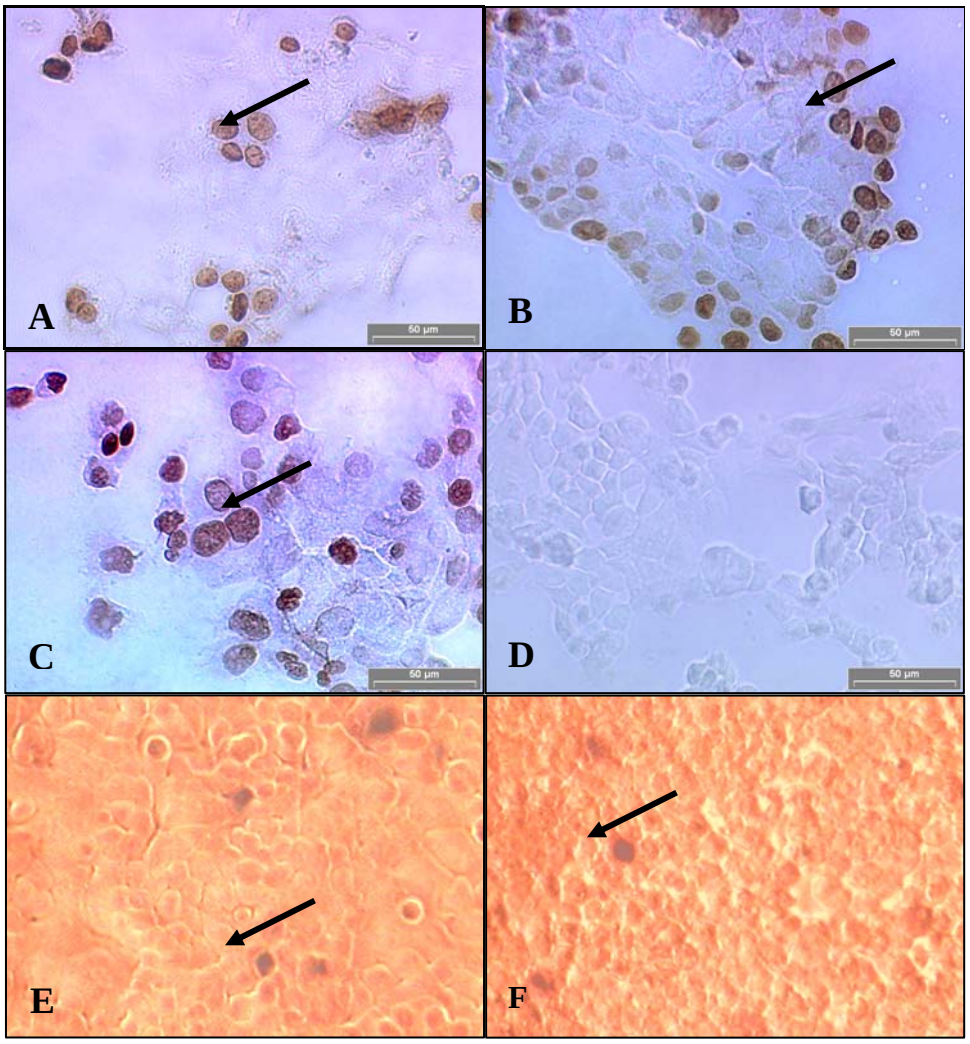

Figure 4 The effects of (A) *Epipremnum pinnatum* chloroform extract (EC_{50} at 72 h, 5.60 µg/ml), (B) DNase I (1 U/ml), (C) etoposide (EC_{50} at 72 h, 1.90 µg/ml) and (D) DMSO 1% (v/v) on T-47D cells as assayed with Deadend™ Colometric Apoptosis Detection System (Promega, USA). Arrows show dark stained 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.

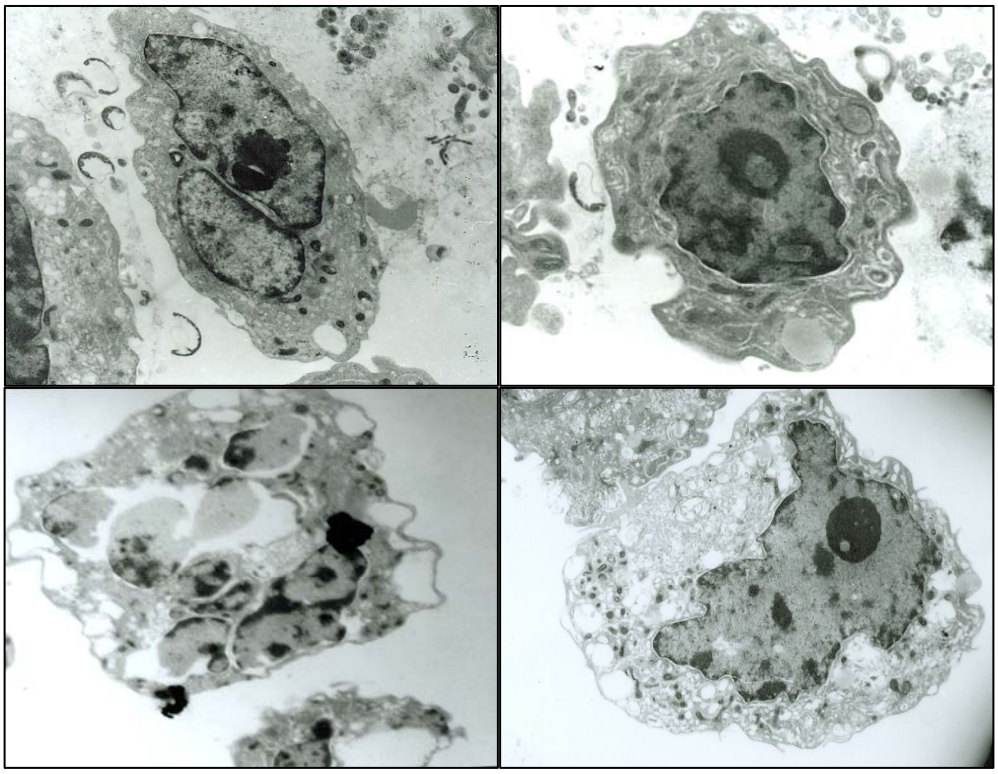

Figure 5 TEM showing the morphological features of T-47D cells treated with *Epipremnum pinnatum* chloroform extract (EC_{50} at 72 h, 5.60 µg/ml), for 24 h. (A) Chromatin clumping (thick arrow), chromatin margination (thin arrow) and apoptotic bodies (a) (B), chromatin clumping (thick arrow), cell blebbing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

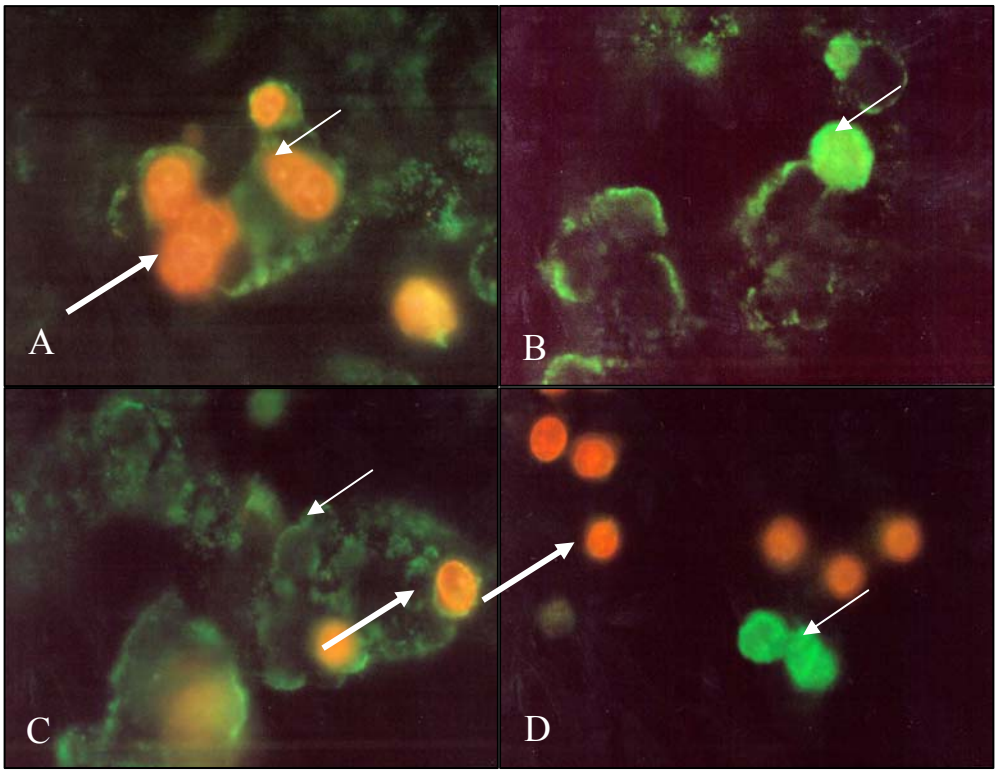

Figure 6 The effects of *Epipremnum pinnatum* chloroform extract (EC_{50} at 72 h, 5.60 µg/ml), on T-47D cell line as stained with Annexin-V-Fluos™ kit (Roche, Germany). The presence of high intensity and weakly scattered annexin V (green fluorescence, thin arrows) and propidium iodide stained cells (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.

4. DISCUSSION

The studies presented in this report revealed the potential anticancer properties of the *Epipremnum pinnnatum* (L.) Engl. 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 is 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 [40], there are studies which reported that over-expression of c-myc can induce programmed cell death depending on circumstances. For example, droloxifene, a tamoxifen derivative induces apoptosis in cultured luteal cells with significant increase of c-myc mRNA expression levels as treatment duration increased [41].

Similarly, an increased in the mRNA expression of caspase-3 was also observed and 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 [42-44]. 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 disrupt survival pathways and disassemble important architectural components of the cell, contributing to the stereotypic morphological and biochemical changes that characterize apoptotic cell death [45]. 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 is not surprising as both pathways are inter-related. The atypical PKC isozymes are activated via the PI-3-K-dependent pathways, which are also the main signaling pathway utilized by the tyrosine kinases [46]. The pro-apoptotic role of caspase-3 gene mediating apoptosis via the protein kinase C pathways is well described in various studies. For example, the tumor necrosis factor α (TNFα) induces cytotoxicity and apoptosis in the rat epithelial cell line, IEC-18, via the activation caspase-3 enzyme and its effects on the activity are reduced by selective inhibition of novel PKCε and δ, indicating that the PKCs mediates the activation of caspase-3 activity [47].

In another example, cDDP [cis-diamminedichloroplatinum (II), an anticancer agent] causes a time and concentration-dependent increase in the generation of the catalytic fragment of novel PKCδ and ε that is associated with activation of caspase-3 and 7 [48]. In addition, one study demonstrated that phorbol 12- myristate 13 acetate (PMA) induces cell death in SNU-16 gastric adenocarcinoma cells via caspase-3 activation and the activity is completely suppressed by Go6983, a specific inhibitor of PKC, indicating that the activation of caspase-3 is mediated via the PKC pathways [49]. Similarly, PKCδ is found to increase the pro-apoptotic effects of caspase-3 in Jurkat-T leukemic cells exposed to ionizing radiation [50].

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 (or known 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 [51-54]. Immunohistochemical experiments were carried out using the annexin V and

propidium iodide staining. 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, concluded the existence of Type II non-apoptotic cell death (vacuolar) as this type of cells appeared to take-up the stain similarly to necrotic cells. The ability of the cells to take-up the propidium iodide stain was probably due to cell porosity [5].

The discovery non-apoptotic cell deaths are demonstrated in MCF-7 breast carcinoma cells induced by TNF-α and tryphostin AG123. It is demonstrated that MCF-7 cell death induced by the EGFR specific tyrosine kinase inhibitor is mediated through the non-apoptotic cell death, in the absence of DNA fragmentation [55]. Tyrosine kinase inhibition has also been reported to produce a non-apoptotic programmed cell death in colon tumor cells [56]. Electron microscope study in human bronchiolar lung cancer cell line cultured with inhibitor of 5-lipooxygenase such as SC41661A and MK886, revealed that the agents produce a form of programmed cell death, which ultrastructural changes are consistent with a Type II non-apoptotic programmed cell death [57]. Some triterpenoid saponins from soybeans are also reported to cause Type II cell death in cultures of human HCT-15 colon adenocarcinoma cells [58].

Recent studies revealed that active self-destruction of cells are not confined to apoptosis alone. Cells actually use different pathways to commit suicide, including the non-apoptotic or Type II PCD, known as vacuolar or autophagic death. Briefly, autophagic cell death is often associated with bulk degradation of proteins and organelles, a process essential for cellular maintenance and cell viability [59]. It is shown to be essential; for differentiation, development as well as cellular maintenance [60]. The most recent reviews have clearly indicated that malignant transformation was frequently associated with the suppression of autophagic cell death [61-65]. Autophagic PCD is also significantly associated with other diseases such as neurodegenerative diseases, cardiomyopathies, bacterial and viral infections [61]. During autophagy, a cup-shaped structure, the preautophagosome, engulfs cytosolic components like organelles to form the autophagosome, which subsequently fuses with a lysosome, leading to the proteolytic degradation of the components [59]. This process usually is 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 [66].

The discovery of the autophagy genes such as *Atg7* and *Beclin1* that were involved in the regulation of this type of cell death and autophagic death induction by some anticancer agents have actually open more avenues for research in this area [59, 61-65]. C-myc gene is also indicated in this type of cell death [5]. 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 growth arrests in T-47D cells via both apoptotic and non-apoptotic programmed cell death. The induction of cell death was probably via caspase-3 and c-myc pathway but was independent of p53. The mRNA expression of the caspase-3 and c-myc gene was likely contributed to the apoptotic and non-apoptotic programmed cell death. Although apoptosis as a mechanism of cell death is desired in cancer therapeutics, the role of non-apoptotic programmed cell death remained unclear. By synergistically producing both type of cell deaths in cancer cells, *Epipremnum pinnatum* (L.) Engl in crude form may be beneficial as a herbal preparation in cancer patients.

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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