Cleistopholine isolated from *Enicosanthellum pulchrum* exhibits apoptogenic properties in human ovarian cancer cells


**Keywords:**
- Apoptosis
- CAOV-3
- Cleistopholine
- *Enicosanthellum pulchrum*
- Natural product
- Ovarian cancer

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**Abstract**

**Background:** Cleistopholine is a natural alkaloid present in plants with numerous biological activities. However, cleistopholine has yet to be isolated using modern techniques and the mechanism by which this alkaloid induces apoptosis in cancer cells remains to be elucidated.

**Hypothesis/purpose:** This study aims to isolate cleistopholine from the roots of *Enicosanthellum pulchrum* by using preparative-HPLC technique and explore the mechanism by which this alkaloid induces apoptosis in human ovarian cancer (CAOV-3) cells *in vitro* from 24 to 72 h. This compound may be developed as an anticancer agent that induces apoptosis in ovarian cancer cells.

**Study design/methods:** Cytotoxicity was assessed via the cell viability assay and changes in cell morphology were observed via the acridine orange/propidium iodide (AO/PI) assay. The involvement of apoptotic pathways was evaluated through caspase analysis and multiple cytotoxicity assays. Meanwhile, early and late apoptotic events via the Annexin V-FITC and DNA laddering assays, respectively. The mechanism of apoptosis was explored at the molecular level by evaluating the expression of specific genes and proteins. In addition, the proliferation of CAOV-3-cells treated with cleistopholine was analysed using the cell cycle arrest assay.

**Results:** The IC₅₀ of cleistopholine (61.4 μM) was comparable with that of the positive control cisplatin (62.8 μM) at 24 h of treatment. Apoptosis was evidenced by cell membrane blebbing, chromatin condensation and formation of apoptotic bodies. The initial phase of apoptosis was detected at 24 h by the increase in Annexin V-FITC binding to cell membranes. A DNA ladder was formed at 48 h, indicating DNA fragmentation in the final phase of apoptosis. The mitochondria participated in the process by stimulating the intrinsic pathway via caspase 9 with a reduction in mitochondrial membrane potential (MMP) and an increase in cytochrome c release. Cell death was further validated through the mRNA and protein overexpression of Bax, caspase 3 and caspase 9 in the treated cells compared with the untreated cells. In contrast, Bcl-2, Hsp70 and survivin decreased in expression upon cleistopholine treatment. Cell cycle was arrested at the G0/G1 phase and cell population percentage significantly increased to 43.5%, 45.4% and 54.3% in time-dependent manner in the cleistopholine-treated CAOV-3 cells compared with the untreated cells at 24, 48 and 72 h respectively.

**Conclusion:** The current study indicated that cleistopholine can be a potential candidate as a new drug to treat ovarian cancer disease.

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**Abbreviations:** HPLC, high performance liquid chromatography; IR, infrared; 1D NMR, one-dimensional nuclear magnetic resonance; MS, mass spectrometry; CO₂, carbon dioxide; IC₅₀, inhibition concentration of 50%; sh, shoulder; NaCl, sodium chloride; HRESIMS, high-resolution electron spray ionisation mass spectrometry; calcd, calculated; CDCl₃, deuterated chloroform; FITC, fluorescence isothiocyanate; mRNA, messenger ribonucleic acid; PCR, polymerase chain reaction; Bcl-2, B-cell lymphoma 2; Caspases 3, 8 and 9, cysteinyl aspartic acid-protease-3, 8 and 9; Hsp70, heat shock protein 70; CAD, caspase-activated DNase.

*Corresponding author.* Tel.: +60123279594; fax: +60379674964.
**E-mail address:** najihahmh@gmail.com, najihahmh@um.edu.my (N.M. Hashim).
Introduction

Cancer is a primary disease that continues to increase annually and cause mortality worldwide. Thus, new discoveries are needed to reduce the morbidity and mortality of cancer (Hail 2005). Ovarian cancer has become the fourth most deadly gynaecologic malignancy and a major cause of death among women in Malaysia (Omar et al. 2006). Moreover, more than 70% patients with late stage ovarian cancer experience deterioration and resistance to conventional chemotherapy drugs (Bellati et al. 2010; Monk and Coleman 2009). Thus, discovering new compounds that can overcome this problem is highly important. A strategy to identify safe compounds with anticancer effects is urgently needed to treat ovarian cancer.

Cleistopholine is anazaanthraquinone alkaloid that was isolated from the *Enicostantherium pulchrum* of the family Annonaceae (Nordin et al. 2012). This family has an abundance of alkaloids, which are highly active components with various biological activities (Leboeuf et al. 1981; Lu et al. 2012). In the presence of a benzene ring, nitrogen atoms and sites in cleistopholine could increase the affinity and hydrogen bonding interaction with specific targets to induce cell apoptosis. This structure indicates that cleistopholine is a potential anticancer agent. However, comprehensive studies on the mechanism by which cleistopholine triggers apoptosis in ovarian cancer cells (CAOV-3) are currently lacking. In the present study, cleistopholine showed high cytotoxic effects against CAOV-3 cells at a very low concentration (μM), which clarifies the potential of cleistopholine as a candidate drug to treat ovarian cancer by killing cancer cells through induction of apoptosis.

Materials and methods

Chemicals and reagents

Dimethylsulphoxide (DMSO), methanol (MeOH), ethanol (EtOH), chloroform (CHCl3) and ethyl acetate (EtOAc) were purchased from Merck Co. (Germany), while silica gel H, 3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazolium bromide (MTT) were procured from Sigma-Aldrich (St. Louis, USA). Trypsin-EDTA 10X, Accutase, RPMI-1640 medium (pH 7.4), penicillin/streptomycin and foetal bovine serum (FBS) were obtained from Nacalai Tesque (Kyoto, Japan).

Plant materials

Roots of *E. pulchrum* were collected from Cameron Highlands mountain forest, Pahang, Malaysia in October, 2011. Permission to collect the samples in the forest was provided by the Director of the Forestry Department of Pahang, Malaysia as previously mentioned by Nordin et al. (2014). The voucher specimen was labelled as SM769 which was identified by the late Prof. Dr. Kamaruddin Mat Salleh (Botany Department, Faculty of Science and Technology, Universiti Kebangsaan Malaysia). The identified specimen was deposited at the Herbarium of Botany Department, UKM, Malaysia. The roots were air-dried and ground to 40–60 mesh size.

Preparation of root extract

Dried roots (100 g) were finely chopped before extraction was carried out using the modified method of Nordin et al. (2012). The EtOAc extract (1.96 g) was further separated using silica gel type H via vacuum liquid chromatography (VLC) to enrich the compounds on the gradient of n-hexane–CHCl3–MeOH. Ten fractions were collected and combined using thin layer chromatography (TLC) analysis to yield five fractions (1–5). Fraction 3 was further purified using preparative HPLC (prep-HPLC).

Separation by prep-HPLC

Prep-HPLC was performed by injecting 2 ml of filtered MeOH fraction 3 onto a Prep Nova-Pak (10 mm × 20 mm × 30 mm) HR C18 reversed-phase HPLC column (Waters, USA), The fraction was eluted using a Gilson pump (322 pump Gilson, USA) at a constant flow rate of 12 ml/min for 95 min. The solvent system consisting of 10% acetonitrile in water was run gradiently up to 100% acetonitrile (v/v). The compound was detected using a Gilson absorbance detector (UV-VIS 156 Gilson, USA) at 254 nm. Cleistopholine (18.9 mg, 0.019% of yield) was collected between 28 and 30 min (Fig. 1).

Cell culture

Human ovarian cancer cells (CAOV-3 and SKOV-3) and immortalized human ovarian epithelial cells (SV-40 Large T Antigen) were purchased from American Type Culture Collection (ATCC, Manassas, USA) and ABM Inc. (Crestwood Place Richmond, Canada), respectively. These three cell lines were originally purchased from the mentioned companies and cultivated in the laboratory of Department of Pharmacy, University of Malaya.

Cytotoxicity assay

This assay was performed using a modified MTT assay (Syam et al. 2014). CAOV-3 cells (100 μl) at a density of 1 × 105 cells/ml were seeded in a 96-well microplate and then treated with cleistopholine (100 μM) in triplicates for 24, 48 and 72 h by serial dilution. The MTT reagent (5 mg/ml) was prepared and a 20 μl MTT solution was added to each well and further incubated for 3 h. The plate was then read at 570 nm using a microplate reader. Two standard drugs, paclitaxel and cisplatin were used as positive controls in the assay.

Assessment of apoptosis morphology using acridine orange (AO)/propidium iodide (PI) double staining

Cleistopholine-induced apoptosis in CAOV-3 cells was investigated using AO and PI double staining (Syam et al. 2014). The morphological changes were observed within 30 min under a UV-fluorescent microscope (Olympus BX60 attached with Q-Floro software) to avoid fading of the fluorescence colour on the cells.

Annexin-V-FITC assay

This assay was performed using an Annexin V-FITC Apoptosis Detection kit I (BD Pharmingen™, San Diego, California, USA). Cells at a density of 5 × 104 cells/ml were plated in a six-well plate and then treated with cleistopholine (61 μM) for 24, 48 and 72 h. The treated cells were harvested and collected using Trypsin-EDTA 10X and centrifuged at 1600 rpm for 5 min. The pellet was resuspended in 1 x assay buffer and a 100 μl aliquot of each sample was transferred into a tube consisting of 5 μl of FITC and 10 μl of PI staining. The suspension was mixed and added with 100 μl of 1 x assay buffer per tube. All tubes were examined by a flow cytometer (BD FACS Canto™II, San Jose, CA, USA).

Caspase 3, 8 and 9 analyses

The assay was performed using a commercial kit (Caspase 3, Caspase 8 and Caspase 9 colorimetric assay: R&D Systems, Inc. USA). CAOV-3 cells at a density of 1 × 106 cells/ml were seeded in a 25 ml flask and treated with cleistopholine (61 μM) for 24, 48 and 72 h. Cells that were induced to undergo apoptosis were trypsinised using Trypsin-EDTA 10X and then centrifuged at
1600 rpm for 10 min. The lysis buffer was added to the cell pellet and then incubated on ice for 10 min. The cell lysate was further centrifuged at 10,000×g for 1 min. After the preparation of cell lysate, the assay was performed in 96-well flat bottom microplate. Each well consisted of 50 μl of cell lysate and 50 μl of 2X reaction buffer 3, 8 or 9. Then, 5 μl of caspase-3, 8 or 9 colorimetric substrate (LEHD-pNA) was added to each reaction well and then incubated at 37°C for 1 h. The reaction mixture was read on a luminescence microplate reader (Infinite M200PRO, Tecan, Männedorf, Switzerland) at an absorbance of 405 nm.

Multiple cytotoxicity assays

Multiple cytotoxicity assays were performed using the Cellomics® Multiparameter Cytotoxicity 3 kit (Thermo Scientific, PA, USA). The assay was performed in triplicates using a 96-well microplate. The 90% confluence cells from the 25 ml flask were seeded in the microplate at a concentration of 5×10^3 cells per well. The cells were treated with cleistopholine at 40, 50 and 60 μM for 24 h and then incubated overnight at 37°C. In brief, several solutions were used and incubated successively, including 100 μl of fixation solution (20 min), 100 μl of 1× permeabilization buffer (10 min) and 100 μl of 1× blocking buffer (15 min) and added to each well containing 50 μl of live cell staining. A total of 50 μl of primary antibody was added to the wells and incubated for 1 h, followed by secondary antibody (50 μl) using the same procedure. The plate was read and evaluated using an ArrayScan HCS Reader (Thermo Fisher Scientific, Pittsburgh, PA, USA).

DNA laddering

A Suicide-Track™DNA Ladder isolation kit (Calbiochem, KGaA, Darmstadt, Germany) was used to analyse DNA fragments (mononucleosomes and oligonucleosomes) formed during apoptosis. The assay was performed in a 25 ml flask in which the CAOV-3 cells were seeded at a concentration of 5×10^4 cells/ml and then treated with cleistopholine for 24, 48 and 72 h. In brief, the harvested cells were collected using Accutase and then centrifuged at 1800 rpm for 5 min. The pellet was gently dispersed in 55 and 20 μl of solutions #1 and #2 (kit components) and then incubated for 1 h at 37°C. The mixture was added with 25 μl of solution #3 (kit components) and then incubated at 50°C overnight. A total of 500 μl of resuspension buffer was added as the final procedure for DNA extraction. To detect the DNA ladder, gel agarose was prepared by adding 1.5% agarose gel into 1 X TAE buffer with the staining reagent supplied in the kit. The gel was run at 50 constant volts until the dye reached 1–2 cm from the end of the gel. The DNA ladder was viewed under a UV light transilluminator and then photographed.

Real-time PCR

Total RNA was extracted from CAOV-3 cells using the RNeasy Mini Kit (Qiagen, Germany). The concentration and purity of RNA were evaluated by absorption ratios of 260/280 UV (Nanodrop 2000 Spectrophotometer, Thermo Scientific). The RNA to cDNA conversion was performed using a Two-Step qRT-PCR Kit, High capacity RNA to cDNA (Applied Biosystems, USA). The gene expression assay was performed in strips where 1 μg/ml cDNA was added to each strip. TaqMan® primers and probe consisting of β-Actin, Bax, Bcl-2, Hsp70, survivin, caspase 3, 8 and 9 genes were used in the assay as supplied from TaqMan® genes expression assay (Applied Biosystem, USA). The real time-PCR program was as follows: incubation at 50°C for 2 min, 95°C for polymerase activation for 20 s, denaturation for 1 s and annealing at 60°C for 20 s. The process of denaturing and annealing was completed in 40 cycles by using StepOne plus real-time PCR machine (Applied Biosystems, USA). Data were examined using the comparative Ct \(2^{-\Delta\Delta Ct}\) method (Wong and Medrano 2005).

Western blot assay

CAOV-3 cells were seeded in a 75 ml culture flask and then treated with cleistopholine (61 μM) at 24, 48 and 72 h. The cells were trypsinised using Accutase and centrifuged at 13,000 rpm for 10 s and then resuspended in 400 μl of PREP™ solution. The cells were lysed after a 20 min incubation at −20°C and then centrifuged at 13,000 rpm for 5 min at 4°C. Total protein (200 μg/ml) was obtained and mixed with loading dye before separation by 12% SDS-PAGE for 90 min. The band that appeared on the gel was transferred to a polyvinylidene difluoride (PVDF) membrane (Bio-Rad) by running it in two bin buffer for 90 min. The PVDF membrane was blocked with 5 ml of BSA (5%) and then incubated for 2 h. Seven primary antibodies obtained from Abcam Inc. (California, USA) were used consisting of β-Actin (1:1000), Bax (1:1000), Bcl2 (1:1000), Hsp70 (1:1000), survivin (1:1000), caspase 3 (1:1000) and caspase 9 (1:1000). The secondary antibody (goat pAb to Rb IgG) was bound to the primary antibody after 2 h of incubation at ambient temperature. The bound antibodies were detected using a detection kit and then exposed for a few minutes to exhibit the bands. The PVDF membrane was viewed and photographed under a UV light transilluminator.

DNA cell cycle

The cancer cells were seeded into a 25 ml culture flask at a density of 1×10^5 cells/ml. The cells treated with cleistopholine for 24, 48 and 72 h were collected using Trypsin-EDTA 10× and then rinsed twice with PBS via centrifugation at 1800 rpm for 5 min. The
pellet was fixed with 700 μl of cold EtOH (90%) and then incubated at 4°C overnight to maintain cell integrity. The EtOH was removed by centrifugation at 1800 rpm for 5 min and then added with 600 μl of PBS to the cell pellet. The fixed cells were mixed with 25 μl of RNaseA (10 mg/ml) and 50 μl of PI (1 mg/ml) and then further incubated for 1 h at 37°C before analysis with a flow cytometer [Ila et al. 2013].

Statistical analysis

All values were reported as mean ± standard deviation (SD) in triplicate experiments. One-way analysis of variance (ANOVA) was used to evaluate the statistical significance data using a SPSS-17.0 package (IBM Corporation, USA), whereas GraphPad prism (version 4.0 Graphpad software Inc, USA) was used to determine the IC_{50} values.

Results

Structure identification of cleistopholine

Cleistopholine (Fig. 2) was elucidated by 1D and 2D NMR spectroscopy [Table 2], as well as ESI mass spectrometry. This compound was also determined previously from other plant species (Waterman and Muhammad 1985).

Cleistopholine: Brown amorphous powder; (MEOH) λ_{max} (log ε); 287, 346 (sh), 415. IR (NaCl) ν_{max}; 1737 (C=O); HRESIMS m/z [M+H]+ 224.0704 (calc’d for C_{14}H_{9}NO_{2}, 223.0633).

Cell growth cytotoxicity assay

The cytotoxic effect against the three cell lines of SV40, CAOV-3 and SKOV-3 was tested for cleistopholine and the standard drugs, paclitaxel and cisplatin [Table 1]. The IC_{50} values of the CAOV-3 and SKOV-3 cells treated with cleistopholine for 24 h were 13.7 ± 0.42 μg/ml (61.4 μM) and 15.0 ± 1.21 μg/ml (67.3 μM), respectively. Meanwhile, the cytotoxic effects against CAOV-3 cells at 48 and 72 h showed a decrease in IC_{50} values of 7.9 ± 1.68 (44.8 μM) and 4.5 ± 0.07 (19.9 μM), respectively (Fig. 3). Cleistopholine also exhibited a comparable effect with cisplatin after 24 h of CAOV-3 cells treatment (Fig. 3). Cleistopholine at >200 μM showed less cytotoxic effect against normal ovarian cells (SV-40).

Quantification of apoptosis using AO and PI double staining

CAOV-3 cells showed morphological changes after treatment with cleistopholine (61 μM) in the AO/PI assay [Fig. 4a]. The cleistopholine effect was observed in the CAOV-3 cells on the basis of the morphological changes that occurred in early apoptosis, late apoptosis or necrosis. Microscopic observation revealed that the untreated CAOV-3 cells had a round shape with a green unbroken nuclear structure. Cleistopholine treatment for 24 h changed the morphology of the CAOV-3 cells indicated by the cell

<table>
<thead>
<tr>
<th>Compound/cell line</th>
<th>IC_{50} ± SD (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAOV-3</td>
<td>SKOV-3</td>
</tr>
<tr>
<td>Cleistopholine</td>
<td>61.4 ± 0.42</td>
</tr>
<tr>
<td>Paclitaxel</td>
<td>0.91 ± 0.01</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>62.8 ± 1.06</td>
</tr>
</tbody>
</table>

Table 1

Cleistopholine IC_{50} concentration of cleistopholine and positive control on human ovarian cancer and normal cells at 24 h in vitro.

Table 2

Spectroscopic data of 1D and 2D NMR in chloroform-d for cleistopholine (δ in ppm, J in Hz).

<table>
<thead>
<tr>
<th>Position</th>
<th>Δ_δ (J in Hz)</th>
<th>Δ_C type</th>
<th>HMBC</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>8.84, d (4.86)</td>
<td>131.2, CH</td>
<td>3, 9a</td>
</tr>
<tr>
<td>3</td>
<td>7.44, d (4.84)</td>
<td>151.6, C</td>
<td>2, 4a, 4</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td>129.2, C</td>
<td></td>
</tr>
<tr>
<td>4a</td>
<td></td>
<td>131.6, C</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>8.31, dt (1.92, 2.58)</td>
<td>127.4, CH</td>
<td>10a, 10</td>
</tr>
<tr>
<td>6</td>
<td>7.75–7.79, m</td>
<td>134.2, CH</td>
<td>7</td>
</tr>
<tr>
<td>7</td>
<td>7.75–7.79, m</td>
<td>134.6, CH</td>
<td>6, 8</td>
</tr>
<tr>
<td>8</td>
<td>8.21, dt (1.98, 2.46)</td>
<td>127.2, CH</td>
<td>8a, 9</td>
</tr>
<tr>
<td>8a</td>
<td></td>
<td>132.6, C</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>184.8, C=O</td>
<td></td>
</tr>
<tr>
<td>9a</td>
<td></td>
<td>150.2, C</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>181.9, C=O</td>
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</tr>
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<td>10a</td>
<td></td>
<td>133.9, C</td>
<td></td>
</tr>
<tr>
<td>4-Me</td>
<td>2.86, s</td>
<td>22.9, CH</td>
<td>4, 3</td>
</tr>
</tbody>
</table>

Notes:

1. Proton was measured at 400 MHz.
2. Carbon was measured at 100 MHz.

Fig. 3. Inhibitory effects of cleistopholine and cisplatin on the CAOV-3 cell line at different times.

Fig. 2. Structure of cleistopholine.
membrane blebbing and DNA fragmentation under bright green fluorescence. The morphological changes, including cell membrane blebbing, apoptotic body formation and presence of reddish-orange coloured cells, were prominent after 48 and 72 h of treatment and indicated late apoptosis. In addition, the number of viable, early apoptotic and late apoptotic cells was counted (Fig. 4b). The number of cells that underwent early apoptosis and late apoptosis increased in a time-dependent manner after cleistopholine treatment. In contrast, the number of viable cells decreased, indicating apoptosis induction in CAOV-3 cells.
Results both was treated Evaluation to secondary in respectively.

However, Annexin Fig. 5. Analysis of Annexin V-FITC in CAOV-3 cells treated with cleistopholine (61 μM) using flow cytometry. [A] Control (untreated), [B] 24 h, [C] 48 h, [D] 72 h, [E] histogram. Results were represented as mean ± SD of three replicates. *P < 0.05 indicates significant difference from the control.

Annexin V-FITC analysis

This analysis was performed to confirm the induction of early apoptosis in CAOV-3 cells after cleistopholine treatment (61 μM). However, other cell stages, including late apoptosis and secondary necrosis were also observed (Fig. 5). After 24 h of exposure, the percentage of early apoptotic cells was 9.1 ± 3.55%. Continuous exposure of the compound for 48 and 72 h increased the number of cells undergoing early apoptosis to 13.7 ± 0.99% and 19.0 ± 1.41%, respectively. Meanwhile, the percentage of viable cells after 24 h of treatment reduced from 87.6% in the untreated cells to 76.4% in the treated cells. The percentages of cells in late apoptosis and secondary necrosis also significantly increased (P < 0.05) from 48 to 72 h compared with the untreated cells.

Evaluation of caspase 3, 8 and 9

Caspase 3, 8 and 9 were investigated in the CAOV-3 cells treated with cleistopholine (61 μM). The action of cleistopholine was observed at 24, 48 and 72 h (Fig. 6). Cleistopholine stimulated both caspases 3 and 9 when the treatment time was increased from 24 to 72 h. As shown in Fig. 6, caspase 8 decreased although not significantly from 24 to 72 h, indicating that caspase 8 was not activated after CAOV-3 cells were treated with cleistopholine. The effect of cleistopholine effect on caspases 3 and 9 indicated the involvement of an intrinsic pathway that triggered cell apoptosis in the cells.

Analysis of multiple cytotoxicity assays

This assay was carried out to investigate four important parameters in apoptosis. Fig. 7a indicates the intensity results of total nuclei, cell permeability, mitochondrial membrane potential (MMP) and cytochrome c. The number of total nuclei (Hoechst dye) decreased after cleistopholine treatment. The MMP also significantly reduced in intensity (P < 0.05) after treatment with cleistopholine at 60 μM (Fig. 7b). The opposite action occurred for the cell permeability and cytochrome c parameters; that is both parameters significantly increased (P < 0.05) in the cells treated with cleistopholine at 50 and 60 μM compared with the untreated CAOV-3 cells (Fig. 7b).
Fig. 6. Colorimetric analysis of caspase 3, caspase 8 and caspase 9 in untreated and treated CAOV-3 cells with cleistopholine (61 μM) at 24, 48, and 72 h. Results were expressed as mean ± SD of three independent experiments. The significance difference is expressed as $P < 0.05$.

Fig. 7. (a) CAOV-3 cells images of untreated and treated CAOV-3 cells with cleistopholine (61 μM) of four dyes, namely Hoechst, cell permeability, mitochondrial membrane potential and cytochrome c. The images were captured from the same field of each row. The CAOV-3 cells exhibited an increase in cell permeability and cytochrome c after being treated with cleistopholine at 24 h (magnification 20×). (b) Quantitative analysis of cleistopholine-mediated apoptosis in CAOV-3 cells. Average intensities were observed simultaneously in CAOV-3 cells for total nuclear intensity, cell permeability, mitochondrial membrane potential and cytochrome c. All data were represented as mean ± standard deviation (SD). Statistical significance was expressed as $*P < 0.05$. 
Western blot analysis

This analysis was performed on CAOV-3 cells to confirm the effect of cleistopholine at the protein level. The study involved six proteins, namely Bax, Bcl-2, Hsp70, survivin, caspase 3 and caspase 9 (Fig. 10). Bax, caspase 3 and caspase 9 were up-regulated, while Bcl-2, Hsp70 and survivin were down-regulated in a time-dependent manner in the treated cells compared with the untreated cells. Significant differences ($P < 0.05$) in protein expression levels were also detected at 72 h for all proteins after cleistopholine treatment between the treated and untreated cells.

Cell cycle analysis

Analysis of cell cycle arrest in CAOV-3 cells was carried out using a flow cytometer (Fig. 11). The number of cells in the G0/G1 phase significantly increased ($P < 0.05$) to 43.5%, 45.4% and 54.3% in a time-dependent manner from 24 to 72 h. However, the population of CAOV-3 cells at the S phase significantly reduced after cleistopholine treatment. The increased number of CAOV-3 cells in the G0/G1 phase indicated that the cells stopped dividing because of cell cycle arrest.

Discussion

Apoptosis or programmed cell death plays a vital role in controlling physiological cell growth and tissue homeostasis (Sreedhar and Csermely 2004). In recent years, most development studies on cell death, particularly that related to cancer has focused on apoptosis (Fulla and Debatin 2006). In the present study, the induction of apoptosis by cleistopholine was evaluated in CAOV-3 cells and was confirmed through the significant inhibition of cell viability (Fig. 3). Cleistopholine isolated from Camanga odorata and Saprosma hainanense exerts cytotoxic effects on other cancer cells, such as hepatocarcinoma cell lines (Hsieh et al. 2001; Wang et al. 2011).

The hallmark of apoptosis was observed in the study of AO/PI via the cytomorphological changes in CAOV-3 cells after cleistopholine treatment. Some of the apoptosis features observed in the cells were similar to those explained by previous researchers (Kurosaka et al. 2003). In the present study, cleistopholine induced apoptosis as indicated by the consistent morphological changes and increase cell number at each phase of apoptosis (Figs. 4a and b). Meanwhile, the induction of early apoptosis in CAOV-3 cells was confirmed through the involvement of Annexin V-FITC (Fig. 5). Annexin V-FITC is an important marker of early apoptosis because it can bind with phosphatidylserine (PS) on the surface of CAOV-3 cells. PS was exposed to Annexin V-FITC after being translocated from the cytosol to the outside cell membrane layer, thereby allowing the cells to remain intact (Vermes et al. 1995). In comparison with AO/PI assay, the Annexin V-FITC assay is more precise in determining the cell population at each stage of apoptosis as well as viable cells.

Induction of apoptosis in CAOV-3 cells via the intrinsic pathway after cleistopholine treatment was confirmed as shown in Figs. 6, 9 and 10. Cleistopholine stimulates the intrinsic pathway which produces the intracellular signals that trigger apoptosis. Upon activation of the intrinsic pathway, distraction occurred in the inner mitochondrial membrane, which opened the mitochondrial permeability (MPT) pores. The mitochondrial trans-membrane potential is also damaged and hence releases the cytochrome c from the mitochondria into the cytoplasm (Du et al. 2000; Saelens et al. 2004). These conditions can be observed in Fig. 7a, proving that cleistopholine induced apoptosis with the involvement of the mitochondria in the intrinsic pathway.

Stimulation of the intrinsic pathway terminates at the execution phase by stimulation of caspase 3 (Saelens et al. 2004). This
pathway is stimulated when cytochrome c is released into the cytoplasm from the mitochondria (Abdul et al. 2013). As shown in Figs. 6, 9 and 10, the activation of caspase 3 stimulates downstream cellular events by stimulating the endonuclease CAD and degrading chromosomal DNA inside the nuclei, which finally leads to chromatin condensation (Mukhtar et al. 2012; Sakahira et al. 1998). Activation of downstream apoptotic signalling on CAOV-3 cells was also observed through the formation of DNA ladders at 48 h of cleistopholine treatment. According to Joza et al. (2001), the presence of DNA ladders indicates DNA fragmentation in cells and thus proves the cleavage of endonuclease products into ~50–300 kb pieces.

The Bcl-2 family is involved in apoptosis induction (Cory and Adams 2002). In general, two proteins promote oncogenesis,
Fig. 11. Flow cytometric analysis on the cell cycle arrest of CAOV-3 cells treated with cleistopholine (61 μM). [A] Untreated cells, [B] 24 h, [C] 48 h, [D] 72 h, [E] graphical analysis of cell cycle arrest in CAOV-3 cells. Results were represented as mean ± SD of three replicates. *P < 0.05 is considered to indicate significant difference compared with the control. G0/G1 (Gap 0 or 1) indicates the resting phase in which the cell has left the cycle and stopped dividing. G1 is the phase in which the cells increase in size. S phase (synthesis) indicates DNA replication, whereas G2/M (Gap2 or mitosis) is the phase in which the cell will continue to develop.
antiapoptotic Bcl-2 and proapoptotic Bax (Liu et al. 2003). These proteins play unique roles by determining whether or not the cell undergoes or terminates apoptosis. The high expression of Bax and low expression of Bcl-2 in the present study demonstrated that cleistopholine triggers apoptosis in CAOV-3 cells. Hsp70 and survivin proteins were also investigated. Heat shock proteins (Hsp) play an important function in cytoreduction in which a high expression of Hsp is associated with apoptosis resistance (Sreedhar and Csmeryl 2004; Newmeyer et al. 2000). Meanwhile, survivin specifically inhibits the induction of apoptosis and activates caspases 3, 7 and 9 (Cheung et al. 2006). As shown in Figs. 9 and 10, the mRNA and protein expression levels of Hsp70 and survivin down-regulated after CAOV cells were treated with cleistopholine, which induced apoptosis in the cells. Inhibition of Hsp70 also activates FasL/FasR to induce apoptosis (Schett et al. 1999).

Cell proliferation was investigated by analysing cell cycle arrest. Cleistopholine disrupts the G0/G1 checkpoints in CAOV-3 cells by continuously increasing cell cycle arrest, followed by a decrease in the number of cells at the S phase, thereby inhibiting the proliferation of CAOV-3 cells. Interference at the G0/G1 phase prevents DNA replication to the next phase. Cell cycle disruption causes DNA damage and therefore prevents CAOV-3 cells from dividing and finally initiates apoptosis (Pietenpol and Stewart 2002).

Conclusion

Cleistopholine may be developed as a new candidate anticancer agent for ovarian cancer. These new findings may overcome the resistance encountered by other drugs used to treat ovarian cancer.

Conflict of interest

Authors declare no conflict of interest.

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References