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

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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 IC_{50} 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 compression 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.

Abbreviations: HPLC, high performance liquid chromatography; IR, infrared; 1D NMR, one-dimensional nuclear magnetic resonance; MS, mass spectrometry; CO_2, carbon dioxide; IC_{50}, inhibition concentration of 50%; sh, shoulder; NaCl, sodium chloride; HRESIMS, high-resolution electron spray ionisation mass spectrometry; calcd, calculated; $CDCl_3$, 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.

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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 an azaanthraquinone alkaloid that was isolated from the *Enicosanthellum 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 ($CHCl_3$) 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–$CHCl_3$–MeOH. Ten fractions were collected and combined using thin layer chromatography (TLC) analysis to yield five fractions (1–5). Fraction 3 was then 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 C-18 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 immortalised 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 cultured 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 1×10^5 cell/ml were seeded in a 96-well microplate and then treated with cleistopholine (100 µl) 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 cytomorphological 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 5×10^4 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 × 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 × assay buffer per tube. All tubes were examined by a flow cytometer (BD FACSCanto™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 1×10^6 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