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Original Article

Apoptotic Activity of Aporphine from *Stephania venosa* on Human Ovarian Cancer Cells

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Abstract *Stephania venosa* is a plant rich of alkaloids of family Menispermaceae. In Thailand, the study on this plant revealed a wild variety of phytochemical constituents. Those including flavonoids, alkaloids, terpenoids, sulfides, and polyphenolics. In this study, we analyzed the anticancer activity according to the antiproliferation and apoptotic activity of the pure constituent isolated from *S. venosa* tuber on human ovarian cancer cells (SKOV3). During phytochemical processes, we concomitantly tested the activity of the isolates and selected the effective fractions for further purification. Crude ethanolic extract and pure constituent were obtained from the tuber of *S. venosa* and evaluated the cytotoxic activity against SKOV3 by MTT assay. Crude ethanolic extract was performed various fractionations and further isolated to obtain pure constituent. Purified constituent and crude extract were demonstrated the significant effect on antiproliferation in a dose dependent manner. Apoptotic effect was confirmed by morphological changes, DNA fragmentation and caspase activation assay. The cytotoxic activity of crude ethanolic extract showed a potent inhibition with an ED_{50} value at 31 μg/ml. Structure of the pure constituent was determined by NMR technique and identified as aporphine. Aporphine from *S. venosa* showed the antiproliferation at ED_{50} value at 6 μg/ml against SKOV3. Results from the morphological changes, DNA fragmentation and caspase activation assay, in this study demonstrated that aporphine could significantly inhibit the treated tumor cell proliferation and cause cells death via apoptosis, whereas those were not found in the untreated cells. We concluded that the pure constituent from *S. venosa* could lead to the further study on the detail mechanisms of action of this active substance for future application as new drug for ovarian cancer.

Keywords: alkaloids, apoptosis, aporphine, cancer, caspase, DNA fragmentation, MTT assay, NMR, SKOV3 cell line, *Stephania venosa*

INTRODUCTION

S. venosa is a member of the Menispermaceae and is a rich source of alkaloids. After the number of reports on alkaloid occurrence was increased, there has doubled growth of interest in alkaloid isolation and biosynthesis. The genus *Stephania* could be a potential source of biologically active compounds which might be used as lead molecules for development of new drugs.[1] In Thailand, the studies on this plant revealed a variety of isolated phytochemical constituents. *S. venosa* is the medicinal plant and its biological activities, especially, the antimalarial activity, acetylcholinesterase-inhibiting activity, and antioxidant activity have been investigated.[2-4]

Preliminary evidence from Moongkarndi and Jongsomboonkusol[5,6] demonstrated that the crude extract from *S. venosa* was able to inhibit tumor cell proliferation in breast cancer cell lines and induce apoptosis and cell cycle arrest in ovarian and breast cancer

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cell lines. These previous reports on *S. venosa* showed highly potential in further cancer therapy for ovarian cancer. Due to only a little number of authentic scientific studies of this plant on anticancer activity, we emphasized to investigate on *S. venosa*.

In this study, we isolated the pure constituent from crude ethanolic extract from *S. venosa* tuber and analyzed the anticancer activity according to antiproliferation and apoptotic activity on ovarian cancer cells.

MATERIALS AND METHODS

Isolation of Pure Compounds from S. venosa

The tubers of *S. venosa* were collected from Chiang Mai Province, Thailand. Isolation of pure compounds from this plant was performed as indicated in Figure 1.

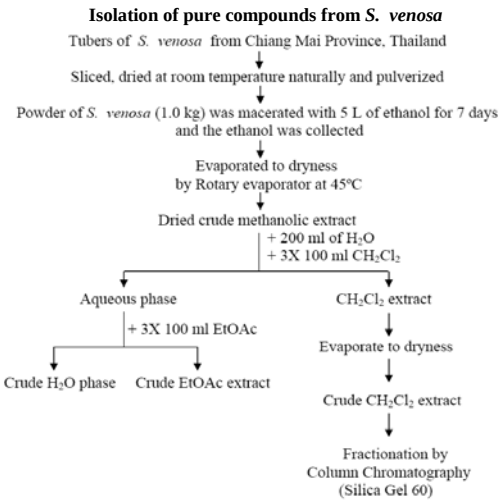


Figure 1. Diagram for extraction and purification of the active constituents from *S. venosa***.**

Antiproliferation Assay

Human ovarian cancer cell line (SKOV3) was obtained from the American Type Culture Collection (ATCC HTB77). Antiproliferation by MTT assay was a conventional method to assess the number of viable cells growing in microtiter plate after treating with substances. SKOV3 cells were treated with various concentrations of crude ethanolic extract for 48 hours. The viable cells could reduce the yellow MTT to insoluble purple formazan crystal by intracellular succinate dehydrogenase.[7,8] The optical density of each well was measured using a microplate reader at 590 nm. The results were shown in line graph between the percentage of cell viability (Y-axis) and the concentrations of sample (X-axis). The ED_{50} could be calculated from this curve.

Morphological Changes Analysis

SKOV3 cells were treated with various concentrations of pure constituents (0, 10 and 25 µg/ml) for 24 hours. After the end of incubation period, the cells were observed by a phase contrast inverted microscope at 400× magnification. Cell membrane blebbing, cell shrinkage and apoptotic body could be observed.

DNA Fragmentation by DNA Ladder Assay

SKOV3 cells were treated with various concentrations of crude ethanolic extract (0, 25, 50 and 100 µg/ml) for 24 hours. After incubation, DNA of treated cells was extracted and demonstrated by agarose gel electrophoresis at 100 volt for 1 hour.

Caspase 9/6 Assay

Caspase 9/6 activities were assayed according to BD ApoAlert™ Caspase-9/6 Assay Protocol.

RESULTS AND DISCUSSION

Aporphine alkaloid has been identified as isolated pure constituent obtained from *S. venosa*. Structures of the alkaloids were determined by NMR technique.

Antiproliferation Assay

Antiproliferation activity of the crude ethanolic extract was determined by MTT assay. The extract showed highly potential cytotoxicity in a dose-dependent manner. The cytotoxic activity was obtained with an ED_{50} value of 31 µg/ml (Figure 2). The isolated pure compound, aporphine, presented the ED_{50} on SKOV3 at 6 µg/ml (Figure 3).

Morphological Changes Analysis

Morphological changes of the extract-treated and untreated SKOV3 cells were demonstrated (Figure 4). The most remarkable changes

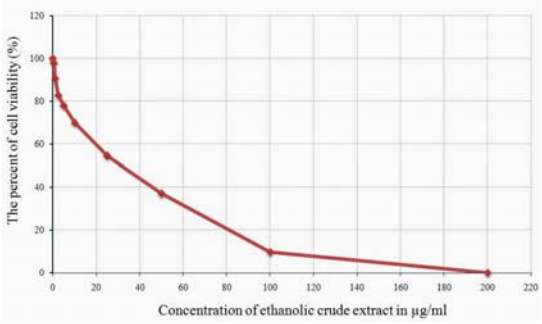


Figure 2. Inhibition of proliferation by the ethanol extract of *S. venosa*. SKOV3 cells were treated with various concentrations of crude ethanolic extract for 48 hours, and cell viability was determined by the MTT assay. The cytotoxic activity was potently inhibited at ED_{50} value of 31 µg/ml.

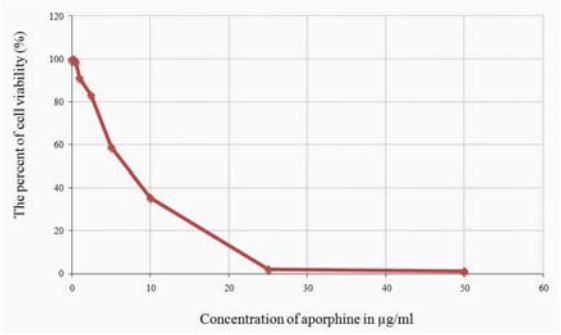
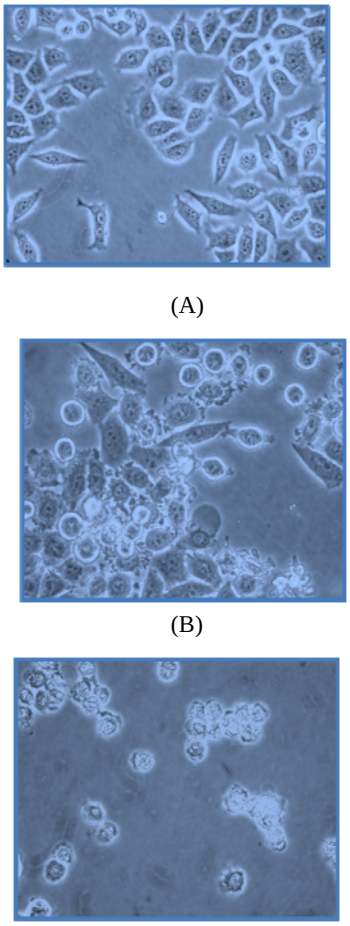


Figure 3. Inhibition of proliferation by various concentrations of the pure compound (aporphine) from fractionated *S. venosa*. SKOV3 cells were treated with 0, 0.5, 1.0, 2.5, 5.0, 10, 25, 50 µg/ml of test materials for 48 hours, and cell viability was determined by the MTT assay. The cytotoxicity showed strong activity at ED_{50} value of 6 µg/ml.



(A) (B) (C)

Figure 4. Morphological changes of SKOV3 cells being observed under phase contrast inverted microscope at 400× magnification: (A) normal untreated cells, (B) cells exposed to 10 µg/ml aporphine for 24 hours, and (C) cells exposed to 25 µg/ml aporphine for 24 hours**.**

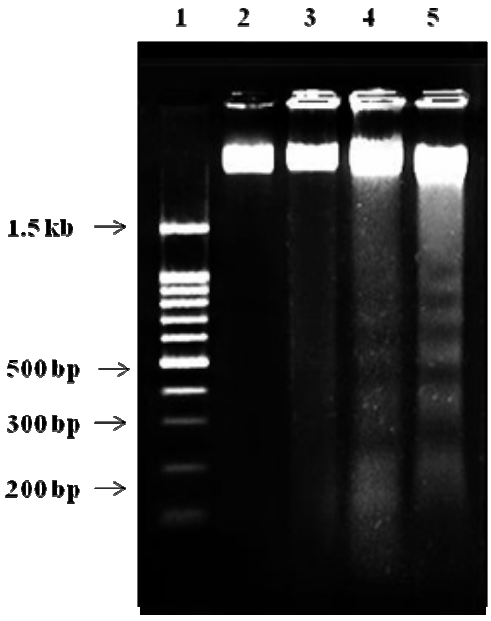
could be observed in extract-treated cells including cell shrinkage and extensive detachment of the cells from the culture surface. These changes which were the characteristics of apoptotic cell death, became visible after 24 hours of extract treatment but were absent in control cells.

DNA Fragmentation by DNA Ladder Assay

DNA fragmentation during apoptosis is very distinct in the terminal stages of the process. When cells undergo apoptosis, DNA will be digested into oligonucleosomal fragments about 180-200 bp.[6,9] DNA fragmentation could be observed in SKOV3 treated with the ethanolic extract at 25 µg/ml, and it became more evident at higher concentrations, *i.e.* 50 and 100 µg/ml (Figure 5).

Caspase 9/6 Assay

Caspase 9/6 activation was determined by fluorometric assay. Caspase-9 has been linked to the mitochondrial death pathway.[10] It is activated during apoptosis. Apoptotic induction



Lane 1: Molecular marker 100 bp + 1.5 kb
Lane 2: Untreated cells
Lane 3: Treated cells - with crude ethanolic extract 25 µg/ml
Lane 4: Treated cells - with crude ethanolic extract 50 µg/ml
Lane 5: Treated cells - with crude ethanolic extract 100 µg/ml

Figure 5. Analysis of genomic DNA fragmentation in SKOV3. Cells (10^6 cells) were induced for 24 hours with crude ethanolic extract (0, 25, 50 and 100 µg/ml). Fragmentation was assessed by agarose gel electrophoresis.

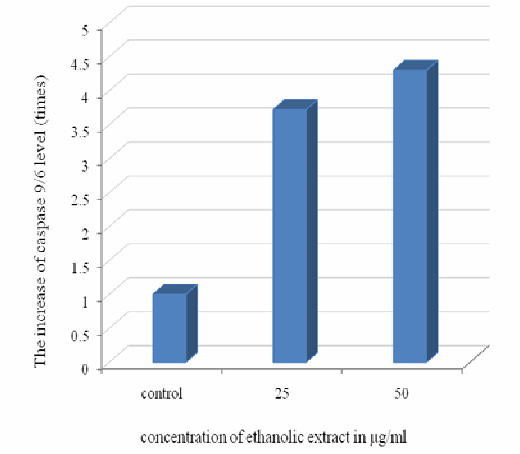


Figure 6. Increase of caspase 9/6 protein expression in SKOV3 after 24 hour treatment with crude ethanolic extract at concentrations of 25 and 50 µg/ml.

was determined by increased caspase 9/6 protein when treating SKOV3 cells with crude ethanolic extract of *S. venosa*. There was a significant increase of caspase 9/6 protein expression, compared to the control (Figure 6).

CONCLUSION

The ethanolic extract of *S. venosa* and the pure constituent, aporphine, exhibited the antiproliferation activity on SKOV3 human ovarian cancer cell line using MTT assay. Aporphine showed strong inhibition activity with an ED_{50} value of 6 µg/ml. By determining morphological changes, DNA fragmentation and caspase activation, it was demonstrated that either the ethanolic extract or aporphine could inhibit cell proliferation and cause cell death via apoptosis. The present results of aporphine obtained from *S. venosa* could lead to the further study on the detail mechanisms of action of this compound for future application as new drug for ovarian cancer.

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