

Terpenoid Bioactive Compound Isolated from Papua Ant Nest Induces the Apoptosis of Human Ovarian Cell Lines (SKOV-3) and Increasing Caspase-9 Activity

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ABSTRACT

Objectives: The aim of this study was to determine the anticancer activity of terpenoid bioactive compound isolated from Papua ant nest in ovarian cancer cell lines (SKOV-3) *in vitro*.

Methods: This was a laboratory experimental study which aims to determine the mechanisms of terpenoid bioactive compound isolated from Papua ant in ovarian cancer cells (SKOV-3) by inducing apoptosis and increasing caspase-9 activity.

Result: At a concentration of 600 µg/ml, terpenoid bioactive compound induced apoptotic process in ovarian cancer cell lines (SKOV-3) with the apoptotic index of 30% at 24 hours, 35% at 48 hours and 37% at 72 hours, respectively, and increased caspase-9 activity of 1.2001 at 24 hours, 1.2896 at 48 hours and 1.5719 at 72 hours, respectively.

Conclusion: This study provides evidence that the terpenoid bioactive compound isolated from Papua ant nest induced apoptotic process in ovarian cancer cell lines (SKOV-3) through an intrinsic apoptotic pathway by increasing caspase-9 activity.

Keywords: apoptotic index, caspase-9, ovarian cell lines (SKOV-3), Terpenoid

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INTRODUCTION

Ovarian cancer is still the most common cause of gynaecological cancer-related mortality, with the highest incidence areas of ovarian cancer are Europe and North America.¹⁻³ Patients with ovarian cancer generally undergo surgery followed by chemotherapy.⁴

Chemotherapy remains the treatment of choice in many malignant diseases. Although the efficacy of chemotherapy for the majority of cancer types has improved over the last three decades, but high toxic effects of chemotherapeutic drugs causing a severe reduction in quality of life are still formidable problems in clinical medicine. And the emergence of resistance to anticancer drugs, in particular multidrug resistance (MDR), has made many of the available anticancer drugs ineffective.⁵⁻⁶

Induction of apoptosis has been recognized as one ideal strategy for cancer chemotherapy. Apoptosis cell death plays a central role in the pathogenesis and disease progression of ovarian cancer as well as in the response to treatment. A balance between rates of cell proliferation and apoptosis is crucial in the regulation of ovarian cancer cells.⁷⁻¹⁰

Apoptosis is programmed cell death, which allows the elimination of cells that have been produced in excess, developed improperly, or sustained genetic damage. Agents with the ability to induce apoptosis in cancer cell have the potential to be used for anticancer therapy and may candidate as alternative ovarian cancer therapy.¹¹

Therefore, it is very important to develop novel potent, but low toxic anticancer reagents, including natural products. Many new treatment strategies targeting apoptosis are feasible and may be used in the treatment of ovarian cancer in several clinical trials.¹²⁻¹⁵

The study of medicinal plants to treat human cancer has increased to explore their therapeutic effect for cancer treatment. Indonesia are very abundant of herbal medicines that can be used for an alternative therapy, such as Papua ant nest. The local people of Papua boiled the ant nest to treat several diseases and it has been used for the treatment of ascaris, cold, hemorrhoids, burn, including cancer.¹⁶⁻¹⁸

The Papua ant nest plant is a herbaceous plant that is new and has potential as an alternative therapy in treating many malignant diseases or cancer, especially the *Myrmecodia* species. It is epiphytic plant of the Rubiaceae family and has 5 genus, but only 2 genus have association with ants. They are *Myrmecodia* (45 species) and *Hypnophytum* (26 species), but only *Hypnophytum fornicarum*, *Myrmecodia tuberosa* and *Myrmecodia pendens* have medicinal value (Figure 1).¹⁸⁻¹⁹



Figure 1. The Papua ant nest

Previous study by Soekmanto et al. (2010) have proved that the extract of Papua ant nest (*Myrmecodia pendens*) has anticancer activity in both human cervix (HeLa) and canine mammary tumor (MCM) cell lines. It was found that the IC_{50} value of Papua ant nest extract was 27.61 ppm (HeLa) and 54.57 ppm (MCM-B2), respectively. These result suggested that the extract of Papua ant nest (*Myrmecodia pendens*) have the capability to inhibit the growth of HeLa and MCM-B2 cells.¹⁸⁻¹⁹ On the background of these findings, we attempted to isolate the Papua ant nest plant (*Myrmecodia pendens*) from propinche of Papua, Indonesia. Continuing our efforts to discover new anticancer agents for ovarian cancer, we have isolate several terpenoid bioactive compound from *Myrmecodia pendens* species, which were shown to be very strong antimicrobial and antioxidant activity.²⁰⁻²¹

In previous paper, we have reported that the terpenoid bioactive compound isolated from Papua ant nest had capability to inhibit the growth of ovarian cancer cell lines (SKOV-3) with IC_{50} of 481 μ gr/ml for 48 hours and 463 μ gr/ml for 72 hours, respectively.²¹

Therefore, it is very necessary to search for a new potential anticancer agents and the precise anticancer mechanisms of this terpenoid bioactive compound. In this studi we investigated the potential anticancer and the precise anticancer mechanisms of terpenoid bioactive compounds in ovarian cancer cell lines (SKOV-3) in vitro.

METHODS

This study has been carried out from June 2014 to January 2015. The Papua ant nest was collected from Papua, Indonesia and isolated at the Department of Chemistry, Faculty of Mathematic and Natural Science, Padjadjaran University, Bandung, Indonesia.

Terpenoid compound isolation

Myrmecodia pendens of Papua ant nest was cleaned, cut, dried, ground and then was extracted with methanol and water. The extraction process was taken over 10 days period (until resulting terpenoids) utilizing liquid ethylacetate, without water and under 35°C temperature. The following describes the work flow:

The corms of the ant nest (about 3 kg) was extracted by sokletation method using 60 mL ethyl acetate as solvent which then produced ethyl acetate extract. This extract was thickened by a rotatory evaporator to remove the solvent, resulting 30 gr of thick ethyl acetate extract. This extract was further separated using silica gel 60 chromatography with gradient n-heksana as a solvent: ethyl acetate 2.5% (100:0 to 80:20, v/v) produced 9 fractions. The 7th fraction (3.8 gr) was purified by using n-hexane to produce 1.8 gr white crystal. In order to determine the purity of this crystal, an analysis was conducted through thin layer chromatography (TLC) gel 60 F254 with n-heksana: aseton (8:2) as its solvent. Then the TLC plate was examined under ultraviolet light at 254 and 365 nm. Furthermore, 10% of acid sulphate was applied to the TLC plate in heated ethanol on a hot plate in order the spots can be visualized. Purplish black spots appeared on the heated TLC plate which indicated the terpenoid compounds. The extracts was subjected to column chromatography, then column were eluted with methanol to obtain a terpenoid fraction, followed by isolation process, we obtained the terpenoid bioactive compound.²¹⁻²²

SKOV-3 Cell culture

The ovarian cancer cell lines (SKOV-3) was supplied by Kalgen laboratory, Kalbe Farma company, Indonesia. Then the cell lines were cultured in Laboratory of Rajawali Hospital, Bandung, Indonesia. All ovarian cancer cell lines (SKOV-3) were grown in RPMI 1640 supplemented with 10% fetal bovine serum (heat-inactivated at 56 °C for 45 minute) and penicillin/streptomycin, in a humidified, 5% CO₂ atmosphere and 37 °C incubator. After all the cells were confluent, the cells were counted using a Neubauer Haemocytometer and resuspended in medium at the final concentration of 5x10⁵ cells/mL and mixed with DMSO medium and treated with terpenoid at the concentration of 200 µg/mL, 400 µg/mL, 600 µg/mL, respectively at 37 °C on plate. Then the ovarian cell lines (SKOV-3) were incubated for 24 hours, 48 hours and 72 hours, respectively. Caspase-9 activity was analyzed according to the manufacture's instructions in the caspase-9 colometric assay kit. Cells were harvested, centrifuged at 125 g for 5 minutes, lysed in 50 µL lysis buffer for 20 minutes, vibrated for 10 seconds, and centrifuged at 10,621 g for 1 minute at 4 °C. After centrifugation, the

supernatant was collected and the protein concentration was determined. Each sample was incubated with 5 μ L caspase-9 substrate at 37 °C for 4 hours and measured by chromatography at 405 nm wavelength.²³⁻²⁴

To determine apoptotic process on ovarian cancer cell lines (SKOV-3) after treatment with terpenoid, we used TUNEL assay to calculate the presence of apoptosis, 1 x 10⁶ cell/mL, were treated with the terpenoid at the concentration of 200 μ g/mL, 400 μ g/mL, 600 μ g/mL for 24 hours, 48 hours and 72 hours, respectively at 37 °C on plate with cover slips. Following incubation, cover slips were fixed on objective glass and morphology of the cell was analyzed under microscope.²³⁻²⁴

RESULTS

The result of this study showed that the terpenoid bioactive compound isolated from Papua ant nest had an anticancer activity in ovarian cancer cell lines (SKOV-3). According to this study, terpenoid bioactive compound was able to induce apoptotic process in ovarian cancer cell (SKOV-3). At the concentration of 200 μ g/mL, terpenoid was able to induce apoptotic process on ovarian cancer cell lines in vitro with the apoptotic index of 18% at 24 hours, 22% at 48 hours and 2% at 72 hours, respectively. At the higher concentration (400 μ g/mL), terpenoid was able to induce apoptotic process on ovarian cancer cell lines in vitro with the apoptotic index of 22% at 24 hours, 24% at 48 hours and 27% at 72 hours, respectively. Further analysis, at the highest concentration (600 μ g/mL), terpenoid was able to induce apoptotic process on ovarian cancer cell lines in vitro with the value of apoptotic index were approximately 30% at 24 hours, 35% at 48 hours and 37% at 72 hours, respectively. The presence of apoptosis (apoptotic index) in ovarian cell lines (SKOV-3) after treatment with terpenoid active compounds isolated from Papua ant nest was shown in Table 1.

Table 1. Apoptotic indexes of ovarian cancer cell lines (SKOV-3) after treatment with terpenoid

Group of treatment	Time of observation		
	24 hours	48 hours	72 hours
Positive control (Carboplatine)	35%	58%	60%
Negative control (DMSO)	5%	8%	9%
200 μ g/mL	18%	22%	22%
400 μ g/mL	22%	24%	27%
600 μ g/mL	30%	35%	37%

As shown in Table 2, the activities of caspase-9 were increased significantly in ovarian cancer cell lines (SKOV-3) after treatment with terpenoid at concentrations of 200 $\mu\text{g/mL}$ as compared with the activities in negative control cells (DMSO). Caspase-9 activity after treatment with terpenoid at concentrations of 600 $\mu\text{g/mL}$ was initially beginning to increase at 24 hours and then peaking at 72 hours. Although the increase in caspase-9 activity after treatment with terpenoid was not as high as compared with the activities in positive control cells (carboplatine). However, the activity of caspase-9 was shown to increase in the negative control cells (DMSO) following incubation for 24 hours and tends to be slightly higher at 48 hours and 72 hours.

Table 2. Caspase-9 activity of ovarian cancer cell lines (SKOV-3) after treatment with terpenoid

Group of treatment	Time of observation		
	24 hours	48 hours	72 hours
Positive control (Carboplatine)	1.31	1.53	1.88
Negative control (DMSO)	0.59	0.62	0.63
200 $\mu\text{g/mL}$	0.99	0.97	0.79
400 $\mu\text{g/mL}$	1.11	1.01	0.98
600 $\mu\text{g/mL}$	1.20	1.29	1.57

DISCUSSIONS

Ovarian cancer has an overall poor prognosis especially in the case of chemoresistance, therefore the development of alternative chemotherapeutic agent is very importance.²⁵ In this study we demonstrate a possible therapeutic mechanisms of terpenoid bioactive compound isolated from Papua ant nest with possesses antineoplastic properties in ovarian cancer cell lines with suppression of proliferation and induction of apoptosis. This is the first study that shows the use of terpenoid bioactive compound in human ovarian cancer cells.^{21,25}

Apoptosis or programmed cell death is an intrinsic cell suicidal mechanisms that plays an important role in the maintenance of healthy tissues. Therefore, searching for agents which trigger apoptosis of tumor cells has become an attractive strategy in anticancer drug.²⁵⁻²⁶

In the course of our search for new bioactive compound as an alternative ovarian cancer treatment from Indonesian medicinal plants, we isolated and investigated the terpenoid bioactive compound from Papua ant nest. Among the bioactive compound isolated from ant nest, flavoids is the most widely studied, especially in recent years. Meanwhile, the study of

terpenoid bioactive compound isolated from Papua ant nest as anticancer for ovarian cell lines has never been done.²⁷⁻²⁸

In the previous study, we reported that terpenoid might has antiproliferative activity against ovarian cancer cell lines (SKOV-3) *in vitro*. It was also found that terpenoid had capability to induce apoptotic process in ovarian cancer cell lines. These findings indicated that terpenoid bioactive compound isolated from Papua ant nest had anticancer properties in ovarian cancer cell lines (SKOV-3) *in vitro*. However, the precise antitumor mechanisms of this compound are unknown.^{21,29}

According to this study, we found that the activities of caspase-9 were increased significantly in ovarian cancer cell lines (SKOV-3) after treatment with terpenoid. However, the precise apoptotic mechanisms of this terpenoid remain unclear, but we believe that the terpenoid induced apoptosis in ovarian cancer cell lines through an intrinsic apoptotic pathway. Moreover, it had been reported that the intrinsic pathway mechanisms requires disruption of the mitochondrial membrane and the release of mitochondrial protein, such as cytochrome-c. Once cytochrome-c is in the cytosol, together with Apaf-1 activates caspase-9, then activates caspase-3, and the latter then induces apoptosis.³⁰⁻³¹

To the best of our knowledge, this is the first report describing the anticancer properties and apoptotic induction by terpenoid bioactive compound in ovarian cancer cell lines (SKOV-3). Induction of apoptosis has been recognized as one ideal strategy for ovarian cancer chemotherapy. Agents with the ability to induce apoptosis in ovarian cancer cells have the potential to be used for anticancer therapy and may candidate as alternative ovarian cancer chemotherapy.

CONCLUSION

In summary, the development of effective therapeutic agents for ovarian cancer is necessary to improve current chemotherapy. Our study demonstrate that the terpenoid bioactive compound from Papua ant nest is an important traditional medicine has strong anticancer properties, which is related to the promotion and induction of apoptosis. To our knowledge, this the first study to report the anticancer effects which pro-apoptotic effect of terpenoid bioactive compound in ovarian cell lines *in vitro*. Regarding the anticancer mechanisms of this bioactive compound, its exact target is still unclear, but we believe that terpenoid might induce apoptosis in ovarian cancer cell lines through an intrinsic apoptotic pathway. Our

finding provide a novel understanding of the anticancer mechanisms of this terpenoid and might be helpful in the developing of new anti-cancer drugs. We believe that based on our results, further studies are required namely *in vivo* experiments to access the potential anticancer activity of these terpenoid in the treatment of ovarian cancer.

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