Journal of Applied Pharmaceutical Science Vol. 10(02), pp 001-006, February, 2020
Available online at http://www.japsonline.com
DOI: 10.7324/JAPS.2020.102001
ISSN 2231-3354

Antiproliferative and apoptosis effect of hyptolide from *Hyptis pectinata* (L.) Poit on human breast cancer cells

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ARTICLE INFO

Received on: 26/09/2019
Accepted on: 24/12/2019
Available online: 05/02/2020

Key words:
Hyptis pectinata (L.) Poit, hyptolide, cytotoxic, apoptosis, antiproliferative, MCF-7 cells.

ABSTRACT

Hyptolide was isolated from the leaves of *Hyptis pectinata* (L.) Poit and was studied in order to discover and develop an anticancer drug. Hyptolide was obtained as a crystal of 87°C–88°C melting point. Spectroscopic identification results show a wave number at 1,735 cm^{-1} indicating the presence of α,β-unsaturated δ-lactone. Gas chromatography-mass spectrometry (GC-MS) analysis provides a single peak in the retention time of 11.701 by m/z value at 239, which indicates explicitly hyptolide. The objective of this research was to evaluate the hyptolide's mechanism of cytotoxic on MCF-7 human breast cancer cells in positive estrogen receptor. The assay test to 3-(4,5-dimethylthiazol-2-yl)-2,5-dphenyl tetrazolium bromide (MTT) showed that hyptolide exhibited cytotoxic effects on MCF-7 and T47D breast cancer cells with an IC_{50} value of 76.76 and 181.55 µg/ml, respectively. Interestingly, the treatment of hyptolide for 24, 48, and 72 hours decreased cell viability on MCF-7 with dose- and time-dependent manner compared to untreated cells. Results of acridine orange-ethidium bromide multiple staining assay revealed that hyptolide induced apoptosis in a dose-dependent manner. It can be concluded that hyptolide possesses antiproliferative effects through apoptosis induction.

INTRODUCTION

Breast cancer frequently occurs among women, impacting 2.1 million women each year. It also causes the highest number of women with cancer-related death (Sasco, 2003; WHO, 2018). Breast cancer is the most common cancer in Indonesia and is a leading cause of mortality, where 21.5 deaths in every 100,000 persons are caused by breast cancer (Mardela *et al*., 2017; Sinaga *et al*., 2018). The most effective treatments for breast cancer are chemotherapy, radiation therapy, and surgery or in special cases, they used in combination (Takimoto and Calvo, 2005). Unfortunately, long-term effects of these cancer treatments have several side effects such as anemia, appetite loss, fatigue, alopecia, toxicity, and drug resistance (Kayl and Meyers, 2006; Tacar *et al*., 2013). Over the past 30 years, the estrogen receptor (ER) has been the most critical target and implicated to breast cancer progression (Ariazi and Jordan, 2006; Paruthiyil *et al*., 2004; Roy and Vadlamudi, 2012). Blocking the action of estrogen has been shown to reduce breast cancer proliferation and incidence in high-risk women by 50%–75% (Lewis-Wambi and Jordan, 2009; Tian *et al*., 2018). The previous study also found that estrogen administration causes breast cancer while antiestrogen prevents it (Hollingsworth *et al*., 1998; Jenie *et al*., 2019). Those findings provide compelling evidence that estrogen and ER have a significant role in the progression of breast cancer. Therefore, based on this reason, the development of new cytotoxic activity in a natural product is significantly crucial for the discovery of safer and more effective chemotherapy agent on ER-positive breast cancer.

One of the herbal plants *Hyptis pectinata* (L.) Poit – popularly known in Brazil as "sambacaita" or "canundiho" (Franzotti *et al*., 2001) – belongs to one of the family lamiaceae and easily found in another tropical as America, Africa, India, and Indonesia. *Hyptis pectinata* (L.) Poit has been proven to have several kinds of effects, such as antinociceptive, antiedematogenic, analgesic, anti-inflammatory, and anticancer (Arrigoni-blank *et al*., 2008; Lisboa *et al*., 2006; Raymundo *et al*., 2011). The

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previous study also reported that *Hyptis pectinata* (L.) poit extract inhibits cancer cell growth on HCT-8, MDA-MB-231, and MCF-7 cells (Barbosa *et al*., 2012; Santana *et al*., 2019; Suzery and Cahyono, 2014). The major compound of this plant is hyptolide (α,β unsaturated δ- lactone) with 2% yield and minor compounds are pectinolide A-G, sambacaitaric acid, and 3-O-methyl-sambacaitaric acid (Falcao *et al*., 2013; Miranda *et al*., 1993). Pectinolide A-C exhibit significant antimicrobial and cytotoxic activities on many types of cancer, specifically human breast cancer cells (Miranda *et al*., 1993). That demonstrates the antitumor potential of this secondary metabolites class. To date, no studies have reported the primary compound of this plant (hyptolide isolated from *Hyptis pectinata* (L.) Poit), which concerns on antitumor activities and suggests hyptolide as an anticancer agent. In this research, we observed the effect of hyptolide isolated from *Hyptis pectinata* (L.) Poit on the cell viability, proliferation, and apoptosis on ER-positive breast cancer cells.

MATERIALS AND METHODS

Plant material

Hyptis pectinata (L.) Poit was collected in October 2014 in Kanayakan village, Bandung West Java, Indonesia (Latitude −7.0460282; Longitude 107.7915393). For biological studies, the leaves were dried in a renewal air oven and circulated at 40°C until it is completely dehydrated. The Biologist at Ecology and Biosystematics Laboratory, Faculty Science and Mathematics, Diponegoro University, Semarang, Indonesia confirmed the identification of the plant (USDA, 2016). A Voucher specimen (MS 100562) was deposited at the Herbarium Biology, Faculty Science and Mathematics, Diponegoro University, Semarang, Indonesia.

Extraction and isolation procedure

Dried powdered leaves (0.710 kg) were extracted by the maceration method using methanol at room temperature. After filtration, the solvent was evaporated to result in the crude extracts of the plant under reduce pressure in a rotary vacuum evaporator. The crude extracts were dissolved in water for 24 hours, and the partitioned water-methanol was evaporated until dry. Hyptolide was isolated in 1.7% yield from extracts of methanol (Achmad *et al*., 1987; Suzery *et al*., 2012).

Cell culture

Michigan Cancer Foundation (MCF-7) and T47D cells were collected from the American Type Culture Collection. MCF-7 and T47D cells were particularly cultured in Dulbecco's Modified Eagle's Medium (Gibco, USA) with 10% (v/v) fetal bovine serum (Gibco, USA), 12.5 µg/ml Amphotericin B (Gibco), 150 µg/ml Streptomycin, and 150 IU/ml penicillin (Gibco). Cells were cultivated at 37°C with 5% CO_2 in a humidified atmosphere.

Cytotoxic assay

The cytotoxicity of hyptolide was tested using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-dphenyl tetrazolium bromide) with slight modifications (Mosmann, 1983). MCF-7 and T47D cells (2×10^4) were seeded in a 96-well microplate and divided into the untreated and treated group. The medium substituted after 24 hours of incubation with a series of hyptolide concentrations. Cells were treated with hyptolide (1,562–100 µg/ml). Untreated cells were regarded as negative controls. After 24-hour-treatment, the medium was removed and substituted with 0.5 mg/ml of MTT (Biovision) and incubated for approximately four hours at 37°C, 5% CO_2. Cells were lysed using 10% stopper of sodium dodecyl sulfate containing 0.01 N HCl and incubated overnight in the dark condition to dissolve formazan salt. After incubation, the absorbance was measured by Enzyme-linked Immunosorbent Assay reader plate at λ 550 nm. The absorbance was transformed to % cell viability by comparing the treated group with the untreated at a particular time course. Linear regression between concentration and % viability of cells giving the equation $y = Bx + A$ was used to calculate IC_{50} value, which was the concentration inhibiting 50% cell proliferation.

Proliferation assay

In the experiments of proliferation level measurement, 5×10^4 MCF-7 cells were seeded in 96-well microplate and treated with hyptolide at several concentrations (1,562–100 µg/ml) for 24, 48, and 72 hours before MTT.

Apoptosis assay

MCF-7 cells grown on coverslips determined the apoptosis induction of hyptolide on MCF-7 cells were included in the 24 microplate wells to obtain a density of 5×10^4 cells/wells and incubated until 50%–60% confluent. Cells were incubated with hyptolide for 48 hours. Culture medium was discarded, and cells were washed with Phosphate Buffer Solution. Coverslips were placed into object-glass and added with 10 ml 1x working solution acridine orange (Sigma)-ethidium bromide (Sigma) and then allowed to stand for 5 minutes. Cell immediately flourescent observed under a microscope (Zeiss MC 80). Apoptosis cells that had lost their integrity of membrane appeared orange and showed morphological features of apoptosis. Cells have been classified as apoptosis based on specific morphological criteria, comprising chromatin condensation and fragmentation, and apoptosis body formation. Living cells fluorescence green (with acridine orange) (Amundson *et al*., 2000).

Statistical analysis

The data presented the mean ± standard error of the mean (SE). The statistical analysis was used a one-way analysis of variance followed by the least significant difference test (Excel 2016 software; Microsoft, Redmond, WA). Statistically significant was the *p*-value less than 0.05.

RESULTS AND DISCUSSION

Results

Isolation of hyptolide from Hyptis pectinata *(L.) Poit extract*

Hyptolide compounds isolated from the leaves of the plant *Hyptis pectinata* (L.) Poit have a melting point of 88°C–89°C. The melting point of hyptolide does not make much difference from the melting point of the compound hyptolide previously reported that are 88.5°C, 88.5°C, and 87°C–88°C (Achmad *et al*., 1987). As we all know the physical constants such as melting points are still used identifying compounds, but many compounds

have the same melting point but different in structure. Infrared spectrophotometer analysis has absorption bands at wave number 1,735 cm^{-1}. It is indicating the presence of clusters α,β unsaturated δ-lactone as the basic framework of the compound hyptolide. Strong type at wave number 1,735 cm^{-1} is the carbonyl group in ketones or esters of conjugated. The results of GC-MS analysis are one peak with a retention time of 11.701 minutes and m/z at 239 (approximate of molecular weight of hyptolide). Thus, the isolated compounds are hyptolide with the structure are shown in Figure 1.

Cytotoxic activity of hyptolide on MCF-7 and T47D cells

Cytotoxicity assay was performed to measure the potency of hyptolide-induced cytotoxicity on MCF-7 and T47D cells. The cytotoxic effect of hyptolide was presented the IC_{50} value and was used to determine the dose for other assays. The results showed that the treatment of hyptolide for 24 hours significantly decreased the viability of the cells with the degree of depletion in a dose-dependent manner on the MCF-7 and T47D. However, the cytotoxic effect was stronger on MCF-7 than on T47D with an IC_{50} value of 76.76 µg/ml and 181.55 µg/ml, respectively (Fig. 2A). This result suggested that the differential expression of protein marked that involved in the cell growth stimulation is more strongly influenced by hyptolide in MCF-7 cells.

Antiproliferation effect of hyptolide on MCF-7 cells

The cytotoxicity data indicate that MCF-7 is more susceptible to be treated with hyptolide than T47D. Therefore, based on these data, we evaluated the effect of hyptolide on MCF-7 cells with variation in time for treatment. Interestingly, the presence of hyptolide on the medium causes highly suppressed cell proliferation with an IC_{50} value of 63.27 and 33.94 µg/ml for 48 and 72 hours, respectively (Fig. 2B). Therefore, we concluded that hyptolide exhibits strong cytotoxic activity in a dose- and time-dependent manner on MCF-7 cells.

Apoptosis induction by hyptolide on MCF-7 cells

To verify the mechanism underlying the cytotoxic effect of the hyptolide treatment on MCF-7 cells, we conducted apoptosis assay with acridine orange-ethidium bromide (AO/EB) multiple staining. In this research, we used one-eighth, one-fourth, half, and IC_{50} value as the concentration of hyptolide. Based on the apoptosis analysis, cells exposed to hyptolide for 48 hours were capable of inducing apoptosis at dose-dependent manner (Fig. 3). Based on the findings, it was evident that the increasing concentration of hyptolide causes a significant reduction in the number of viable cells. In addition, some cells had typical apoptotic characteristics such as plasma membrane blabbing. However, the number of cells stained red increases. This finding suggests that most of the cell deaths occurred primarily through apoptosis.

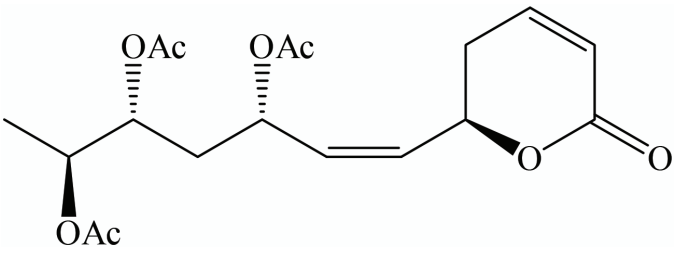

Figure 1. Chemical structures of hyptolide compounds isolated from *Hyptis pectinata*.

DISCUSSION

The purpose of this research was to introduce hyptolide as a natural chemotherapeutic agent to inhibit the proliferation of ER-positive breast cancer cells. The concepts of natural chemotherapy are to improve efficacy and reduce the side effects of a chemical chemotherapeutic agent. Hyptolide is among the natural agents that can be potentially developed to be anticancer. However, studies of the anticancer activity of hyptolide have never been attempted. On the other hand, exploration of the anticancer activity of hyptolide extract was reported in several cancer cells. *Hyptis pectinata* extract has been shown to prevent cancer growth in several cancer cells such as MDA-MB-231 (Santana *et al*., 2019) and HCT-8 cells (Barbosa *et al*., 2012). However, hyptolide, one of the major isolates compound from *Hyptis pectinata*, has not been observed for cytotoxic activity. Thus, based on this result,

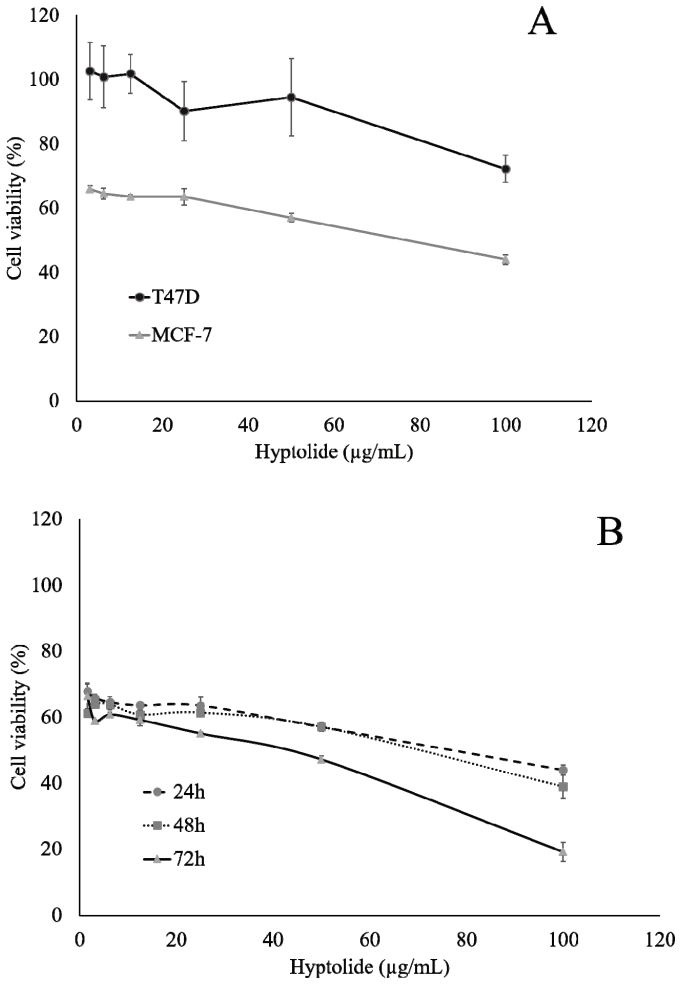

Figure 2. Effect of Hyptolide on the proliferation of MCF-7 and T47D cells. (A) MCF-7 cell (2×10^4 cells/ml) and T47D cell were culture in presence of hyptolide (3.125 – 100 µg/ml) for 24 hours. (B) MCF-7 cell ((2×10^4 cells/ml) in 96-well plates for 24 hours to adapt, then treated with hyptolide concentrations of 1.5625 – 100 µg/ml, then incubated again for 24, 48, and 72 hours. Profiles of cell viability expressed mean ± SD of three experiments. IC_{50} obtained from the linear regression calculation of cell viability versus log concentrations with $p < 0.05$.

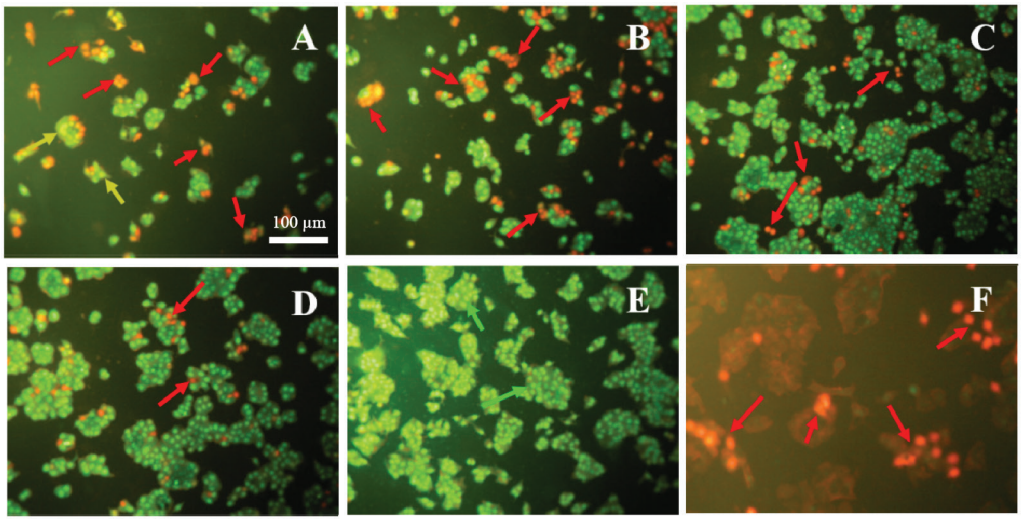

Figure 3. Apoptosis induction of hyptolide on MCF-7 cells. Apoptotic morphology detection by AO/EB fluorescent staining of MCF-7 cells treated with the Hyptolide. A IC_{50}; b ½ IC_{50}; c ¼ IC_{50}; d 1/8 IC_{50}; e-negative control; f- positive control. Green arrows: live cells, greenish yellow: early apoptotic cells, orange red: late apoptotic cells, red: dead cells (some cells are fragmented and become faded). This figure denotes the results of at least 3 independent experiments (Original magnification 40×).

our group had successfully isolated the hyptolide from *Hyptis pectinata* (L.) Poit extract, and we evaluated the cytotoxic activity of hyptolide using MCF-7 and T47D ER-positive human breast cancer cells. First, we tested the cytotoxic activity of hyptolide in different concentrations and incubation time on two models of ER-positive breast cancer cells. The single hyptolide treatment showed the cytotoxic effect on MCF-7 cells with an IC_{50} value of 76.76 µg/ml for 24 hours. We then compared the growth inhibitory actions of hyptolide on another breast cancer cells that is T47D (the ER-positive breast cancer reportedly p53 mutant, while MCF-7 are p53 wild-type) (Holliday and Speirs, 2011). Interestingly, cytotoxicity of hyptolide is more effective in MCF-7 than in T47D cells (IC_{50} 181.55 µg/ml) (Fig. 2A). In this study, two types of breast cancer cells showed different responses to treatment with hyptolide. In the same treatment level, 50 µg/ml of hyptolide, cell viability of MCF-7 is lower than T47D. The results suggest that MCF-7 is more susceptible than T47D to be treated with hyptolide. It shows that characteristics of MCF-7 are sensitive to the hyptolide. Perhaps this is due to the effect of ER expression (ER), expression of p53 protein wild type, and over-expression of Bcl-2 one of protein marked on apoptosis (Amundson *et al*., 2000; Berger *et al*., 2013; Butt *et al*., 2000; Li *et al*., 2004; Ma *et al*., 2013).

Thus, based on these results, we observed cytotoxicity in time variation on MCF-7 cells. Interestingly, the presence of hyptolide on the medium, for 48 and 72 hours, causes highly suppressed cell proliferation and exhibits strong cytotoxic activity in dose- and time-dependent manner on MCF-7 cells (Fig. 2B). One explanation is that there might be a decrease in cell viability caused by the presence of unfunctional ER, induction of p53 protein and caspase 3 (Amaral *et al*., 2010; Jamalzadeh *et al*., 2017; Schüler-Toprak *et al*., 2017). Another report said that α-tubulin inhibition is also caused by hyptolide treatment related to its activity on cell proliferation (Asy *et al*., 2019). However, this phenomenon by which hyptolide suppresses cell proliferation through α-tubulin inhibition causing the induction of tumor suppressor gene p53 should be clarified further.

To obtain a further investigation of the cytotoxicity' mechanism of hyptolide, we observed apoptosis induction of hyptolide by AO/EB multiple staining. AO/EB multiple staining showed that the differential absorption of the AO and EB fluorescent DNA-binding dyes was to determine apoptosis (nonviable) and viable cells. These dyes were used to identify apoptosis cells and to distinguish between cells in the early or late stages of apoptosis stages based on membrane integrity (García-Rodríguez *et al*., 2013). Early apoptosis cells will have fragmented DNA that gives many green colored nuclei as EB could not penetrate the cells, but chromatin condensation can be visualized as bright green patches in the nuclei. Late apoptosis and necrotic cell DNA would be fragmented and orange-red stained as the EB intercalates into the DNA. Thus, an apoptosis cell will be stained by EB over-whelms AO staining contain a bright orange nucleus. Therefore, using this system one can distinguish between early and late apoptosis cells. Viable cells with intact membranes will have a uniformly stained green nucleus because the AO intercalates into the DNA.

We explored the effect of hyptolide on physiological changes focusing on the profile of apoptosis (Fig. 3). In this study, the treatment of MCF-7 cells with a low concentration (one-eighth IC_{50}) of hyptolide slightly induced apoptosis but did not affect the number of cells grow. In contrast, the high concentration of hyptolide (one IC_{50}) highly induced apoptosis and suppresses cells grow compared to the untreated one. Similar results were performed by previous studies in which reported (Tamura *et al*., 2009; Usui *et al*., 2004) that pironetin (have a similar structure with hyptolide) could inhibit the growth of HT1080 and 3Y1 cells by apoptosis induction. The apoptosis induction on ER-positive breast cancer cells is due to the role of ER in inhibiting cell growth (Jenie *et al*., 2019; Rouhimoghadam *et al*., 2018). According to this finding, cell viability response that under hyptolide treatment

showed a dose-dependent phenomenon and most of the cell deaths occurred predominantly through apoptosis.

In the present study, we found the mechanism of cell death by hyptolide through apoptosis induction. However, the molecular mechanisms involved in hyptolide-induced inhibition in the progression of cancer cells need to be further explored. Nevertheless, we suggest the use of hyptolide as a novel approach to suppress tumorigenesis by using induced apoptosis mechanism.

CONCLUSION

In the present study, authors have investigated the cytotoxic effect of hyptolide on two types of estrogen-positive human breast cancer cells. The results demonstrated that the cytotoxic effect of hyptolide on MCF-7 is stronger than T47D cells using the MTT assay. Authors also found hyptolide highly suppressed cell proliferation in the dose- and time-dependent manner on MCF-7 cell. This effect was mediated through apoptosis induction by analysis of AO/EB multiple staining assay. More importantly, our findings suggest that hyptolide has the potential to be developed as a natural chemotherapeutic agent with better effects and possibly fewer side effects.

ACKNOWLEDGMENT

We express our gratitude to Fundamental Research Grant Ministry of Research Technology and Higher Education, The Republic of Indonesia who funded this research.

CONFLICT OF INTEREST

The authors declare that there is no conflict of interest relevant to the contents of this article.

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How to cite this article:
Suzery M, Cahyono B, Amalina ND. Antiproliferative and apoptosis effect of hyptolide from *Hyptis pectinata* poit on human breast cancer cells. J Appl Pharm Sci, 2020; 10(02):001–006.