Anti-leukaemia of fermented product of methanol extract *Hyptis pectinata* (L.) Poit leaf

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Anti-leukaemia of fermented product of methanol extract *Hyptis pectinata* (L.) Poit leaf

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Abstract. *Hyptis pectinata* (L.) Poit is a traditional medicinal plant that has long been used in some countries such as in Northeast Brazil, Ghana, Thanzania and West Indies, while in Indonesia, not much is known. Hiptolida is the main compound of *Hyptis pectinata* (L.) Poit leaf which has potential as anticancer. Biotransformation by fermentation is one approach to increase bioactive activity of plants. This study aims to determine the anti-leukemia activity of fermented product of methanol extract of leaf *Hyptis pectinata* (L.) Poit. Fermentation is carried out by the addition of methanol extracts in the early of death phase of *Apergillus niger* culture on potato dextrose broth media and harvested in the mid-phase of death. The fermented product was purified to get crystals. The crystal obtained is 37.8 mg, white in the form of a needle with a melting point range of 102-103°C. Based on toxicity test on leukaemia cell P388, fermented product extract has IC_{50} at: 84,21 µg/mL. While the toxicity test of methanol extract without fermentation obtained IC_{50} at: 21,01 µg/mL. Based on UV spectroscopy analysis the fermented product has a wavelength absorption capacity of 249.8 nm which is much different from pure hyptolide. Based on the FTIR data, it was also found that the -OH group that widened in the band 3394 cm^{-1} which previously was not present in the hyptolide compound, was thought to have formed a transformant compound. The results showed that the extract methanol leaves of *Hyptis pectinata* (L.) Poit has better anti-leukaemia activity compared to transformation from fermented products.
Keywords: Biotransformation, *Hyptis pectinata* (L.) Poit, Anti-leukaemia.

1. Introduction

Hyptis pectinata (L.) Poit is *Lamiaceae* family spread in the topic area and belongs to aromatic shrubs. The plant has used as traditional medicine [1]. The use of that plant is to treat indigestion, skin infection, nasal congestion, fever, cramps, inflammation, pain [2]. In Tanzania, it is used to treat boils; and in West Indies, stew of *Hyptis* leaf is drunk as tea and distraction of stomachache [1].

In Indonesia, especially in west Java and West Sumatra, *Hyptis pectinata Poit* is wild plant which is growing fertile on a place with enough sunlight [3]. The pharmacology investigation of *Hyptis pectinata Poit* has conducted in vivo and in vitro. It is proven by Suzery *et al.* [4] through BSLT test of methanol extract of *Hyptis pectinata Poit* leaf on the death of *Artemia salina Leach* larvae from several non-polar fractions. Cytotoxic activity is also tested through MCF-7 cell [5]. In bioorganic chemistry field, the finding of compound α.β unsaturated lactones is one of the interesting things; besides having a role in biology activity, the compound is also used to synthesis need. Year after year, isolation of pure compound of *Hyptis pectinata Poit* is developed in several countries especially isolation of hyptolide.

Fermentation is one of the approaches to increase compound bioactive activity. The increasing is because of compound biotransformation process. Biotransformation by using microbe is more specific than using pure chemistry and sustains addition, removal, or modification on certain functional groups of complex molecules [6]. *Aspergillus niger* is a microorganism from one of the moulds which is safe and categorized as GRAS (Generally Recognized as Safe) by FDA (Food dan Drug Administration) in America Madigan *et al.* [7]. In its metabolism, *Aspergillus niger* can produce citric acid, so that it is mostly used as fermentation model. Second metabolite compound is also made by modifying synthetic path of primary metabolite compound, or several substrates coming from primary metabolite [6]. According to Suzery et al. [4], hyptolide anticancer compounds from *Hyptis pectinata Poit* leaf can be isolated using methanol solvents. Based on these studies, this study aims to determine the anti-leukemia activity of fermented product of methanol extract of *Hyptis pectinata Poit* Leaf.

The research was conducted at the Laboratory of Organic Chemistry and Biochemistry, Faculty of Science and Mathematics, Chemistry Department of Diponegoro University. The renewal of the research was having analysis and anticancer test of murine leukemia P388 on fermented product of methanol extract of *Hyptis pectinata Poit* leaf at the stationer last phase. The research was begun by preparation of *Hyptis pectinata Poit* extract, preparation of *Aspergillus niger* spore, addition of *Hyptis pectinata Poit* leaf extract at the stationer last phase of *Aspergillus niger*, isolation and identification of fermented product and cytotoxic test of anticancer leukaemia P388 on fermented product of *Hyptis pectinata Poit* extract.

2. Materials and Method

2.1. Equipment

Equipment used in the research: Rotary Vacuum Evaporator (Re-2010), water bath HH-6 (DFS KW-1000DB), autoclave (Napto model 25x-2, seri 0013354), Laminar Air Flow (NUAIRE), Hemasitometer (Quiunjing xb.k.25), Centrifugation (Centrific model PLC-05), Shaker Incubator TIT (TS-330 A), UV light at 254 nm dan 365 nm Spectroline ENF-24/F, Fourier Transform Infrared Spectroscopy/ FTIR (8201 PC Shimadzu), UV Spectroscopy (mini 1240 Shimadzu), Fisher John Melting Point Apparatus, and kit MTT *Assay* (Bio-Rad).

2.2. Materials

Materials used in the research were Aquades, methanol p.a (Merck), ethyl acetat p.a (Merck), chloroform p.a (Merck), ether p.a (Merck), H_2SO_4 10%, Na_2SO_4 p.a, TLC plate silica gel 60 F_{254} (Merck), alcohol 70%, twen 80, PDB (Potato Dextro Broth) Difco, Agar p.a (Merck), and NaOH 10%, murine leukemia P-388 cells (The Japan Foundation for Cancer Research), RPMI 1640 media (Rosewell Park Memorial Institute) Gibco, MTT 3-(4,5 dimethyilthiazol-2-yl)-2-5-diphenyl tetrazolium bromide) Sigma, FBS (Fetal Bovine Serum) 2 % Sigma, kanamycin (Sigma), SDS (Sodium Deodecyl Sulfate) Sigma, DMSO (Dimetyl Sulfonamida) merck, HCl 0.01 N p.a (Merck).

2.3. Procedures

2.3.1. Extraction of Hyptis pectinata Poit Leaf. Hyptis pectinata Poit leaf was obtained from East Dago, West Java. There were 314 grams of simplicia *Hyptis pectinata Poit* leaf were macerated with 5 Litres of methanol solvent so that it was be perfectly extracted. Then, the extract was filtered and concentrated by rotary evaporator until formed thick extract [3].

2.3.2. Preparation of Aspergillus niger culture. Rejuvenation was conducted by taking one ose of *Aspergillus niger* on PDA (Potato Dextrose Agar) media. The culture was incubated at a temperature of 30ºC during 3-4 days until formed hyphae. Next, continued by making starter culture on PDB (Potato Dextrose Broth) media. Starter was observed until seen 1 x 10^7 spore/mL [8].

2.3.3. Biotransformation with Aspergillus niger. The growth profile of *Aspergillus niger* referred to research method conducted by Rejeki *et al.* [9]. Fermentation pointed at research of Miyazawa *et al.*

[10] and Arakawa *et al.* [8] with a little bit modification. 300 mL liquid media of PDB (Potato Dextrose Broth) [11] were got into 3 erlenmeyers of 250 mL, and each erlenmeyer contained 100 mL of PDB media. Two erlenmeyers were performed as beginning and last control of the addition *Hyptis pectinata Poit* extract, while 1 erlenmeyer was as fermentation sample. The addition 1 gram of *Hyptis pectinata* extract was done at the seventh day based on the growth curve [9]. At the extract addition, it was taken 1 erlenmeyer as a control before fermentation. After added extract, incubation was continued until the eleventh day (accordance with death phase). Fermentation was stopped by extraction.

2.3.4. Extraction and Separation of Fermented Product. Extraction of fermented product was centrifuged at 2000 rpm during 25 minutes. The filtrate and sediment were separated. On the filtrate, it was added by $NaSO_4$ until truly saturated [8], then filtered by Buchner funnel and *vacuum*. The obtained filtrate was extracted by using separating funnel producing ethyl acetate solvent. The filtrate result was concentrated by rotary evaporator until obtained thick extract. Then, the extract was separated and performed compound isolation of fermented product.

2.3.5. Isolation and Identification of fermented Product. The first step to separate fermented product was conducted analysis of TLC (Thin Layer Chromatography) with eluent chloroform: ether (1:1). The spot measurement on silica gel GF_{254} was observed by UV (λ) detector of 254 nm and 365 nm. Crude extract was washed by ether continuously so that it became crystal. The compound isolates purity was identified by TLC and melting point determination with Fisher John Melting Point Apparatus. Furthermore, to find out the functional groups in a single compound can be analysed using FTIR.

2.3.6. Anti-Leukaemia Activity Test of Fermented Product of Hyptis pectinata Poit. Anticancer test was conducted at laboratory KOBA-ITB using method of MTT [3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide] Assay. The principle of this method was changing colour of MTT from yellow to blue. Cytotoxic activity in this method was stated as IC_{50}. There were 10 µL sample with 7 different concentrations (0.1; 0.3; 1; 3; 10; 30; 100 µg/mL) which added into murine leukaemia P388 cells in *microplates* and saved in incubator CO_2 as 72 hours. Then, 20 µL MTT colour reactor (5 mg/µL) per well were increased into culture media, shaken using *microplates*, and incubated as 4 hours. Added 100 µL SDS solvent 10 %, HCl 0.01 N and formazan crystal into each well. Optical density (OD) was measured by using *Microplate Spectrophotometer Reader* (Tohso, MPR-A4i) at λ 550 and 700 nm. All steps were conducted in triplo. IC_{50} value was obtained by the extrapolation line 50 % of control uptake at the uptake curve on several sample concentrations using semi-algorithmic graph.

3. Result and Discussion

3.1. Fermented Product of Hyptis pectinata Poit Extract

The addition of *Hyptis pectinata Poit* extract was conducted at the stationer last phase (the 7th day) based on the previous observation [9]. Stationer phase is phase profile with horizontal line. At the stationer beginning phase, there was change of enzyme composition, and enzyme that was needed to have synthesis of secondary metabolite. While, at the stationer last phase, microbe consumed nutrition and removed metabolism products in the form of secondary metabolite of what it was excreted [12]. Generally, organism formatted filament fungi (spore) produced enzyme at the stationer beginning phase, and at that time, secondary metabolite was produced at the stationer last phase or the beginning of death phase and synthesized to fulfil complementary need and extracellular products. Based on the result, there were 108.4 mg of crude fermented product extract which had extracted with the brown one and oily.

3.2. Isolation and Identification of Fermented Product

To find out the existence transformation on the fermented product, it was performed TLC analysis. Through the observation, it was obtained that there were some spots stating the number of compounds of fermented product.

Table 1. Rf Crude Fermented Product (HPF) on ethyl acetate extract

Eluent	*Rf* Hiptolida	*Rf* HPP	*Rf* HPF	*Rf* KA	*Rf* KB
Ether : Chloroform (1:1)	0.48	0.07	0.03	0.68	0.91
		0.17	0.21		
		0.22	0.26		
		0.44	0.75		
		0.48			
		0.52			
		0.71			

Note HPP : Methanol Extract of *Hyptis pectinata Poit* leaves without fermentation
HPF : Fermented methanol extract of *Hyptis pectinata Poit* leaves until the 11th day
KA : (Control A) Culture extract before increasing methanol extract of *Hyptis pectinata* Poit leaves
KB : (Control B) Culture extract without increasing methanol extract of *Hyptis pectinata* Poit leaves until the 11th day

Based on TLC preliminary test of what has been done, the analysis using ether: chloroform (1:1) eluent products good separation, this is known because at this separation produces 7 spots on HPP. Hyptolide was one of the biggest components of *Hyptis pectinata Poit* [13] on Rf : 0.48. Resulted spot on crude HPF showed the difference between Rf and standard hyptolide, thus it was expected that it was transformation at crude HPF. The addition of the -OH group in hyptolide greatly increases the polarity of these compounds, so they can increase reactivity as an anticancer agent.

Aspergillus niger culture was also able to produce secondary metabolite, to get information related to the culture condition before and after transformation, on the analysis, it had spot comparison between KA and KB. Identification KA and KB described that resulted spot had different polarity than spot crude HPF, thus it was estimated that the spot was the result of transformation HPP without the influence of KA and KB. In this case, culture product was referred to similar eluent (chloroform: ether), so that there was possibility if KA and KB were identified on the different eluent.

The result of purity *crude* HPF on ether solvent resulted white crystals of 37.8 mg in the form of needle with melting point of 102-103°C (then it was called by THPF/transformant of HPF). TLC analysis was conducted by variety eluents; the best result showed that there was 1 single spot with Rf :0.27 on the eluent (ether : chloroform). The result stated that THPF was in polar thing and the polarity could be as a parameter to form new compound. The result of spectroscopy UV analysis showed there was uptake numbered 249.8 nm, than λ hyptolide of 212.0 nm [14]; 212.5 nm [3]. Based on the FTIR data, it was also found that the -OH group that widened in the band 3394 cm^{-1} which previously was not present in the hyptolide compound, was thought to have formed a transformant compound. The results have strengthened the presence of THPF transformation with differences in the amount of uptake, bandwidth and hyptolide standard compounds.

3.3. Cytotoxic Test of Fermented Product

Murine leukemia P388 was one of the types of leukemia cancer cell which was used in cytotoxic test. Usually, murine leukemia P388 cell was used on pre-screening stage to find out the new compound with cytotoxic activity. It was because murine leukemia P388 cell had clinically sensitivity greater than 95% on anticancer compound, therefore, through cancer cell, it could be found new compounds which was toxic on cancer cell although the compound activity was not significant [15].

Anticancer activity of isolate THPF was conducted *in vitro* on murine leukemia P388 cell using MTT *Assay* method. To determine the compound activity THPF, it could be performed by comparing the cytotoxic activity of methanol extract of *Hyptis pectinata Poit* without fermentation. The following was

the result of toxicity and IC_{50} value of crystal THPF of fermented product and *Hyptis* extract without fermentation:

Table 2. The result of cytotoxic test and IC_{50} value THPF and *Hyptis pectinata Poit* extract without fermentation on murine leukemia P388 cells.

Sample	Concentration µg/mL	Replication			Mean	IC_{50} µg/mL
		1	2	3		
THPF (Transformant of fermented *Hyptis Pectinata* Poit)	100	0.331	0.295	0.057	0.228	84.21
	30	0.894	0.981	0.766	0.88	
	10	0.809	0.846	0.880	0.845	
	3	0.675	0.620	0.677	0.657	
	1	0.660	0.436	0.640	0.579	
	0.3	0.587	0.583	0.490	0.553	
	0.1	0.664	0.590	0.566	0.607	
Methanol extract of *Hyptis pectinata Poit* without fermentation	100	0.011	-0.014	-0.020	-0.007	21.01
	30	0.253	0.272	0.329	0.284	
	10	0.446	0.458	0.488	0.464	
	3	0.518	0.580	0.578	0.558	
	1	0.646	0.603	0.680	0.643	
	0.3	0.665	0.634	0.690	0.666	
	0.1	0.567	0.544	0.625	0.578	

Toxicity test of isolate THPF resulted the risibility activity of 84.21 µg/mL, while methanol extract of *Hyptis pectinata Poit* without fermentation showed the risibility activity was 4 times higher than compound THPF which was fermented product, 21.01 µg/mL. It means that isolate THPF was one of the compound components of *Hyptis pectinata Poit* other than hyptolide that had no lactone ring on the position of unsaturated α,β,-δ and aliphatic side-chain position that was very essential on the decreasing increasing of biological activity of bioactive compound [16], thus it could be a basic of the existence of decreasing activity on isolate THPF of fermentation result. Based on the literature, it had been found out *argentilacton* compound of isolation result of *Hyptis pectinata Poit* extract without fermentation that had risibility activity on murine P388 cell numbered 21.40 µg/mL. It showed that pure compound and *Hyptis pectinata Poit* extract did not give the significant effect on the risibility of murine P388 cell. The following was the comparison of cytotoxic activity of isolate compound HPF of fermentation result and some *Hyptis pectinata Poit* extracts without fermentation on murine P388 cancer cell and MCF-7 cell based on the literature.

Table 3. The comparison of cytotoxic test and IC_{50} value of isolate HPF and some *Hyptis pectinata Poit* extracts without fermentation on P388 cell cancer and MCF-7 cell based on the literature:

Research Result	IC_{50} (µg/mL)	Literature	IC_{50} (µg/mL)
Crystal HPF	84.21 (leukemia P388 cells)	*Argentilacton* [16]	21.40 (leukaemia P388 cells)
Methanol extract of *Hyptis pectinata Poit*	21.01 (leukemia P388 cells)	Methanol extract of *Hyptis pectinata Poit* [5]	18.90 (MCF-7 cells)
		Chloroform extract of *Hyptis pectinata Poit* [17]	2.2 (leukemia P388 cells)

The American National Cancer Institute stated that an extract had had strong cytotoxic activity as anticancer agent if it had IC_{50} < 30 ppm [18]. While according to Ueki *et al.* [19], a compound had had anticancer activity if it had IC_{50} less than 100 µg/mL. Based on the analysis, it prove that IC_{50} on methanol extract of *Hyptis pectinata Poit* and crystal THPF were categorized in a good anticancer.

4. Conclusion

It was expected that isolate THPF was one of the compound components of *Hyptis pectinata Poit* leaves which didn't have lactone ring at unsaturated α,β,-δ and aliphatic side-chain position which was very essential on the decreasing and increasing of biological activity of bioactive compound, thus it needed structure determination step.

Gratitude

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