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Impact of Fermentation on Hyptolide and Phytochemical Composition of *Hyptis pectinata* (L.) Poit

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Abstract

Solid State Fermentation has been employed for the biotransformation of the herbal plant *Hyptis pectinata* (L.) Poit is widely distributed in tropical regions of Brazil and is also present in Indonesia. The plant has been used to treat anti-inflammatory, anti-cancer, bacterial infection, and wound healing. This study aims to investigate the effects of using a solid-state fermentation method to ferment the leaves of *Hyptis pectinata* (L.) Poit towards the composition of phytochemicals and hyptolides. This study was carried out in several stages, including fermentation of *Aspergillus niger* leaves, sample extraction with methanol, determination of total phenolic and total triterpenoid content, phytochemical screening, and analysis of hyptolide using HPLC. The research results showed the growth of *Aspergillus niger* on the leaves of *Hyptis pectinata* (L.) Poit got into the logarithmic phase at 36 hours. Triterpenes and phenolics were present in the phytochemical profile, while alkaloids, tannins, and flavonoids were absent. The total phenolic and triterpenoid content increased by 33.68% and 17.78% respectively. HPLC analysis showed a decrease in hyptolide content in the fermented product. These results indicate the potential of fermented *Hyptis pectinata* (L.) Poit as an additional cancer therapy.

1. Introduction

Hyptis pectinata (L.) Poit is a plant widely distributed in America, especially in Brazil. However, it can also be found in the highlands of Indonesia. In Brazil, this plant is extensively cultivated and used as an anti-inflammatory medicine for bacterial infections and wound healing. This plant is often used in India and Mexico to relieve fever, skin diseases, and stomachaches [1]. In Indonesia, there is no reported empirical evidence related to the use of this plant by local communities because it is visually similar to weeds that grow wild in various places [2]. Research on the potential of *Hyptis pectinata* (L.) Poit in Indonesia has revealed its significant properties, demonstrating anti-cancer, anti-proliferative, anti-plasmodial, and apoptotic effects on cancer cells [3, 4, 5].

Biotransformation is the process of modifying or altering chemical compounds involving microorganisms or enzymes. Biotransformation can be carried out using microorganisms (fermentation), enzymatic, and chemoenzymatic [6]. Biotransformation is known to enhance the bioactivity of bioactive compounds because this process can degrade cell walls so that they can break down bound compounds into free ones or convert complex compounds into safer ones. *Aspergillus niger* is a non-pathogenic fungus that is often used in production because it is known not to produce toxic compounds (mycotoxins), and its metabolism is capable of producing citric acid. Biotransformation with this fungus is also known to be involved in increasing the antioxidant bioactivity of flavonoid compounds [2]. Hyptolide is the focus of this research because it is reported that this compound is contained in *Hyptis pectinata* in large quantities [7].

Solid-state fermentation (SSF) is an easy and cost-effective fermentation method [8]. However, it is widely reported to be able to increase levels of bioactive compounds. The controllable humidity in SSF provides an

optimal condition for *Aspergillus niger* to produce more than 19 cell wall-degrading enzymes such as α-amylase, β-glucosidase, cellulase, pectinase, protease, lignocellulose, and others, with the potential to enhance the phenolic content of a substrate [9]. The solid-state fermentation method applied to moringa leaves using *Aspergillus niger* has been reported to increase the total phenol and total flavonoid content and increase antioxidant activity [10]. Conducting the fermentation of *Hyptis pectinata* (L.) Poit leaves with *Aspergillus niger* in a solid-state fermentation (SSF) process is an intriguing endeavor. This exploration aims to assess the potential bioactivities against anti-degenerative diseases, a facet that has not been reported previously. The analysis of hyptolide was conducted due to its high concentration in the *Hyptis pectinata* plant. Consequently, the biotransformation of this plant can potentially influence the hyptolide content.

2. Experimental

2.1. Materials and Equipment

Hyptis pectinata (L.) Poit leaves were obtained from harvesting at Bandungan, Central Java, Indonesia. The materials were methanol, *Aspergillus niger* IPBCC.08.610, potato dextrose broth (PDB), Folin–Ciocalteu reagent, Na_2CO_3, $AlCl_3$, CH_3COONa, gallic acid, ursolic acid, H_2SO_4, HCl (analytical grade), gelatine 1%, NaCl 10%, $FeCl_3.6H_2O$, Mg powder, chloroform, anhydrous acetic acid, amyl alcohol, Mayer's reagent, and Dragendorff's reagent.

2.2. Fermentation and Extraction Product of *Hyptis pectinata* (L.) Poit Leaves with *Aspergillus niger*

The leaves of *Hyptis pectinata* (L.) Poit were washed with water. Subsequently, they were dried with a food dehydrator and ground using a food processor. The resulting powder was obtained, which was utilized in the next step. Spore counts were conducted on the *Aspergillus niger* fungi. Fungal spores were measured using a 0.5 McFarland standard (at 625 nm, absorbance range of 0.08-0.1 in a UV-Vis Spectrophotometer) to generate a standard microbial inoculant with an approximate spore concentration of $0.5-2.5\times10^3$ spores/mL.

Hyptis pectinata (L.) Poit leaves were weighed to 10 grams and placed in a glass jar. Subsequently, 50 mL of distilled water (in a ratio of 1:5) was added to achieve the optimal humidity for Solid State Fermentation (SSF), ranging between 50-80%. The glass jar was then inoculated with 1% *Aspergillus niger*, whose spores had been quantified, and incubated at 37°C for 36 hours during the logarithmic phase. The anticipated logarithmic phase of the fungus was evidenced by the proliferation of hyphae on the surface of the sample.

The fermented leaves were macerated using 100 mL of methanol (1:10 ratio) for 24 hours and then shaken using a rotary shaker at 125 rpm. The extract was filtered in a Buchner funnel assisted using a vacuum pump for 10 to 15 minutes. The filtrate was subsequently stored for further analysis.

2.3. Phytochemical Screening

Alkaloid, saponin, flavonoid, phenolic, terpenoid, and tannin tests follow Bhardwaj's method [11].

2.3.1. Alkaloid Test

Three mL of extract was added with a few drops of HCN 2 N. The solution was divided into three equal parts. Each section was tested with a few drops of Mayer's reagent and Dragondorff's reagent. Positive characteristics that contain alkaloids were the formation of a white layer in Mayer's reagents and red brick deposits in the Dragendorff's reagent.

2.3.2. Phenolics Test

One mL of extract was added with a few drops of 5% iron chloride solution. The dark green color indicated the presence of phenolic compounds.

2.3.3. Flavonoid Test

One mL of extract was added with 5 to 10 drops of 2 N HCl and magnesium powder. Then, the mixture was heated for 2 minutes. After that, 2 mL of amyl alcohol was added to the mixture. Positive identification of flavonoids was indicated by the formation of a pink, yellow, or orange color.

2.3.4. Saponin Test

One mL of extract was dissolved with distilled water, shaken vigorously, and allowed to stand for 10 minutes. The presence of saponins was indicated by stable froth after HCl 2 N drops.

2.3.5. Terpenoids Test

One mL of the filtrate was heated until evaporation, followed by adding two drops of acetic anhydride. Subsequently, three drops of concentrated H_2SO_4 were added to form a distinct layer. The development of a reddish-brown color at the interface indicated positive results for the presence of terpenoids.

2.3.6. Tannin Test

One mL of filtrate was added with a few drops of 1% gelatin and 10% NaCl. A precipitate gelatine would be formed to show positive results for the presence of tannins.

2.4. Determination of Total Phenolic Content

The total phenolic content test was conducted following the method outlined by Ibrahim *et al.* [12] with modifications. A 0.25 mL sample was added with 1.25 mL of distilled water and 1.25 mL of Folin-Ciocalteu reagent. Samples were incubated at 25°C for 15 minutes, and 1 mL of Na_2CO_3 was added and incubated in the dark for 30 minutes at 25°C. Absorbance was measured at a wavelength of 765 nm. The gallic acid solution was used as a standard curve solution. The standard curve was used to determine the phenolic content of the sample.

Table 1. The phytochemical screening of biotransformation product

Sample	Secondary metabolite compound					
	Tannin	Phenolic	Terpenoid	Flavonoid	Alkaloid	Saponin
F0	-	++	+	-	-	-
F36	-	+++	++	-	-	-

2.5. Determination of Total Triterpenoid Content

The total triterpenoid content test was performed following the method outlined by Wei *et al.* [13] with some modifications. A 100µL of the sample was heated until it evaporated in the bath. A 500µL of glacial vanillin-acetate 5% (w/v) was added with 900 µL of sulfuric acid, followed by homogenization and heating for 30 minutes at 70°C. The solution was cooled, and 3.6 mL of glacial acetate was added. Absorbance was measured at a wavelength of 553 nm. The ursolic acid solution was used as a standard curve solution. The standard curve was used to determine the triterpenoid content of the samples.

2.6. Hyptolide Analysis Using High-Performance Liquid Chromatography

Quantitative analysis of the hyptolide content using HPLC (Shimadzu, LC-20AD) with the following specifications: wavelength of λ = 245 nm, RP-18 Endcapped Hibar C18 column (250 mm × 4.6 mm, 5 µm), flow rate of 1 mL/min, injection volume of 20 µL, methanol 100% as mobile phase, and UV-Vis Prominence detector (SPD-20A) [7].

3. Results and Discussion

Fermentation was carried out on the leaves of *Hyptis pectinata* (L.) Poit and extraction were performed using methanol as the solvent. Qualitative phytochemical screening was also conducted to identify the types of chemical compounds present. From the results of phytochemical screening, the total phenol and triterpenoid content of both fermented and non-fermented extracts were calculated. HPLC analysis was performed to determine the levels of hyptolide in the extracts obtained from the fermentation and non-fermentation processes of *Hyptis pectinata* (L.) Poit leaves. HPLC was employed to assess the impact on the hyptolide content in plants subjected to biotransformation using the SSF method in this study.

3.1. Fermentation and Extraction Product of *Hyptis pectinata* (L.) Poit Leaves with *Aspergillus niger*

Fermentation was carried out for 36 hours until it reached the logarithmic phase and was extracted using methanol. Generally, the fungal growth profile in the logarithmic phase is characterized by the appearance of white hyphae and becomes blackish in the stationary phase. The fermented product is F36, and the non-fermented product is F0.

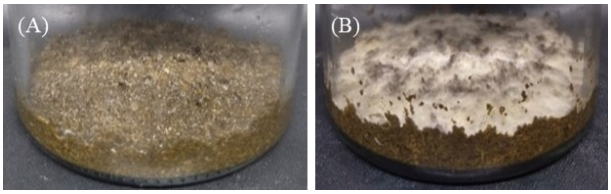

Figure 1. *Hyptis pectinata* (L.) Poit leaves: (A) non-fermented; (B) after 36 hours of incubation

Figure 1A shows that the leaves of *Hyptis pectinata* (L.) Poit exhibit a dark green color, the substrate emits a tea-like aroma, and there is an absence of hyphae. Figure 1B shows a white mycelium that covers the entire surface of *Hyptis pectinata* (L.) Poit leaves in 36 hours (logarithmic phase) of fermentation using *Aspergillus niger*, the aroma of the tea faded, and the color of the substrate turned brownish-green. The hyphal growth indicates that the fungus successfully lives in the substrate media (*Hyptis pectinata* leaves). In this study, fungal growth was limited to the logarithmic phase only. The growth of these hyphae is influenced by nutrition from plant cell wall degrading enzymes produced by *Aspergillus niger*, such as pectinase, cellulase, hemicellulase, endoglucanase, exoglucanase, and others [14].

Extraction was conducted after 36 hours of fermentation, utilizing methanol as the solvent for 24 hours. This process involved agitation with a rotary shaker, chosen for its ability to reduce the maceration duration compared to the lengthier static maceration method. Methanol is selected as the solvent due to its universal solvent properties, enabling the attraction of both polar and semi-polar substances [7].

3.2. Phytochemical Screening of F0 and F36 *Hyptis pectinata* (L.) Poit

Phytochemical screening employed qualitative testing to evaluate the presence of additional chemicals in the biotransformation product of *Hyptis pectinata*. Table 1 shows the phytochemical test results for *Hyptis pectinata* leaves. The preliminary screening of methanol extracts from both non-fermented and fermented *Hyptis pectinata* leaves revealed the presence of beneficial compounds such as phenolic and terpenoid. The increase in phenolic and terpenoid content after fermentation may be attributed to the potential degradation of plant cell walls during the process, facilitating the release of these compounds still bound to the cell walls. However, the extract did not show the presence of flavonoids, tannins, alkaloids, and saponins. This absence could be due to either the minimal quantity of these substances, preventing a proper reaction with the reagent, or the inherent absence of these chemicals in *Hyptis pectinata* leaves [15].

Table 2. Total phenolic content of methanol extract of *Hyptis pectinata* (L) Poit leaves

Sample	Total phenolic content (GAE/g sample)
F0	275.859
F36	368.775

3.3. Determination of Total Phenolic and Total Triterpenoid Content of F0 and F36

The assessment of the total phenolic content in F0 and F36 involved conducting a test based on the principle of forming a blue molybdenum-tungsten complex. This complex is produced through a colorimetric oxidation and reduction reaction of the hydroxyl group in phenolic compounds under alkaline conditions facilitated by Folin-Ciocalteu [16]. Gallic acid was used as a standard, and total phenolic content was measured at a wavelength of 765 nm [17]. The total phenolic content of the fermentation product methanol extract is shown in Table 2.

The total phenolic content in Table 2 shows an increase in total phenolics in the fermented product. Fermentation can increase the phenolic content by 33.68%. Many polyphenolic compounds in plants are found in a bound state, generally forming ether bonds with lignin and ester bonds with carbohydrate/protein structures. The cellulase enzyme, generated by *Aspergillus niger*, plays a pivotal role in hydrolyzing these bonds. The elevation in phenolic levels can be linked to the degradation of plant cell walls facilitated by cell wall-degrading enzymes produced by *Aspergillus niger*, including cellulase, lignocellulose, xylanase, and others. This enzymatic action enables the release of phenolic compounds initially bound to the cell walls, transforming them into free phenolic compounds [18].

The β-glucosidase enzyme, produced by *Aspergillus niger*, is instrumental in converting glycosides into simpler compounds, specifically aglycones. This enzymatic activity plays a crucial role in the breakdown of carbohydrates, facilitating the liberation of phenolic compounds. The total triterpenoid contents in F0 and F36 were determined based on the principle outlined by Wei *et al.* [13], with slight modifications. The total triterpenoid content of biotransformation products is shown in Table 3.

The total triterpenoid content in Table 3 shows an increase in total triterpenoids in fermented products. Fermentation can increase the triterpenoid content by 17.78%. The observed elevation in triterpenoid levels during the logarithmic phase of the two extracts is attributed to the lignolytic enzymes produced by *Aspergillus niger*. These enzymes have the capability to degrade cell walls, consequently leading to an augmentation in triterpenoid levels [2].

Table 3. Total triterpenoid content of methanol extract of *Hyptis pectinata* (L) Poit leaves

Sample	Total triterpenoid content (UAE/g sample)
F0	58.064
F36	68.387

3.4. Hyptolide Analysis Using High-Performance Liquid Chromatography

The abundance of hyptolide in the *Hyptis pectinata* plant was analyzed using HPLC to ascertain the anticipated increase in levels during the biotransformation process. The HPLC analysis of hyptolide crystals as a standard exhibited a single peak at a retention time of 2.27 minutes with a purity of 100% and a concentration of 1000 ppm as the standard. The hyptolide standard chromatogram, methanol extract of non-fermentation product (F0), and methanol extract of fermentation product at 36 hours (F36) are shown in Figure 2.

The chromatogram reveals the presence of hyptolide in the methanol extract of the non-fermented product (F0), with a peak observed at a retention time of 2.265 minutes and a concentration of 41.618 ppm. In contrast, the methanol extract of the fermentation product at 36 hours (F36) exhibits a peak at a retention time of 2.268 minutes, with a concentration of 18.096 ppm. This suggests a decrease in the hyptolide compound by 56.517%. Figure 2 indicates that fermentation may lead to a reduction in toxicity, aligning with findings from prior research. This decrease in toxicity is presumably attributed to the biotransformation process, wherein hyptolide, a toxic compound, undergoes breakdown into potentially less toxic compounds and its derivatives [19]. The chromatogram also reveals additional peaks that are not well-separated, likely attributable to the less effective separation of the methanol mobile phase [7].

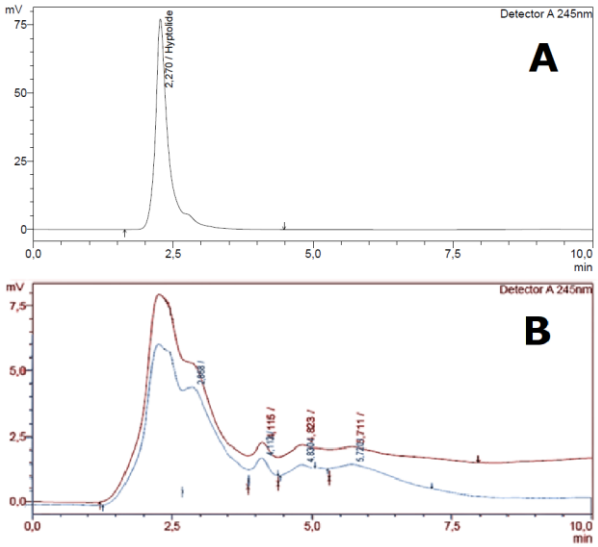

Figure 2. HPLC chromatogram of hyptolide: (A) standard, (B) F0 (unfermented - red), and F36 (fermented for 36 hours - blue)

4. Conclusion

The logarithmic phase of *Aspergillus niger* using the SSF method on *Hyptis pectinata (L)* Poit leaves was attained at 36 hours of fermentation. The qualitative analysis of methanol extract showed the presence of phenolics and terpenoids. Total phenolics and triterpenoids significantly increased after fermentation, indicating that the SSF method is optimal for the biotransformation of *Hyptis pectinata* leaves with *Aspergillus niger*. According to the HPLC results, the hyptolide content of *Hyptis pectinata* (L.) Poit decreased after fermentation. This reduction is likely attributed to the biotransformation process, wherein hyptolide undergoes breakdown into other compounds and potentially unknown derivatives. The fermented product of *Hyptis pectinata (L)* Poit leaves can be studied further for their potential bioactivities.

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