



## Total Hyptolide of Indonesian *Hyptis pectinata* extracts in a various solvent using HPLC and UV-Vis spectroscopy and their toxicities

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### Abstract

The bioactive compound of hyptolide has been isolated from an Indonesian plant *Hyptis pectinata* using various solvents. The pure compound was then used as a standard in quantitative analysis using HPLC and UV-Vis spectroscopy in ethanol ( $E_{Eth}$ ), ethyl acetate ( $E_{Ea}$ ), acetone ( $E_{ac}$ ) and hexane ( $E_{hex}$ ) extract each earned 3.14%, 0.01%, 1.33% and 0.04% (dry weight per sample). The standard curve of hyptolide using UV spectroscopy has been obtained with the coefficient of relationship ( $r$ ) of 0.997. However, the use of spectroscopy was not recommended for the standardization of hyptolide in the extract due to interference from other compounds that absorb the same wavelength. Furthermore, the toxicity test using the Brine Shrimp lethality test shows  $LC_{50}$  value of 92–181 ppm, which was in  $E_{Ea}$  compared to  $E_{Eth}$ ,  $E_{ac}$ , and  $E_{hex}$ .

### 1. Introduction

Hyptolide, one of the bioactive compounds from *Hyptis pectinata* Poit (Lamiaceae), is most frequently used as the target of research. The development of research on the compound covers three aspects, which are the aspect of insulating and structural analysis, aspects of assay activity as well as the aspects of functional group transformations to produce the derivatives compounds. Gorter [1] was the first researcher who has managed to isolate the pure compound of hyptolide. The molecular formula of hyptolide was proposed as the  $C_{18}H_{24}O_8$ . Other researchers examine the structure of its stereochemistry molecules [2, 3, 4]. From the aspect of the activity and the selective against targets, it was reported that hyptolide Lys-352 amino acid  $\alpha$ -tubulin has a structural analog with the natural product from pironetin [5, 6]. Within the scope of the synthesis, the double bonds of alkenes in hyptolide have been transformed into epoxides and naphtha derivatives [7, 8].

Research using the extract of *Hyptis pectinata* for assay activity on liver mitochondrial respiration [9], antinociceptive [10], antibacterial [11], and anticancer

[12] has been reported. These activities closely related in the presence of hyptolide or other compounds of the type  $\alpha$ ,  $\beta$ -unsaturated  $\delta$ -lactones in its mixture. Unfortunately, the amount of hyptolide in the extract is still very little reported, whereas it is generally understood that the amount of bioactive compounds is significant in testing activities.

Our interest is to analyze the content of hyptolide compounds in various preparations extracted from the *Hyptis pectinata* plant so that the number of bioactive compounds in hyptolide extracts can be known with certainty. In addition, the results of this study are expected to provide information about the best solvents used to extract hyptolide compounds. Comparison of UV spectroscopy and HPLC methods are also discussed to get an illustration of the possible use of UV spectroscopy for the analysis of hyptolide in the mixture. Finally, the standardized extract testing activity was carried out with the Brine Shrimp Lethality Test (BSLT).

## 2. Methodology

This research was conducted in several stages. *Hyptis pectinata* Poit was extracted in ethanol, ethyl acetate, acetone, and *n*-hexane solvents. Each extract was analyzed for the total hyptolide contents using UV-Vis spectroscopy and HPLC. The hyptolide activity test was carried out using the *Brine Shrimp Lethality Test* (BSLT) method.

### 2.1. Material and Tool

The material used in this study were the *Hyptis pectinata* plants that were collected from Kanayakan Bandung Indonesia, ethanol, methanol, distilled water, ethyl acetate, acetones, hexane, chloroform, rotary vacuum evaporator (IKA@ RV 10 Basic), UV Spectrophotometer (Shimadzu, UV-1601), HPLC (Shimadzu, LC-20AD).

### 2.2. Extraction of Hyptolide in Various Solvents

The sunlight dried leaves of *Hyptis pectinata* for eight days. The dried *Hyptis pectinata* (15 g each) were extracted in 20 mL ethanol, ethyl acetate, acetones, and *n*-hexane solvents for 24 hours. The filtrate was then separated and concentrated by the rotary vacuum evaporator. The extracts of ethanol ( $E_{eth}$ ), ethyl acetate ( $E_{Ea}$ ), acetones ( $E_{ac}$ ), and hexane ( $E_{hex}$ ) were then stored for hyptolide analysis.

### 2.3. Isolation of Hyptolide

The isolation of hyptolide was based on the method conducted by previous research [2]. From 500 g of dried material, it was obtained a 5.21 g crystal (1.7%). The hyptolide crystals were tested its purity through melting point test and HPLC analysis. The hyptolide crystal was then used as a standard for total hyptolide contents analysis on different solvent extracts using HPLC and UV-Vis spectroscopy.

### 2.4. Analysis of Total Hyptolide Contents Using HPLC

Hyptolide standard stock solution was made by weighing 12 mg of hyptolide crystals and then dissolved in 10 mL chloroform (1200 ppm). Further, the standard stock solution of hyptolide was diluted with a varied concentration of 800 ppm, 600 ppm, 400 ppm, 200 ppm. After that, it was analyzed using HPLC. Extracts of *Hyptis pectinata* Poit derived from each solvent was taken 32.50 mg and then dissolved in 50 mL chloroform (650ppm), then analyzed using HPLC. The column used was Vertex, Eurospher 100 5-C18, 150x4, 6 mm, Me-OH/H<sub>2</sub>O (90/10) mobile phase, 1 mL/minute flow rate, 8.9 MPa, UV 245 nm detector, and 20  $\mu$ L injection volume.

### 2.5. Analysis of Total Hyptolide Contents Using UV-Vis Spectroscopy

Analysis of total hyptolide contents using UV-Vis Spectroscopy was conducted by the calibration curve method. Each extract of *Hyptis pectinata* Poit derived from

each solvent was taken 10 mg and then dissolved in 100 mL chloroform (100 ppm), then analyzed using UV-Vis.

### 2.6. Brine shrimp lethality assay

*In vitro* assay, the lethality of *A. saline* was used to detect cell toxicity [13, 14]. Brine shrimp eggs were placed in seawater (3.8% w/v sea salt in distilled water) and incubated at 24°C to 28°C in front of a lamp. Eggs were hatched within providing a large number of 48 h plural larvae (*nauplii*). A convenient number of *nauplii* were placed in vials containing 5 mL of seawater and increasing concentrations of *Hyptis pectinata* extracts (0.1-500ppm). Control was made with the same seawater without the addition of the extract. Alive *nauplii* were counted after 16 h, and the lethal concentration ( $LC_{50}$ ) was calculated. Lethality assays were evaluated by statistical computer programs (Finney) to determine the  $LC_{50}$  values and 95% confidence intervals. All other data were expressed as mean  $\pm$  SD.

## 3. Results and Discussion

The discovery of pure compounds in a given plant has a meaning that is very strategic because it can be used as material for assay activity, for the transformation into a new compound and a drug dose for standardization using plant extracts. Besides, researchers in Indonesia are currently being excited doing bioactive compounds that are detected and quantifies in plant material. The results of this research have been abstracted for the manufacture of Standard Operation Procedures (SOP) prospective cultivation in order to obtained harvest product and post-harvest, which has the most active component for assay activity, both preclinical and clinical. *Hyptis pectinata* was used as a target plant in our laboratory because it is a tropical plant and reportedly has a prospective hyptolide compound for the treatment of various diseases [6].

### 3.1. Extraction of Hyptolide in Various Solvents

Dried simplicia with a moisture content of 7.6% was macerated with ethanol, acetone, ethyl acetate, and *n*-hexane followed with separation techniques by [2]. The result was a green solution on ethanol, acetone, and ethyl acetate filtrate, whereas *n*-hexane filtrate was yellow. This solution was resulting in crude with the same color. Ethanol was a solvent that gave the highest yield compared to other solvents (Table 1).

**Table 1.** Yield in extracts of *Hyptis pectinata* Poit in various solvents

No.	Solvent Type	Weight of Extract (g)	Extract yield (%)
1	Ethanol	0.7909	5.27
2	Ethyl acetate	0.3205	2.136
3	Acetone	0.238	1.587
4	<i>n</i> -hexane	0.07	0.467

### 3.2. Isolation of hyptolide

Isolation of hyptolide from ethanol extract produced white crystals, needles shaped with a total of 1.7% yields obtained. This result was smaller compared with isolated crystals in previous research [1]. The alleged hyptolide isolate had a melting point of 86–87°C. The previous studies reported that hyptolide had a boiling point of 55°C [10], 55°C [2], 87–88°C [2]. The existence of a single peak,  $t_r = 1,750$  minutes on HPLC chromatograms showed that the compound of isolation was a pure compound.

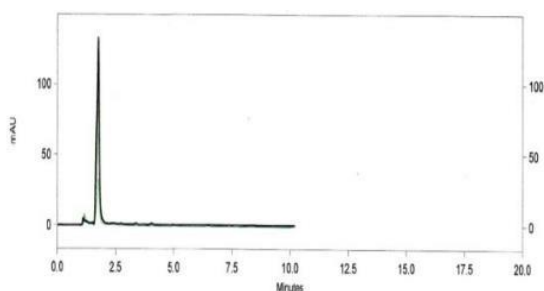


Figure 1. The single peak on the HPLC spectrum of isolated Hyptolide

The data spectrum by UV absorption of isolate provided a wavelength at 212,5 nm, which not much different from previous research [3]. The spectra of HPLC show peaks on 1.750 minutes of retention time, proving that the pure compound isolated was hyptolide.

### 3.3. Analysis of Total Hyptolide Contents Using HPLC

The first step, carried out four times the injection of pure hyptolide to obtain the retention time of hyptolide on the HPLC instrument. The results showed high reproducibility (1.750, 1.750, 1.767 and 1.733 minutes). Then standard solutions of hyptolide were made which is 200 ppm, 400 ppm, 600 ppm, and 800 ppm. Then, linearity and the amount of area obtained were analyzed and it was found that the relationship between concentration and area was  $y = 2x + 155.1905$ . The standard hyptolide curve shows excellent linearity in the concentration range of 200–800 ppm, with a correlation coefficient of 0.997.

The HPLC chromatogram of Acetone extracts ( $E_{ac}$ ) to standard hyptolide was shown at a retention time of 1.750 min with an area of 486.794, at a wavelength of 245 nm. On the same extract concentration,  $E_{Et}$ ,  $E_{EA}$ , and  $E_{Hex}$  all have a smaller area compared to  $E_{ac}$ , which means acetone extract contains most hyptolide (table 2). From these results, it can be seen that the difference of solvent greatly affects the total hyptolide in the extracts.

Table 2. Yield in extracts of *Hyptis pectinata* Poit in various solvents

No.	Extract	Peak area	% hyptolide in extract
1	EEth	486794	59.51
2	EEA	578443	71.15
3	EAc	678348	83.83
4	EHex	449752	54.80

We can measure the effectiveness of the solvent in extracting hyptolide from dry *Hyptis pectinata* samples by multiplying the hyptolide percentage in the extract with its yield. Extraction with ethanol was the best because it would be obtained 3.14% hyptolide, compared to extracts of ethyl acetate (1.52%), acetone (1.33%), and hexane (0.04%), respectively. Unfortunately, research regarding the solvent influence on the bioactive compounds that have a lactone framework has not been reported yet, so we have difficulties in comparing these results with the literature. However, other researchers have used ethanol for efficient isolation of hyptolide compounds from *Hyptis pectinata* [1]. The pattern of this kind of research can be applied to evaluate how to obtain  $\alpha$ ,  $\beta$ -lactones the plant *Hyptis* from others, such as Argentilactone, Boronolide, and so forth.

It is important to note that at a wavelength of 245nm, a UV detector was also capable of detecting four other components in each extract analyzed (Figure 3), such as pectinolyde A–F in *Hyptis pectinata* [15, 16], though the compounds gave peaks at a different retention time on HPLC spectrum.

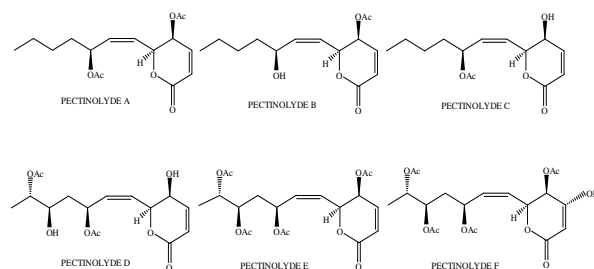


Figure 2. Some compounds are  $\alpha$ ,  $\beta$ -unsaturated lactones than that found in hyptolide *Hyptis pectinata*

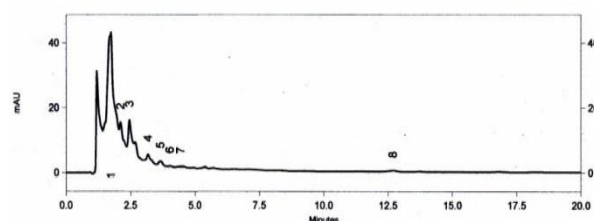


Figure 3. HPLC spectrum extracts from ethanolic.

### 3.4. Analysis of Total Hyptolide Contents Using UV-Vis Spectroscopy

UV/Vis spectroscopy is a method that often used for the analysis of bioactive compounds in traditional medicine preparations of extract. For example, the analysis of the Curcuma content in the extract can be applied with the assumption the other two have a molar or absorption extension that is similar to the standard Curcuma. Such analysis has been applied as a quick method to monitor the analysis of post-harvest products in some institutions in Indonesia [17, 18, 19].

The standard curve of hyptolide with UV spectroscopy (Figure 4) has been obtained with the equation of the line  $y = 0.00116x - 0.024$ . The correlation coefficient showed a very high linearity (0.997). The

relationship between analytic responses is proportional to the concentration measured from making a calibration curve, so this curve can be applied to determine the hyptolide content in *the Hyptis pectinata* Poit.

However, ultraviolet spectroscopy analysis showed that the content of hyptolide in all extracts was more than 100%. This shows that the absorbance is shown at the maximum wavelength, not only originating from hyptolide, but there are other compounds that provide absorption at a wavelength of 212 nm. The compounds contained in the *Hyptis pectinata* Poit plant and have conjugated double bonds so that it was estimated to contribute to the absorption of the group are flavonoid compounds, alkaloids, triterpenoids, and lactone compounds [11, 16, 20]. There was a strong suspicion that some of these compounds have a molar extension that was larger than hyptolide.

**Table 3.** Hyptolide content *Hyptis pectinata* in Poit using the UV

No.	Solvent Type	Absorbance (A)	Hyptolide extract (%)
1	Ethanol	0.492	444.83
2	Ethyl acetate	0.398	363.8
3	Acetone	0.527	475
4	n-hexane	0.620	555.2

The presence of other compounds that have conjugated double bonds similar to the structure analyzed is a disadvantage of UV / VIS spectroscopy. The pectinolida compound in *Hyptis pectinata* [21], which has the same conjugation as hyptolide, is thought to provide a disturbance that contributes to absorption at 215.5 nm wavelength in the form of an electronic transition over arising from unsaturated acetone  $\alpha, \beta$ .

### 3.5. Extracts Cytotoxicity by Brine Scrimp Lethality

A search for new anticancer drugs has taken many different approaches. The brine shrimp lethality bioassay is an efficient, rapid, and inexpensive test that requires only a small number of samples. The technique was easily mastered, costs little, and utilizes a small amount of test material. Meyer *et al.* [22] have been successively the self-employed for *in-vivo* bioassay lethality-fractionation of an active cytotoxic guide.

The cytotoxic activity of *Hyptis pectinata* extract was investigated *in vitro* against brine shrimp (*Artemia salina*). The results are presented in Table 3. All crude extracts of *Hyptis pectinata* produce  $LC_{50}$  values of less than 200 g/mL, which are considered active against saltwater shrimp. This bioassay has a good correlation with cytotoxic activity in some plant extracts because some samples show significant lethality in saltwater shrimp [23]. In this report, all *Hyptis pectinata* extracts show good saltwater shrimp lethality.

**Table 4.**  $LC_{50}$  of *Hyptis pectinata* Poit in various solvents

No.	Extract	$LC_{50}$ (ppm)
1	Ethanol	181.639
2	Ethyl acetate	92.548
3	Acetone	135.854
4	n-hexane	128.455

## 4. Conclusion

Total hyptolide content in extracts using HPLC in ethanol ( $E_{Eth}$ ), ethyl acetate ( $E_{EA}$ ), acetone ( $E_{ac}$ ), and hexane ( $E_{hex}$ ) extracts were obtained 3.14%, 0.01%, 1.33%, and 0.04% respectively (per sample dry weight). However, the use of UV spectroscopy was not recommended for standardizing hyptolide extract because there was interference from other compounds that give absorption at the same wavelength. Furthermore, the toxicity test with *the Brine Shrimp Lethality Test* showed an  $LC_{50}$  value of 98–181ppm.

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