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Identification of Alkaloid Compounds from Cytotoxic Active Fraction in *Peperomia pellucida*

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Abstract: *Peperomia pellucida* attracts a lot of interest due to its bioactivity and has been used in various folk medicine. In this report, n-hexane, dichloromethane, and ethyl acetate fraction of *Peperomia pellucida* extract were examined for its cytotoxic activity against *Artemia salina* larvae using the BSLT method. The ethyl acetate fraction showed higher cytotoxic activity than n-hexane and dichloromethane. The LC_{50} of ethyl acetate, n-hexane, and dichloromethane fractions was 39.665, 278.920, and 60.808 ppm, respectively. Mayer and Dragendorff tests give a positive result to the alkaloid constituent in ethyl acetate fraction. The alkaloids in *Peperomia pellucida* were extracted using ethyl acetate and further separated using chromatography techniques. Two alkaloid components were identified in ethyl acetate fraction from *Peperomia pellucida* as Piperine and Nigramide N by using LC-MS/MS.

Keywords: *Peperomia pellucida, cytotoxic assay, phytochemical screening, alkaloid, LC-MS/MS*

Introduction

Peperomia pellucida belongs to the Piperaceae family and Peperomia genus. It spreads all over the tropical country and has been utilized in traditional communities to cure various illnesses such as rheumatism, arthritis, and headache [1], [2]. In Indonesia, the traditional treatments of Peperomia pellucida are mostly related to high uric acid contents in the body [3].

Previous research shows that *Peperomia pellucida* exhibits cytotoxic activity against *Artemia salina* [4], [5]. Further studies confirm its cytotoxic activity by using various cell lines, including HEK 293 (*Human Epithelial Kidney cell line*), HeLa (*Human cervical cancer cell line*), HePG2 *(Human hepatic carcinoma cell line*), and *human breast adenocarcinoma* (MCF-7)[6], [7]. However, the specific compounds that responsible for the cytotoxic activity are not clear yet.

Several secondary metabolites have been successfully isolated and identified in *Peperomia pellucida*. Most of them are O-containing compounds such as peperomin A, B, C, and E [8], 3',4',dihydroxy-3-5-dimethoxy flavon-7-O-β-rhamnose [9], 3',4', 7-tri-O-methoxy flavone [10], friedeline, β-sitosterol, herniarin, scopoletin [11]. Pheophytin and β-sitosterol-D-glucopyranoside were also identified in *Peperomia pellucida* [12]. Additional studies need to be conducted to identify other secondary metabolites in *Peperomia pellucida* and relate to its bioactivity.

Not many studies deal with the alkaloid constituents in *Peperomia Pellucida*. In 2017, Fachriyah and co-workers studied the alkaloid in *Peperomia pellucida* which relates to the xanthin inhibition activity [13]. Another report indicates that *Peperomia pellucida* contains 29.59 mg alkaloid with piperine as a control [14]. Those papers did not mention the identified alkaloid constituent in their works. In this report, two alkaloid constituents in the most active fraction against *Artemia salina* larvae were identified using LC-MS/MS.

Method

Materials

Peperomia pellucida, ethanol, ethyl acetate, n-hexane, aquades, chloroform, ammonia 25%, sulfuric acid 98%, hydrochloric acid 2 M and 1 M, Reagent Dragendorff and Mayer, Magnesium powder, amyl alcohol, H_2SO_4 98%, $FeCl_3$ 1%, NaOH 1M.

Equipment

Glassware, UV lamp 245 and 365 nm (CAMAG UV Cabinet 4), rotary vacuum evaporator, LC-MS/MS (Xevo G2-XS Qtof).

Fractionation of *Peperomia pellucida* Extract

Dried *Peperomia pellucida* was macerated by ethanol as a solvent for 24 hours. *Peperomia pellucida* residue was separated from its filtrate to get the ethanolic extract after 24 hours. This procedure was repeated to get the nearly clear ethanolic extract. The ethanolic extract was concentrated using a rotary evaporator to get the crude extract. The chlorophyll content in the crude extract was removed and fractioned by n-hexane, dichloromethane, and ethyl acetate. Each fraction was concentrated to prepare the solution for the cytotoxic activity assay.

Cytotoxic Activity Assay

The cytotoxic activity assay was conducted by preparing brine water to hatch the *Artemia salina* larvae. N-hexane, dichloromethane, and ethyl acetate fractions were dissolved in the same brine to get 2500 ppm of a stock solution by adding 1 drop Tween. These solutions were further diluted with brine to make 1000, 100, and 10 ppm of test solutions. To these solutions, 10 larvae were added and incubated for 24 hours under light conditions. After 24 hours, the living larvae were calculated. The procedure was triplicate to determine the LC_{50} by probit analysis using SPSS.

Phytochemical Screening

The most active fraction was screened its phytochemical components using various specific reagents. Mayer's and Dragendoff's reagents were used to identify the alkaloid compound which gives white and red precipitation as a positive result. Magnesium powder, HCl 1 M, and amyl alcohol were used to observe the flavonoid constituent which gives red color as a positive result. To identify saponin, the active fraction was shaken to form stable bubbles. The triterpenoid and steroid formed blue-green and red-violet after the addition of H_2SO_4 98% as a positive result. The tannin contents were identified as blue or green color after the addition of $FeCl_3$ 1%. Quinone was identified as red color after NaOH 1 M addition.

Alkaloid Extraction

The alkaloid content in *Peperomia pellucida* was separated by the same solvent that gives the highest cytotoxic activity against *Artemia salina* larvae. The alkaloid separation follows the same chromatography procedure as [13].

Alkaloid Identification

The separated alkaloid constituent was identified by LC-MS/MS. The elution used the reverse phase procedure with water and methanol as eluent. The molecule candidates matched to the library and elucidated base on its fragmentation.

Results and Discussion

The maceration process using ethanol resulted in 100 mL of crude extract. The fractionation steps of the chlorophyll-free crude extract follow the gradient of solvent polarity starting from n-hexane as the least polar to the more polar dichloromethane and ethyl acetate. Therefore, the least polar compounds such as triterpenoid will be dissolved and fractioned in n-hexane [15]. In contrast, dichloromethane and ethyl acetate are rich in non-polar to semi-polar and semi-polar to polar compounds, respectively.

The *Artemia salina* larvae lethality test can be used to preliminary evaluate the cytotoxic activity of plant extract. The cytotoxicity of n-hexane, dichloromethane, and ethyl acetate fraction are shown in Table 1. Based on the LC_{50}, all the fraction shows toxicity due to the LC_{50} value that less than 1000 ppm [16]. Ethyl acetate fraction exhibits the highest toxicity against *Artemia salina* larvae, among other fractions. The LC_{50} of n-hexane, dichloromethane, and ethyl acetate are 278.920, 60.303, and 39.665 ppm, respectively.

Table 1. The value of LC_{50} of n-hexane, dichloromethane, ethyl acetate fraction.

Fraction	LC_{50} (ppm)
N-hexane	278.920
Dichloromethane	60.808
Ethyl Acetate	39.665

The effect of the secondary metabolic constituents and the cytotoxic activity was evaluated by phytochemical screening. This method allows observing the secondary metabolite in ethyl acetate fraction that responsible for the cytotoxic activity. The result of phytochemical screening in the ethyl acetate fraction is shown in **Table 2**. The phytochemical screening shows that the ethyl acetate fraction contains alkaloid, flavonoid, steroid, and tannin, which revere these secondary metabolic are

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semi-polar. This result is in line with Riris et al. [17]. Various semi-polar secondary metabolic give different effects on the lethality of *Artemia salina* larvae. Alkaloids are predicted to act as a toxin to the larvae after the biotransformation process [18]. This report focus on identifying the alkaloid compounds in *Peperomia pellucida*. Ethyl acetate was used to extract and separate alkaloids in *Peperomia pellucida*.

Table 2. Phytochemical screening of ethyl acetate fraction

Secondary Metabolite	Result
Alkaloid	+
Flavonoid	+
Steroid	+
Triterpenoid	-
Saponin	-
Tanin	+
Quinone	-

The separated alkaloid constituents were eluted and analyzed using LC-MS/MS to identify the compound base on its molecular weight. The chromatogram of the separated alkaloid shows several peaks in **Figure 1**, which means there are still many compounds in the sample. Due to the minimum information about alkaloids in the Piperaceae family, the discussion only covers two compounds in this report.

Based on the analysis, the peak at 4.37 min has the ionic molecular base peak $[M+H]^+$ 286.1433 m/z, as shown in **Figure 2**. A similar result was also identified in another report [19]. Therefore, the molecular weight of this peak is identified as 285.1433 g/mol and predicted as Piperine. The prediction was supported by fragments 175 and 143 m/z [19]. The structure of Piperine is shown as an inset in **Figure 2**. The evidence of Piperine in the separated alkaloid is proven by the fragment match of mass spectroscopy in the library in **Table 3**. Piperine is also one of the chemical constituents in *Piper longum,* which is in the same family as *Peperomia pellucida* [20]. The chemotaxonomy can support the identification of Piperine in *Peperomia pellucida*.

Table 3. Fragment match of mass spectroscopy from retention peak of 4.37 min.

Component Name	Candidate 286,1433	Mass
m/z	286,1433	
Elemental Composition	$C_{17}H_{19}NO_3$	
i-FIT Confidence	100 %	
Common Name	Piperine	
Fragment Matches	3	

Figure 3 shows the spectrogram of the peak with the retention time of 4.94 min. This peak has an ionic molecular protonation $[M+H]^+$ with a base peak of 270.1120 m/z. The molecular weight of this peak is predicted as 269.1120 g/mol. Based on the

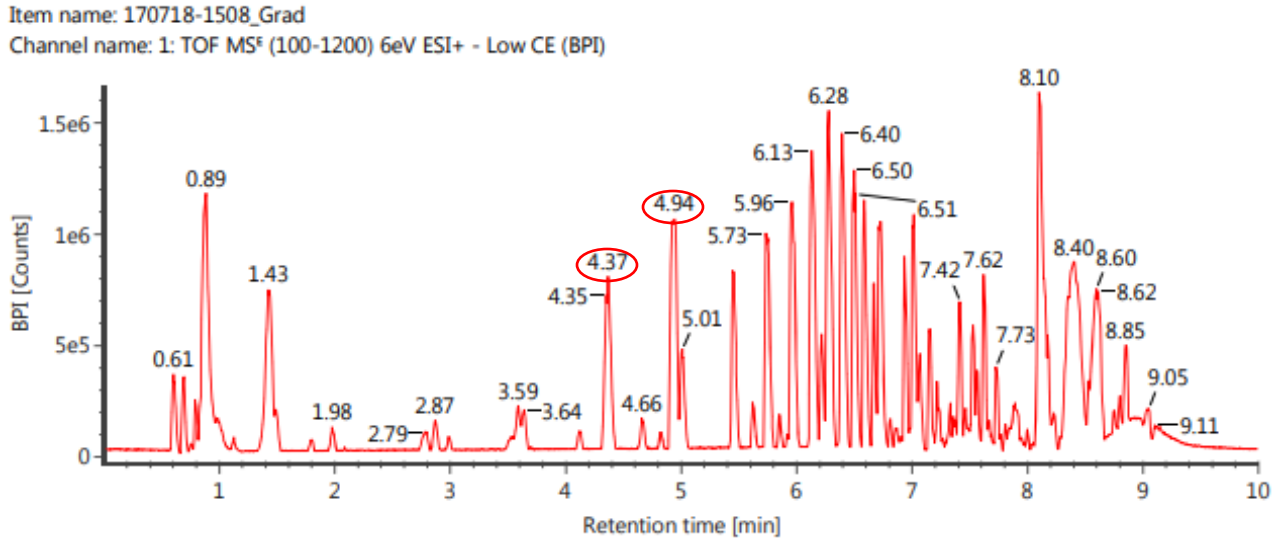

Figure 1. Chromatogram of separated alkaloid

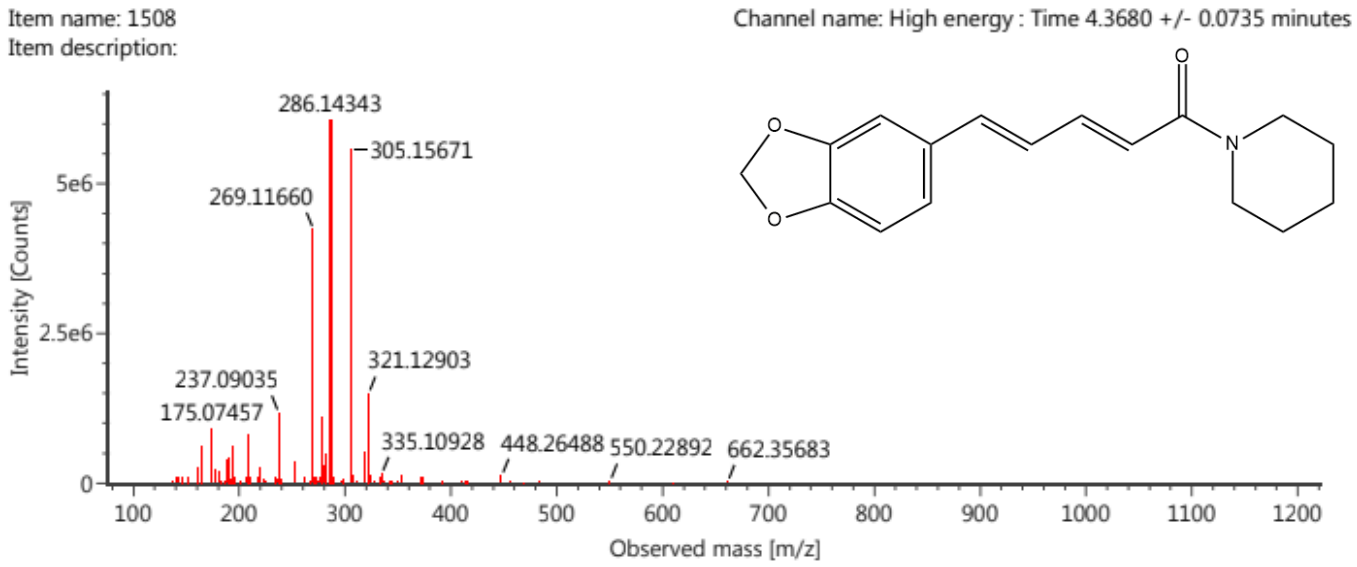

Figure 2. Spectrogram of the peak with retention time 4.37 min

chemotaxonomy approach, the molecule with 269.1120 g/mol is predicted as N-Bezoyl Phenylalanine. This compound has been identified in *Piper aurantiacum,* which belongs to the same family as *Peperomia pellucida* [21]. The structure of N-Bezoyl Phenylalanine is shown in **Figure 4**.

The structure of N-Benzoyl Phenylalanine with the carboxylate moiety is relatively more polar than Piperine; thus during the elution process using the reverse phase method, N-Benzoyl Phenylalanine should appear before Piperine. However, the chromatogram in **Figure 1** shows a different result. Piperine appeared before N-Benzoyl Phenylalanine with retention times are 4.37 min and 4.94 min, respectively. From the data can be inferred that the compound which appears 4.94 min might not be Benzoyl Phenylalanine.

At the retention time of 4.94 min, there is another probability of molecular ionic, which is $[M+H]^+$ 509.2585 m/z and thought to have a molecular weight of 508.2574 g/mol. An alkaloid compound with a molecular weight of 508.2574 g /mol is predicted as Nigramide N. This compound was successfully isolated from the *Piper nigrum,* which is in the same family as *Peperomia pellucida* [22].

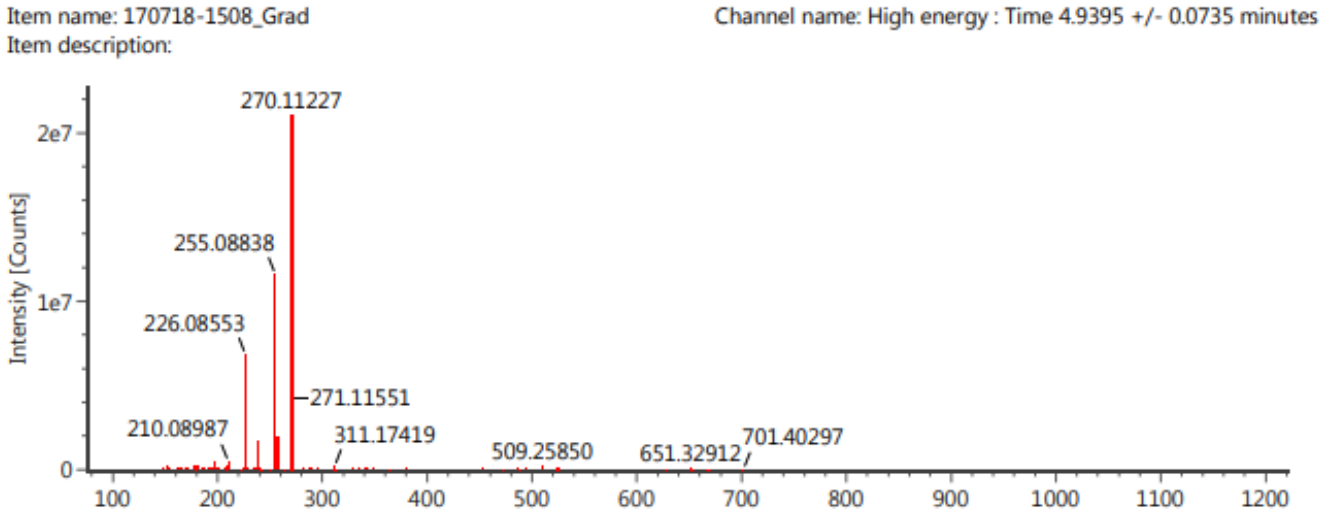

Figure 3. Spectrogram of the peak with a retention time of 4.94 min

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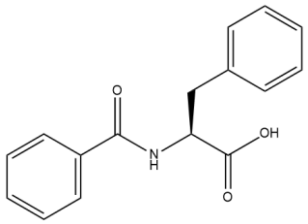

Figure 4. Structure of N-Benzoyl Phenylalanine

Nigramide N has a lesser polar character than Piperine, which preventing the compound to be eluted before Piperine. The non-polar character can be derived from the long alkyl chain and bulky structure of Nigramide N. Therefore, the compound with a retention time of 4.94 min is predicted as Nigramide N.

The ionic fragmentation with 270.1120 m/z is estimated a fragment of the Nigramide N. Nigramide N might be unstable, thus fragmented. One of the stable fragmentations is thought to have m/z 270.1122. Therefore, 270.1122 m/z is observed as a base peak in the spectrogram. The possibility of fragmentation of Nigramide N compounds is shown in **Figure 5**. Based on the analysis, the separated alkaloid contains Nigramide N.

Conclusions

The cytotoxic activity assay of n-hexane, dichloromethane, and ethyl acetate was successfully conducted. The ethyl acetate fraction shows high cytotoxicity with the LC_{50} value of 39.665 ppm. Hence, the less polar fraction exhibits lower cytotoxicity, n-hexane and dichloromethane have LC_{50} 278.920 and 60.808 ppm. In this study, two alkaloid constituents of *Peperomia pellucida* had been succesfully identified by using LC-MS/MS. Compound with retention time 4.37 min was identified as Piperine. Another alkaloid compound was identified as Nigramide N with a retention time of 4.94 min. Further research needs to be conducted to correlate between the alkaloid constituent in *Peperomia pellucida* and cytotoxic activity. Moreover, further purifications are needed to estimate the alkaloid constituents in *Peperomia pellucida*.

Conflicts of interest

There are no conflicts to declare

Acknowledgments

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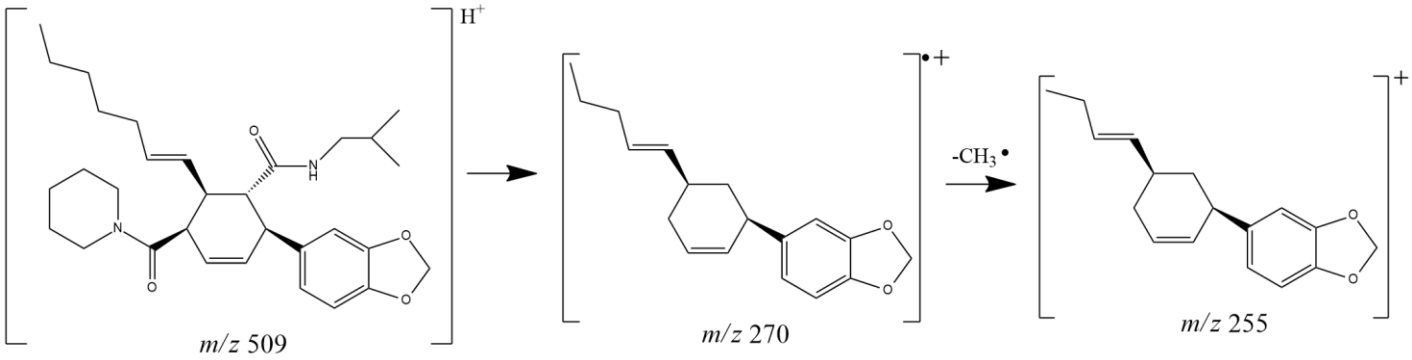

Figure 5. Predictions of Nigramide N fragmentation

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