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Isolation and Qualitative Test of Piperine from Black Pepper (Piper nigrum L.) Methanol Extract

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Abstract
Black pepper (*Piper nigrum* L.) is a spice commodity rich in alkaloids, in the form of piperine which gives a spicy taste and has various pharmacological activities such as anti-inflammatory, antioxidant, and anticancer properties. This study aims to isolate and perform qualitative validation of piperine compounds from black pepper fruit methanol extract through a series of phytochemical stages. Soxhlet extraction using methanol solvent yielded a viscous extract with a 10.3% yield. Furthermore, piperine separation was carried out by adding a 10% KOH solution in methanol to neutralize fatty acid and resin impurities, allowing neutral piperine to be separated. The purification stage through crystallization, washed with cold methanol, resulted in an isolate in the form of yellow needle-shaped crystals with a yield of 0.22%. Through phytochemical screening, the crystal was found to contain alkaloids because it gave a positive reaction to Mayer, Dragendorff, and Bouchardat reagents. Thin Layer Chromatography (TLC) analysis showed a single spot with an Rf value of 0.74, in accordance with literature standards. More specific identification using UV-Vis spectrophotometry showed maximum absorption at a wavelength of 342 nm, and melting point test results at 129.9°C matched literature values, confirming the purity of the piperine isolate. Therefore, the overall procedures proved effective for piperine isolation.

Keywords: Black pepper, Piperine, Isolation, Qualitative test

Introduction

Black pepper (*Piper nigrum* L.) is a spice that plays an important role in Indonesia's economy, where Indonesia is one of the largest pepper exporters in the world with a global demand reaching 29% (1). The high interest in black pepper is influenced by its chemical content, especially the alkaloid piperine, which is the main component and gives the characteristic spicy taste of black pepper. In addition to piperine, other chemical components of black pepper include pipericine and isopiperoleine (2).

Piperine is a mono cyclic prism-shaped alkaloid compound derived from alcohol with a melting point of 128-130°C. Piperine is also well soluble in ethanol and ether but almost insoluble in water (3). Analysis of piperine content in black pepper using HPLC shows levels of 3-6% (4). However, this content can vary due to many environmental factors that affect it, such as climate and the growing location (5).

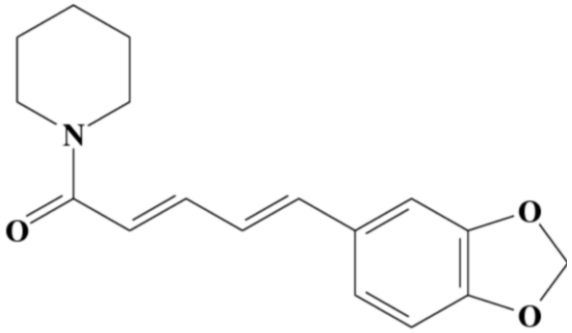

Figure 1. Chemical structure of piperine (6)

Piperine can vary by a few percent in black pepper fruits and directly affects the spiciness level and aromatic character. In addition to piperine, black pepper also contains essential oils, resins, and various secondary metabolites that provide a distinctive aroma as well as biological potential such as antioxidant activity. Its essential oil content is also responsible for the pepper aroma, with piperine as the main component of pepper (5). Piperine, as an alkaloid compound, is also thermostable because its boiling point is quite high, preventing the alkaloid groups in its structure from being damaged during heating (7).

Black pepper in traditional medicine is generally used as a remedy after childbirth when mixed with ginger and honey. Piperine from black pepper is claimed to have antihyperglycemic activity (8), anti-inflammatory (9), anticancer (10), antifungal, antimutagenic, protective effects on the stomach and liver (11), antioxidant (12), and others. Black pepper in the form of tea is also effective for relieving headache, sore throat, and digestive problems (5).

Based on the analysis results using the TLC-densitometry method (13), Soxhlet extraction is a more effective method than maceration for extracting piperine. Soxhlet extraction is a continuous extraction method where the sample is extracted by condensed solvent, making it more solvent-efficient than maceration, which requires a large amount of solvent (14). In addition, Soxhlet extraction can be used for thermolabile compounds (15). However, its drawback is that free radical compounds can be formed during solvent sonolysis, causing some sensitive sample materials to degrade due to oxidation (16). Therefore, in this study, the Soxhlet extraction method was chosen.

The aim of this research is to isolate piperine as the main compound from black pepper. This isolation process goes through stages of extraction, separation, purification, and crystallization, followed by a series of qualitative tests to validate the presence of piperine in the final crystals. In isolating secondary metabolite compounds, the choice of solvent used is one of the most important factors. The isolation of piperine using 96% ethanol or methanol is known to yield high purity (17). This underlies the use of methanol in this study.

Materials and Method

Research Materials

The materials used in this study include black pepper fruit simplicia, methanol (Lab Grade), chloroform, Mayer's reagent, Dragendorff's reagent, Bouchardat's reagent, 10% KOH in methanol, and a Dragendorff spray reagent.

Extraction of Black Pepper Fruit

The black pepper crude drug is ground using a blender and the powder is filtered using a sieve to separate it from impurities. Place 30 g of the weighed black pepper powder into each of 3 filter paper packages that have been sewn to form small bags. Perform Soxhlet extraction on the three bags with 1.3 L of methanol solvent until a clear extract is obtained. The obtained extract is then concentrated using a rotary evaporator and water bath to evaporate the solvent until a thick extract remains. Weigh the thick extract by subtracting the weight of the empty dish from the weight of the dish containing the thick extract, then calculate the % yield.

Testing of Extract Characteristics

1. **Organoleptic Test**
   Observe the shape, color, smell, and taste of the thick extract. Compare with the descriptions available in the literature.

2. **TLC Test**
   Mark the upper and lower boundaries about 1 cm from the edge with a pencil on a silica gel GF 254 plate as the stationary phase. Take a capillary tube and spot the thick extract dissolved with a little methanol at the lower boundary of the silica gel plate. Soak the silica gel plate in a chamber containing a small amount of chloroform and methanol eluent (5:5) that has been saturated with filter paper. Make sure the spots of thick extract are not submerged in the eluent.

If the solvent has climbed up to the upper limit of the plate lift, observe under UV light at 254 and 366 nm for spot marking. Spray the plate with Dragendorff's reagent to mark the spots, then heat using a water bath. Observe again under UV light at 366 nm and calculate the Rf value.

3. **Phytochemical Screening**
   Phytochemical screening is carried out on the alkaloid content of black pepper. Prepare 3 test tubes, each containing 0.5 grams of thick extract dissolved in 2 ml of methanol. The first test tube is given a few drops of Mayer's reagent. A positive reaction is indicated by the formation of a white or yellow precipitate (18)(19).

   The second test tube was given a few drops of Dragendorff's reagent. A positive reaction is indicated by the formation of a red or orange (brick red) precipitate. The third test tube was given a few drops of Bouchardat's reagent. The formation of a brown precipitate will indicate a positive reaction. The test sample can be declared positive for containing alkaloids if at least two positive reactions occur from these 3 tests (19).

4. **Identification of Maximum Absorption Wavelength Using UV-Vis Spectrophotometer**
   Dissolve a small amount of thick extract in methanol and perform testing on a UV-Vis Spectrophotometer in the range of 200-400 nm. Perform dilutions until an absorbance in the range of 0.2-0.8 is obtained. Observe the maximum wavelength that appears in the spectrum.

Separation with 10% KOH

Prepare a 10% (w/v) KOH solution by dissolving 10 g of KOH in 100 ml of methanol. Add 10% (w/v) KOH to the concentrated extract, stir until dissolved, and refrigerate until a precipitate forms. Once the precipitate has formed, separate the precipitate and the solution by decantation to collect the solution.

Purification by Crystallization

Store the solution obtained from the previous decantation step in the refrigerator until crystals form at the bottom of the container. Prepare cold methanol to wash the crystals, avoiding the use of room-temperature methanol so the crystals do not dissolve. Repeatedly wash with cold methanol until clear crystals are obtained. Weigh the crystals obtained.

Testing the Characteristics of Crystals

1. **Organoleptic Test**
   Observe the shape, color, smell, and taste of the obtained crystals.
2. **Purity Test with TLC**
   Follow the same steps as the TLC test with the concentrated extract. The sample used for spotting is a small amount of crystal dissolved in methanol. Once the solvent has traveled to the top limit, lift the plate. Observe under UV light at 254 and 366 nm for marking spots. Spray the plate with Dragendorff's reagent and then heat it using a water bath. Observe again under UV light at 366 nm and calculate the Rf values.

3. **Phytochemical Screening**
   Phytochemical screening was carried out on the alkaloid content of black pepper. Prepare 3 test tubes, each containing a small amount of crystals dissolved in 2 ml of methanol. Test for alkaloid content using the same procedure as was done on the previous thick extract.

4. **Identification of Maximum Absorption Wavelength Using a UV-Vis Spectrophotometer**
   Dissolve a small amount of crystals in methanol and test using a UV-Vis spectrophotometer in the range of 200-400 nm. Perform dilutions until absorbance is in the range of 0.2-0.8. Observe the maximum wavelength that appears in the spectrum.

5. **Melting Point Test**
   The melting point test is performed using a digital melting point apparatus with a heating rate of 50°C/min starting from 100-150°C. Use a capillary tube to take a small amount of crystal at the tip as a sample. Insert the capillary tube into the slot of the apparatus. Observe the sample through the magnifying lens on the instrument. Record the temperature when the sample melts.

Result and Discussion

In this study, soxhletation was used as the extraction method because it is considered more efficient and effective in terms of time and the amount of solvent used. This method has also been proven to isolate more piperine from pepper than the maceration method (20). Methanol is also a solvent commonly used in various black pepper extractions and is effective for isolating piperine compounds (21). The total number of soxhletation cycles carried out until a clear extract was obtained was 69 cycles.

The soxhletation method, as a hot extraction method, utilizes heating the solvent until it evaporates and then condenses again to continuously dissolve the sample, so there is no need to constantly use fresh solvent. These repeated cycles ensure that the active compounds can be optimally extracted from the sample matrix. The more cycles conducted, the greater the likelihood that the solvent will dissolve the target compound until the extraction reaches a near-equilibrium condition.

The extraction result was then concentrated by evaporating the solvent using a rotary evaporator equipped with a vacuum to accelerate the process. Further concentration was carried out on a water bath until a thick extract was obtained. From 90 g of black pepper fruit powder subjected to Soxhlet extraction, a yield of 10.3% was obtained. This yield value meets the standard for a good yield, which is more than 10%. This yield calculation serves as a measure of how optimally the secondary metabolites are carried by the solvent (22).

Figure 2. Thick Black Pepper Extract

Testing of Extract Characteristics

Organoleptic tests were carried out on the form, color, smell, and taste of the thick extract and were found to be in accordance with the literature. The observation results are presented in Table 1.

Table 1. Results of Organoleptic Test of Thick Extract

Characteristic	**Literature** (23)	**Observation**	**Conclusion**
Form	Thick extract	Thick extract	In accordance with literature
Color	Dark brown	Dark brown	In accordance with literature
Smell	Distinctive	Distinctive	In accordance with literature

Taste	Spicy	Spicy	In accordance with literature

The next test was carried out using TLC. A rapid separation time is one of the advantages of TLC. TLC has a larger capacity and its absorption system is good for separating hydrophobic compounds such as piperine (24). When the TLC plate was observed under both 254 nm and 366 nm UV light, a single spot with an Rf of 0.96 was visible. However, tailing was observed, indicating that the isolated compound was not yet pure and that other impurities or compounds were present. In addition, the Rf value also did not match the Rf value of piperine reported in the literature, which is 0.35 (23).However, this is reasonable because the sample was still a thick extract that had not undergone separation and purification, so piperine had not been purely isolated.

In the phytochemical screening, it was only carried out on the alkaloid content because piperine, as the target compound, is an alkaloid. The alkaloid test was conducted using three reagents, all of which showed positive results, indicating that the concentrated extract sample is positive for containing alkaloids (19).The results of the alkaloid screening of the black pepper concentrated extract are presented in Table 2.

Table 2. Results of Alkaloid Screening of the Thick Extract

Compound	Reagent	Observation	Conclusion
Alkaloid	Mayer	White precipitate formed	+
	Dragendorff	Orange precipitate formed	+
	Bouchardat	Brown precipitate formed	+

Alkaloid testing is based on the ability of an acidified extract to form an insoluble salt complex as a precipitate when mixed with certain precipitating reagents. Chemically, the precipitate occurs when the metal complex ions or bismuth/iodine salts contained in these reagents react with the alkaloid ions present in the test solution (25). A positive result from adding Mayer's reagent (containing potassium tetraiodomercurate (II)) produces a white precipitate formed due to the alkaloid ions reacting to form an insoluble coordination complex. Dragendorff's reagent (containing potassium tetraiodobismuthate) gives a positive reaction in the form of an orange to deep red precipitate. Meanwhile, the addition of Bouchardat's reagent (iodine solution in potassium iodide) shows a positive reaction if a brown precipitate forms (25,26).

When a concentrated extract sample was observed in the UV-Vis spectrum, the maximum wavelength was found at 212 nm with an absorbance of 0.715. This wavelength does not match the literature, which reports approximately 334 nm (23) to 343 nm (27). Other

peaks are also visible, indicating the presence of impurities, so further separation and purification are still required. The spectrum view can be seen in Figure 3.

Figure 3. UV-Vis Spectrum Results of the Concentrated Extract

Separation with 10% (w/v) KOH

The separation of piperine from the thick extract is carried out by dissolving it in 10% (w/v) KOH-methanol and left until a precipitate forms. Black pepper methanol extract not only contains piperine but also fatty acids, resins, and other impurities that are more acidic than piperine. KOH will neutralize the acidic compounds, forming potassium salts that easily precipitate in methanol solvent, whereas piperine is neutral (amide alkaloid) and does not react with KOH. Decantation is carried out to take the solution that has isolated piperine for use in the next stage. Impurities will be trapped in the precipitate (24).

Purification by Crystallization

The solution from the previous decantation stage is stored in the refrigerator until crystals form. The crystals are separated from the solution and washed with cold methanol to obtain pure crystals. This washing is repeated until the crystals become clear. Crystallization is one of the simplest and most effective methods for purifying compounds.

Crystal Characteristic Test

Organoleptically, the obtained crystals are needle-shaped and yellow in color, with a weight of 0.2 g. The crystal yield compared to the initial raw material weight is 0.22%. The yield size serves as an indicator of the effectiveness of the metabolites extracted from the sample. The crystal yield value is, of course, smaller than the yield of the concentrated extract.

Figure 4. Black Pepper Crystals

The TLC test using a chloroform and methanol eluate (5:5) yielded a single spot with an Rf value of 0.74. This Rf value resembles the Rf value of piperine crystals from the literature, which is 0.73 (24). This correspondence indicates that piperine was successfully isolated, based on the TLC results. The single spot also shows that there are no other impurities present. After spraying with Dragendorff's reagent, the spot became more visible under a 366 nm UV lamp, appearing blue in color.

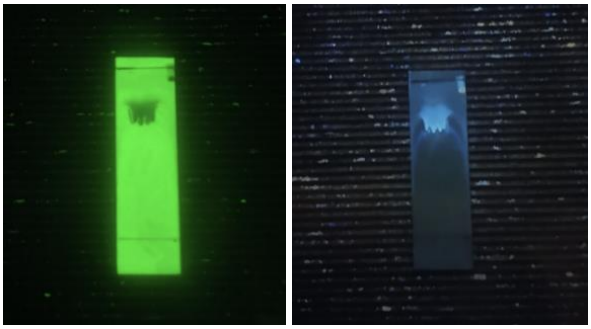

Figure 5. TLC test results of crystals under 254 nm UV lamp (left) and 366 nm UV lamp (right)

The next test used a UV-Vis spectrophotometer to identify the maximum wavelength in order to determine the presence of piperine. The maximum wavelength was found at 342 nm. This value is similar to the wavelength of piperine in methanol solvent from previous studies, which showed maximum absorption at wavelengths ranging from 334 nm (23) to 343 nm (27). In addition, no other significant peaks were observed in the UV-Vis spectrum, indicating that piperine was well isolated as a single, pure compound.

Finally, a melting point test was conducted on the crystals. Based on visual observation, the crystals began to melt at 129.9°C. This melting point is within the range of piperine melting points reported in several journals, i.e., 128-130°C (3,28–30). This agreement indicates the presence of pure piperine isolate in the crystals.

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Research Article
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Figure 6. UV-Vis Spectrum Results of Black Pepper Crystals (Bottom Graph)

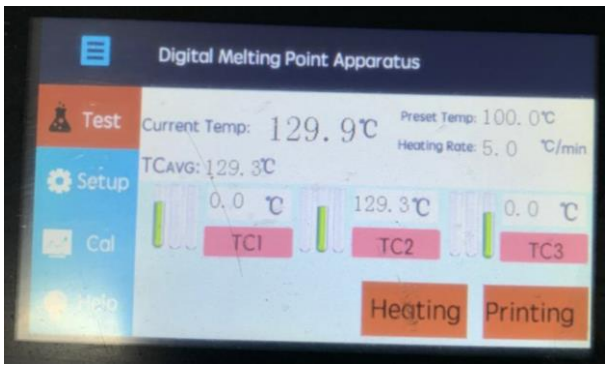

Figure 7. Crystal melting point test results

Conclusion

Based on the results of organoleptic tests, Rf values from thin-layer chromatography (TLC), alkaloid phytochemical screening, maximum wavelength in the UV-Vis spectrum, and the melting point of the crystals, the presence of piperine compounds in the crystals is indicated. This statement is made because the results obtained resemble those reported in previous literature. Therefore, Soxhlet extraction of black pepper using methanol, separation with 10% KOH, and purification through crystallization is effective for isolating piperine compounds.

Conflict of Interest Statement

The authors declare no conflict of interest related to this work.

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