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Development of New Isolation and Quantification Method of Piperine from White Pepper Seeds (*Piper Nigrum* L) Using A Validated HPLC

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ABSTRACT

The majority of active pharmaceutical ingredients in pharmaceuticals are obtained from natural ingredients, one of which is white pepper. White pepper seeds (*Piper nigrum* L.) contain an alkaloid compound named piperine, which has a broad spectrum of pharmacological effects. This research aimed to isolate the piperine from white pepper seeds to obtain piperine in a more economical, simple, and effective way so that it can be applied to large-scale production in the pharmaceutical industry. Extraction and purification of piperine were carried out using n-hexane and cyclohexane, respectively. The isolated piperine samples were tested for their purity with a set of melting point apparatus, bidimensional TLC, and HPLC. The compound structure was then analyzed using FTIR and NMR spectroscopy. Cyclohexane was successfully used to remove the resinous matter from the n-hexane extract of white pepper to produce piperine with 94.57 ± 0.82% of purity. The results of the melting point measurement, FTIR, ^{1}H-NMR, and ^{13}C-NMR spectra of the isolated piperine were similar to that of the piperine reference substance and agreed with data found in the literature.

Keywords: white pepper, piperine, HPLC, NMR

INTRODUCTION

Indonesia is a tropical country with rich plant diversity. There are around 30,000 plants in Indonesia, and approximately 3,000 of those have been reported to have medicinal benefits. Still, only a few Indonesian plants have been used in the industry of traditional medicines. One of such plants is white pepper (*P nigrum*). It contains essential oils (1-2.5%) and alkaloids (5-9%), of which the major constituents are piperine (1.7-7.4%), chavicine, and piperidine. (Khan *et al*., 2010)

Piperine has many pharmacological benefits, including for the treatment of pain during menstruation, tuberculosis, sleep disorders, respiratory infections, and arthritis, as well as anticonvulsant, antiulcer, anti-inflammatory, antibacterial, antioxidant, anticancer, antidepressant, and antiplatelet aggregation (Ee *et al*., 2010; Han *et al*., 2008; Koul and Kapil, 1993; Li *et al*., 2007; Mujumdar *et al*., 1990; Srinivasan, 2007). For example, piperine can be used to treat osteoarthritis by restraining the activity of enzymes responsible for the synthesis of leukotriene and prostaglandin, NO (Nitric Oxide), and TNF-*α* (tumor necrosis factor-α) (Umar *et al*., 2013). Several studies have also reported that piperine significantly inhibits the activation of NF-*κ*B induced by IL-1β, as well as inhibiting COX-2, PGE2, iNOS, NO, and MMPs that play a role in osteoarthritis (Li *et al*., 2017; Ying *et al*., 2013).

Currently, efforts to isolate piperine compounds have been widely reported. However, methods to obtain pure compounds require complicated, ineffective steps, and lack economic value, especially when produced on a large scale. So far, the most widely used method of isolating piperine compounds is by using ethanol, followed by washing using potassium hydroxide to remove resinous matter (Chaudhri, 2017; Rahman *et al*., 2011; Saha *et al*., 2014). However, the use of

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potassium hydroxide can catalyze the hydrolysis of piperine to piperidine and piperic acid (Ikan, 2013). Therefore, further studies into the more appropriate method for isolating piperine on a large scale are still needed.

This study aimed to isolate piperine from white pepper seeds in a simple, economical, and effective manner by avoiding the alteration of the active substance. In this study, isolation of piperine was carried out using the Soxhlet method to minimize the amount of solvents used for the extraction. The solvent selection approach was based on piperine's non-polarity, which was easier to extract using non-polar solvents.

MATERIALS AND METHODS

Instrumentation

The instruments used in the research include a set of Soxhlet extractors, melting point apparatus (Stuart Scientific, UK), UV254 lamp, FTIR spectrophotometer (Thermo Scientific Nicolet iS10), HPLC Hitachi L-2420 UV-Vis detector with Luna® 5µM C18 100 Å LC Column 250 x 4.6mm Phenomenex, and NMR spectrophotometer (JNM-ECZ500R, 500MHz Super Conductive Magnets).

Materials

White pepper was collected from farmers in Sorowako, South Sulawesi. Piperine reference compound was obtained from E.Merck, China, with a purity of ≥98%. Silica gel 60 F254 plates for thin-layer chromatography (TLC) were obtained from E.Merck, Germany. All pro analysis solvents such as methanol, ethanol, n-hexane, cyclohexane, ethyl acetate, acetic acid were obtained from E.Merck.

Extraction and purification of piperine

White pepper powder was extracted using Soxhlet apparatus and n-hexane in the first method and ethanol in the second one. The extract was tested on a silica gel TLC plate with n-hexane – ethyl acetate (7:3) as a mobile phase to choose the best extraction method by comparing TLC profiles of the extracts to that of the piperine reference substance. The selected menstruum was evaporated until only half of the volume left and then stored in the refrigerator to form crystals, which were then referred to as the semi-purified white pepper fraction. After the filtration process, the obtained crystals were washed by cyclohexane. The insoluble cyclohexane fraction was dried at 25^{o}C and tested by TLC, HPLC, and melting point measurement. The compound structure was analyzed using FTIR and NMR spectroscopy.

Melting point

The melting point of isolated piperine was measured using a set of melting point apparatus. A melting point test was carried out on isolated piperine and piperine reference substances to compare the obtained results.

Bidimensional Thin-Layer Chromatography

The isolated piperine in the methanolic solution was spotted on TLC silica gel 60 F254 plates. It was first eluted with n-hexane – ethyl acetate (9:5) and then with cyclohexane – chloroform (1:4). The chromatogram was visualized under UV 254 and 366 nm.

FTIR spectroscopy

FTIR spectroscopy test was carried out to determine types of chemical bonds of sample constituents in the range of wavelengths of 400-4000 cm^{-1}. FTIR testing was performed using an Mb3000 FTIR spectrophotometer combined with the intuitive Horizon MBTM FTIR software. The samples, isolated piperine, and piperine reference substances were prepared beforehand in a 1% KBr powder mixture and followed by pressing into transparent slices for analysis.

Purity test with HPLC

The purity test of isolated piperine was carried out using HPLC Hitachi L-2420 with isocratic elution, UV detector at 340 nm, a mobile phase of methanol: water (75:25), stationary phase Luna® 5 µM C18 100 Å LC Column 250 x 4.6 mm Phenomenex, flow rate 1mL/min, column temperature of 25°C, and sample injection of 20µL (Chithra *et al*., 2014; Hamrapurkar *et al*., 2011). Samples were dissolved in methanol.

NMR Test

^{1}H and ^{13}C NMR spectra were obtained from a test using NMR JNM-ECZ500R spectrophotometer, 500MHz Super Conductive Magnet. Samples were dissolved in deuterated methanol (CD3OD) containing trace amounts of tetramethylsilane and then inserted into the NMR spectrometer. The spectra of isolated piperine were compared to those of the reference substance.

RESULT AND DISCUSSION

Thin Layer Chromatographic Profiles of Extract Obtained by Extractions

The thin layer chromatographic profile of extract obtained by extraction with n-hexane and that with ethanol (Figure 1). The TLC profile (Figure 1) shows that the n-hexane extract contained impurities only in the upper part of the piperine spot, while the ethanolic extract might contain both non-polar substances and more polar substances in the lower part of the piperine spot. This is due to the nature of the n-hexane solvent which is non-polar and suitable to attract non-polar compounds. Meanwhile, ethanol solvents contain polar and non-polar groups, suitable for extraction of polar and non-polar compounds, especially compounds that have hydroxyl groups (Do *et al*., 2014; Escorsim *et al*., 2018). The ethanolic extract contained more piperine than that of the n-hexane with the same duration of extraction. Based on the TLC profiles (Figure 1) of the ethanolic and n-hexane extract, the ethanolic extract produced the same spot with more concentration in comparison to the n-hexane extract. This shows that the ethanolic extract contained more piperine than the n-hexane extract because the penetration power of ethanol was stronger than the n-hexane (Baümler *et al*., 2016). The amount of piperine in the n-hexane extract could increase and achieve the same level as those of the ethanolic extract if the duration of extraction is increased three times longer than the latter (Hamrapurkar *et al*., 2011; Khan *et al*., 2017; Subramanian *et al*., 2016). On the other side, the elimination of polar substances in the ethanolic extract may be performed by fractionation using n-hexane, but it would take more extraction steps. The elimination of polar substances in the ethanolic extract can also be achieved by evaporation of solvent followed by the addition of potassium hydroxide alcoholic solution. The resinous material will be precipitated. This last-mentioned procedure can only be performed on a laboratory scale due to the inconvenient use of potassium hydroxide on a large scale, which will cause environmental pollution. Based on the above-mentioned conditions, the n-hexane extraction was the best method for piperine extraction. In this research, the extraction with Soxhlet apparatus was performed on 300g white pepper powder, and after the removal resinous matter with cyclohexane yielded 2.49g of isolated piperine or 0.83% yield. The organoleptic properties of the isolated piperine were crystalline powder, yellowish-white, odorless, and pungent taste.

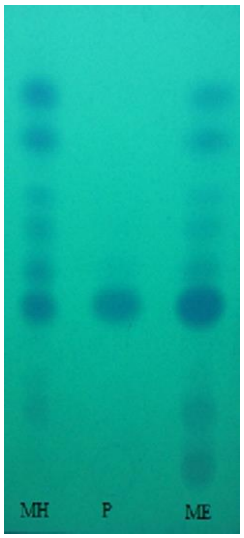


Figure 1. TLC profile of white pepper extracts. Stationary phase: Silica gel F254, Mobile phase: n-Hexane:Ethyl acetate (7:3), Detection: UV_{254} . MH: *n*-Hexane extract, ME: Ethanolic extract, P: Piperine reference substance.

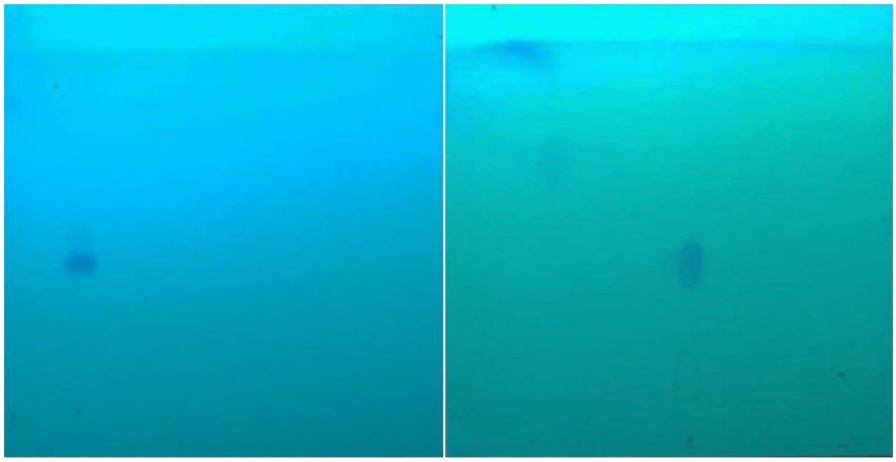
Identification Isolated Crystal

The isolated crystal from *P. nigrum* and piperine reference substance had melting points of 123-124°C and 123-123.8°C, respectively. It can be concluded that the isolated crystal had similar properties to the piperine reference substance obtained from E.Merck.

Bidimensional Thin-Layer Chromatography

The bidimensional TLC aimed to ensure the purity of the isolated piperine using the chromatogram results. The test was expected to produce one spot on the first elution and the second elution using a different mobile phase system (Figure 2).

The results of the isolated piperine with bidimensional TLC (Figure 2) shows that the first elution produced one spot with an Rf value of 0.42, while the second elution produced one spot with an Rf value of 0.38. Based on the results of the purity test using bidimensional TLC, it can be concluded that the isolated piperine obtained was pure.



(a) (b)

Figure 2. The results of bidimensional TLC under UV 254 nm (a) eluted with n-hexane-ethyl acetate (9:5) (b) eluted with cyclohexane-chloroform (1:4)

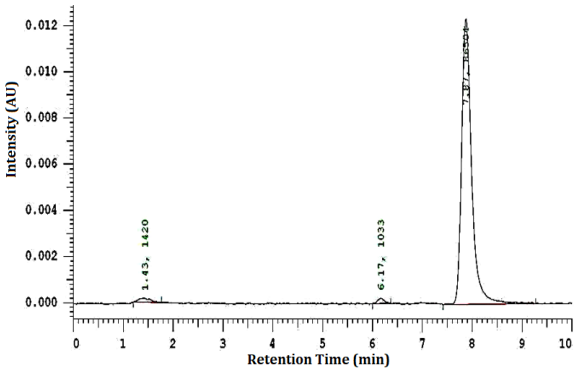


Figure 3. Chromatogram of isolated piperine at 340 nm

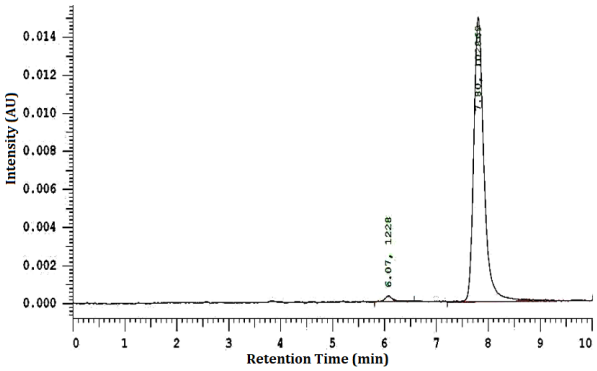


Figure 4. Chromatogram of piperine reference sub stance at 340 nm

Purity test using HPLC

The isolated piperine from hexane extract was analyzed and identified by HPLC using a previously validated method (y=148.11x+8208.6, r ≥ 0.9999). Pure piperine was used as a reference for identification using HPLC. The obtained results (Figure 3) showed one peak corresponding to the isolated piperine in comparison to the reference substance (Figure 4), which appeared at the same retention time (7.88min), and the percent area of isolated piperine and piperine reference substance were 97.24% and 98.76%, respectively. Meanwhile, the peak that was apparent at 6.10 minutes was possibly piperic acid compounds, which were more polar than piperine compounds (Zarai *et al*., 2013). According to the quantification results using the validated analytical method, the isolated sample contained 94.57 ± 0.82% piperine.

FTIR analysis

FTIR analysis was performed to prove that the isolated piperine and the piperine reference substance have similarities in their spectra to confirm the functional groups and determine the molecular structure of the isolated piperine. The peak positions and functional groups in the piperine molecule (Figure 5) (Table I). The peak positions and interatomic bonds of the piperine structure are as follows: the peaks at 2800-3000, 1635, 1495-1589, 1030-1257, and 1134 and may indicate the presence of CH functional groups, C-H2, O=C-N, C=C, =C-O-C, and C-O-C, respectively (Shingate *et al*., 2013; Silverstein and Bassler, 1962). The similarity proved that the isolated piperine has a similar structure to the reference substance.

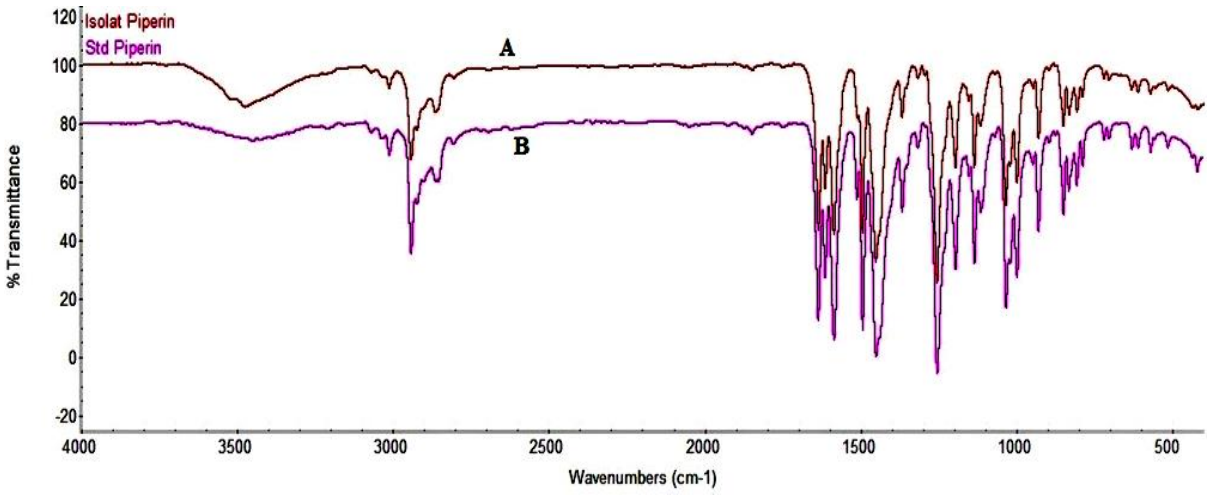


Figure 5. FTIR spectra of isolated piperine (A) and piperine reference substance (B)

Table I. The comparison of IR spectra of isolated piperine and piperine reference substance

Type of phenomenon	**piperine reference substance IR values**	**Isolated piperine IR values**
Aromatic C-H stretching	3008.95	3008.95
Symmetric and asymmetric stretching of C=C diene	1635.64	1635.64
Aromatic stretching of C=C (benzene ring)	1589.34	1581.63
	1495	1495
Stretching of –CO-N	1635.64	1635.64
Asymmetric and symmetric CH2 stretching, aliphatic C-H stretching	2939.52	2939.52
	2854.65	2862.36
CH2 bending	1442.75	1442.75
Asymmetrical stretdhing =C-O-C	1257.59	1257.59
	1195.87	1195.87
Symmetrical stretching =C-O-C	1033.85	1033.85
C-O stretching in-plane bending of phenyl C-H	1134.14	1134.14
C-H bending of trans –CH=CH-	995.27	995.27
Out-of-plane C-H bending 1,2,4- trisubstituted phenyl (two adjacent hydrogen atoms)	848.68	848.68
	802.39	802.39

NMR analysis

NMR analysis was carried out to determine the chemical structure and the purity of the isolated compound. Structural elucidation results were obtained based on NMR analysis, while the purity of the piperine compound (Figure 6) was confirmed by ^{1}H and ^{13}C-NMR spectral data.

The results of the ^{13}C-NMR spectrum of the isolated piperine (Figure7) showed that there were 17 C atoms with the following signals: δ 25.71 (C-13), δ 27.05 (C-14), δ 28.04 (C-14), δ 44.70 (C-15), δ 48.28 (C-15), δ 102.87 (C-12), δ 106.83 (C-7), δ 109.83 (C-9), δ 120.79 (C-2), δ 126.56 (C-8), δ 140.319 (C-5), δ 132.57 (C-6), δ 124.04 (C-4), δ 144.74 (C-3), δ 149.92 (C-10), δ 149.95 (C-11), and δ 167.89 (C-1) (Table II). The signal of methanol solvent was observed around 48.64 - 49.67 ppm. All ^{13}C-NMR values were similar to the previous studies (Alves et al., 2019). The use of different solvents in the NMR analysis in the previous studies (Alves *et al*., 2019) gave a slight shift in the ^{13}C-NMR signals produced. Signal peaks in the range of 0-50 ppm represented carbon-carbon single bonds, peaks in the range of 101 ppm represented carbon-oxygen atoms in the molecule.

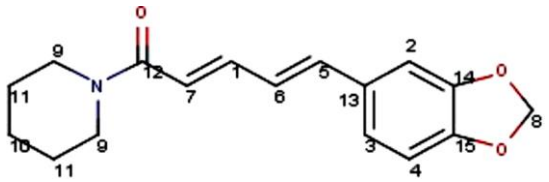


Figure 6. The molecular structure of isolated piperine

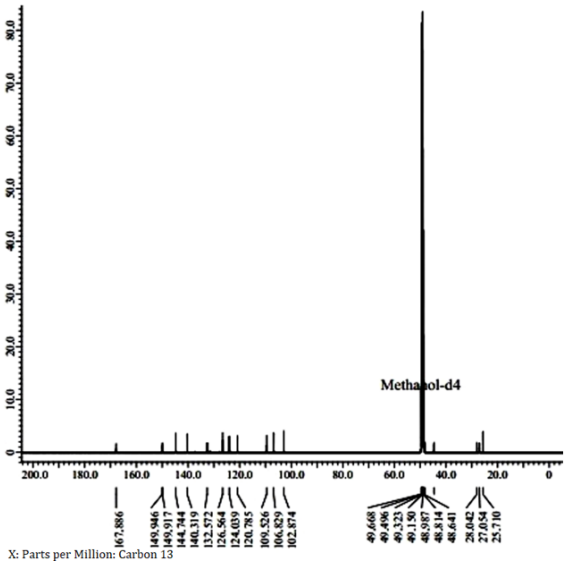


Figure 7. ^{13}C-NMR spectrum of isolated piperine

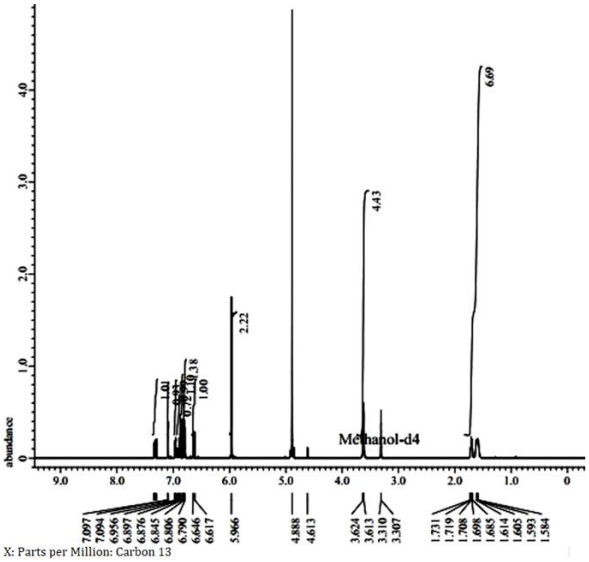


Figure 8. ^{1}H-NMR spectrum of isolated piperine

Table II.^{13}C-NMR and ^{1}H-NMR spectra of the isolated piperine

No Atom	^{13}C-NMR δ(ppm)	^{1}H-NMR δ(ppm)
13	25.710	1.584 – 1.614 , multiplet, 2H
14	27.054	1.685 – 1.731 , multiplet, 2H
14	28.042	1.685 – 1.731 , multiplet, 2H
15	44.696	3.613 – 3.635 , multiplet, 2H
15	48.277	3.613 – 3.635 , multiplet, 2H
12	102.874	5.966 , singlet, 2H
7	106.829	7.094-7.097 , multiplet, 1H
9	109.526	6.845 , doublet, 1H
2	120.785	6.617 , doublet, 1H
8	126.564	6.876, doublet, 1H
5	140.319	6.806 , multiplet, 1H
6	132.572	-
4	124.039	6.790 , multiplet, 1H
3	144.744	7.296 – 7.347 , doublet of doublet, 1H
10	149.917	-
11	149.946	-
1	167.886	-

Peaks in the range 100-150 and 167.89 corresponded to carbon-carbon double bonds and carbon-oxygen double bonds, respectively

The ^{1}H-NMR spectrum of the isolated piperine (Figure8) showed 19 protons with the following signals: δ 1.599 (2H, m, H-13), δ 1.708 (2H, m, H-14), δ 3.624 (2H, m, H-15), δ 5.966 (2H, s, H-12), δ 7.0956 (1H, m, H-7), δ 6.845 (1H, d, H-9), δ 6.617 (IH, d, H-2), δ 6.876 (1H, d, H-8), δ 6.806 (1H, d, H-5), δ 6.790 (1H, m, H-4), and δ 7.3215 (1H, ddd, H-3). The methanol solvent signal was observed around 3.31 ppm and 4.88 ppm. The ^{1}H-NMR piperine signal was similar to the previously reported NMR of the piperine compound (Alves *et al*., 2019).

CONCLUSION

Cyclohexane has been successfully used to remove resinous matter from the n-hexane extract of white pepper in the production of isolated piperine with 94.57 ± 0.82% of purity. The melting point, ^{1}H-NMR, and ^{13}C-NMR spectra of the isolated piperine were similar to those of piperine reference substances in the literature. The simple isolation method and the technical grade solvent used in this study open up the possibility to apply the method for industrial scale production. Moreover, the isolated piperine can potentially be used as an active pharmaceutical ingredient (API) in pharmaceutical dosage forms.

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