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Determination of the Potential Antioxidant Activity of Isolated Piperine from White Pepper Using DPPH, ABTS, and FRAP Methods

Nindya Kusumorini[1], Akhmad Kharis Nugroho[2*], Suwijiyo Pramono[3], Ronny Martien[2]
[1] Doctoral Program in Pharmaceutical Science, Faculty of Pharmacy, Universitas Gadjah Mada
[2] Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada
[3] Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada
Corresponding author: Akhmad Kharis Nugroho: Email: a.k.nugroho@ugm.ac.id
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ABSTRACT

White pepper (*Piper nigrum L*) is a native plant of Indonesia that has been used for generations as a spice and traditional medicine. White pepper contains the main alkaloid compound, namely piperine which has antioxidant activity as shown in the previous studies. The purpose of this study was to determine the potential antioxidant activity of isolated piperine compared with n-hexane extract of white pepper using DPPH, ABTS, and FRAP test methods. The isolated piperine was obtained from extraction using Soxhlet with n-hexane as a solvent, followed by purification with cyclohexane. The evaluation of antioxidant activity was carried out using the DPPH method to see free radicals, while the ABTS and FRAP methods evaluated antioxidant activity. Antioxidant activity was expressed as IC_{50} value and Trolox compound was used as a positive control of antioxidant activity. The analysis results show isolated piperine had antioxidant activity in the ABTS test with the results of 4.35 ± 0.004 mg/mL and 2.53 ± 0.08 mg/mL, respectively and FRAP test was 10.53 ± 0.06 mol TE/g sample and 6.86 ± 0.08 mol TE/g sample, respectively. Isolated piperine and n-hexane extract of white pepper did not show their activity as free radical scavengers. The antioxidant activity of isolated piperine was lower than that of n-hexane extract of white pepper. The presence of other compounds in white pepper shows a synergistic interaction in increasing the antioxidant activity of white pepper extract.

Keywords: white pepper; piperine; antioxidant; DPPH; ABTS; FRAP

INTRODUCTION

Free radicals are a trigger for cell damage in the body that can cause various diseases. Cells in the body can be damaged due to the oxidation process caused by chemical compounds obtained from the environment, such as air pollution, chemicals, alcohol, and cigarette smoke (Purwantiningsih et al., 2019). Antioxidant compounds inhibit oxidation reactions by breaking the chain reaction and turning it into a more stable product (Kumar and Pandey, 2013). All aerobic organisms have antioxidant defense systems to counterbalance the harmful effects caused by free radicals. However, the amount is limited. It is necessary to add antioxidant compounds from outside sources. Antioxidant compounds can be found naturally in fruits, vegetables, and spices (Kedare and Singh, 2011). It can also be found in the form of synthetic antioxidants such as butylated hydroxyanisole (BHA), butylhydroxytoluene (BHT), tertiary butyl hydroquinone (TBHQ), and propyl gallate (PG) (Koksal and Gülçin, 2008; Litescu et al., 2011). However, the use of synthetic antioxidants must be limited because they are toxic. Therefore, previous studies on antioxidant activity have been developed in various plants and their parts.

Biodiversity in Indonesia has the potential as a natural antioxidant, one of which is white pepper (*Piper nigrum* L). Various studies have shown that the compounds contained in white pepper have antioxidant activity (Gülçin et al., 2004; Nahak and Sahu, 2011; Vijayakumar et al., 2004; Vijayakumar and Nalini, 2006; Zarai et al., 2013). Phytochemical screening of aqueous extracts, ethanol extracts, and methanol extracts of white pepper revealed the presence of various secondary metabolites, including alkaloids, glycosides, terpenoids, steroids, flavonoids, tannins, and anthraquinone compounds (Nahak and Sahu, 2011) which are thought to play an important role in the antioxidant activity of white pepper.

Various studies on the antioxidant activity of white pepper extracts such as water

extract, ethanol extract, and methanol extract have been carried out, but the determination of the antioxidant activity of single compound piperine from white pepper using DPPH, ABTS, and FRAP methods has not been carried out. This study was intended to determine the antioxidant activity of piperine isolated from an n-hexane extract of white pepper using the DPPH method for free radical reduction and the ABTS and FRAP methods to determine antioxidant activity. In this study, Trolox was a positive control. Trolox is an antioxidant that is synthesized from a vitamin E derivative that is easily soluble in water. Trolox is widely used as a comparison compound in various antioxidant tests.

MATERIALS AND METHODS

Materials

Isolated piperine (purity ≥95%) was isolated from white pepper (Sorowako, South Sulawesi). Piperine reference substance was obtained from E. Merck, China, with a purity of ≥ 98%. All pro analysis solvents such as methanol, ethanol, n-hexane, cyclohexane, acetic acid, and hydrochloric acid were obtained from E. Merck. All reagents for antioxidant assays, such as DPPH (1,1-diphenyl-2-picrylhydrazyl), ABTS (2,2'-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt), TPTZ (2,4,6-tri(2-pyridyl)-s-triazine), and Trolox were obtained from Sigma Aldrich, while the $K_2S_2O_8$, $FeCl_3.6H_2O$, and $FeSO_4.7H_2O$, reagents were obtained from E. Merck.

Instrument

The instruments used in this study were a set of Soxhlet apparatus and UV-Vis spectrophotometer (UH5300, Hitachi).

Methods

Preparation of white pepper powder

White pepper fruit that had been dried in an oven for 24 hours was finely grounded with a blender and then the resulting powder was sieved with a size of 40 mesh.

Extraction and purification of piperine compounds

White pepper fruit powder was extracted using a pro-analytical hexane solvent using the Soxhlet method by placing as much as 120 g of dry powder simplisia in the Soxhlet tube. The Soxhlet tube was placed between the round bottom flask and the cooler. The extraction process begins by pouring the solvent through the top of the Soxhlet apparatus as much as 500 mL and the heater was turned on. The extraction process was stopped after the circulating solvent changed color from yellow to colorless. Furthermore, the filtrate was then evaporated at room temperature up to half and stored in the refrigerator until crystals were formed. The crystals formed were filtered and dried at room temperature of 25°C. The yellow crystals formed were washed using cyclohexane, then the insoluble crystals were dried at room temperature of 25°C. The results obtained were isolated piperine with a purity of 95% and had been published previously (Kusumorini et al., 2021).

Antioxidant Activity Test:

DPPH Assay

The antioxidant activity of the sample is carried out by mixing 1.0 mL DPPH 0.4 mM and the test sample of 1.0 mL at a concentration of 100, 200, 300, 400, 500, 1000 µg/mL and adding ethanol to 5.0 mL, left at temperature room (25°C) for 30 minutes in a dark place (Floegel et al., 2011). Absorbance was determined after 30 minutes and read at λ 517 nm. Three replications were performed for antioxidant determination. Free radical scavenger activity was calculated from the difference in absorbance of the test solution and the control solution containing DPPH and ethanol, calculated by the formula $(1 - \frac{As}{Ac}) \times 100\%$, where As is the absorbance of the sample and Ac is the absorbance of the control. Then, the IC50 value was determined using a linear regression equation.

FRAP Assay

The FRAP method was determined using a modification of the test method of ferric reducing/antioxidant power (FRAP) (Benzie & Strain, 1996). The FRAP reagent contained

2.5 mL of 10 mM tripydyltriazine reagent (TPTZ) solution in 40 mM HCl, 2.5 mL of 20 mM of $FeCl_3.6H_2O$ solution and 25 mL of 0.3 M acetate buffer pH 3.6. The newly made FRAP reagent was reacted with 3 mL FRAP with a 1 mL sample and incubated at 37°C for 30 minutes. Then read the absorbance at a maximum wavelength of 596 nm with a UV-Vis spectrophotometer. The blank solution used

contained water incubated at 37°C for 30 minutes. $FeSO_4.7H_2O$ and Trolox standard solutions were made for the manufacture of standard curves, which were then used to determine the equivalent antioxidant capacity of Trolox (Shui and Leong, 2006).

ABTS Assay

$ABTS^{\bullet+}$ solution was prepared by reacting 5 mL of 7 mM ABTS solution in water and 88 µL of 140 mM (final concentration 2.45 mM) of potassium persulfate ($K_2S_2O_8$) solution in water. Subsequently, the ABTS and $K_2S_2O_8$ solutions mixture were left for 12-16 hours at room temperature (25°C) to produce blue $ABTS^{\bullet+}$solution. The $ABTS^{\bullet+}$ solution was then dissolved in deionized water to produce an absorbance of 0.7 ± 0.05 at λ 734 nm. The antioxidant activity of the sample was determined by mixing 1.0 mL of the sample in 2 mL of $ABTS^{\bullet+}$solution and allowed to stand at room temperature for 6 minutes in a dark place (Mareček et al., 2017). Free radical capture activity was determined by the formula $(1 - \frac{As}{Ac})$ x 100%. Then, the IC50 value was determined using a linear regression equation.

Statistical Analysis

All data were expressed as mean ± standard deviation (SD). The results of DPPH and ABTS test expressed in IC50 values which were analyzed using a linear regression equation. Computerized data described statistically with Microsoft Excel v.10.0 (Microsoft, USA). Antioxidant abilites are categorized into 4, namely the strongest antioxidants with IC50 value of <0.05 mg/mL, a strong antioxidant with IC50 value of 0.05-0.1 mg/mL, a moderate antioxidant with IC50 value of 0.1-0.15 mg/mL, and weak antioxidant with IC50 value of > 0.15 mg/mL.

RESULT AND DISCUSSION

In previous studies, aqueous extract, ethanol extract, and methanol extract of white pepper (*Piper nigrum* L) showed their activity as antioxidants. However, research on single compounds that play a role in antioxidant activity is still limited. In this study, researchers determined the antioxidant activity of the main compound in white pepper, namely piperine and n-hexane extract of white pepper using the DPPH, ABTS, and FRAP methods in vitro. The method is considered quite simple, easy, fast, and only requires a UV-Vis spectrophotometer.

DPPH radical scavenging activity

DPPH (1.1 diphenyl-2-picrylhydrazyl) is a stable free radical with absorbance characteristics at 517 nm. It is one of the most commonly used substrates to evaluate antioxidant activity (Yamaguchi et al., 1998). The working principle of the DPPH method is the donation of hydrogen atoms or electrons from antioxidant compounds that bind to free electrons in radical compounds, causing a change from free radicals (diphenyl-picrylhydrazyl) to non-radical compounds (diphenyl-picrylhydrazine) (Zarai et al., 2013). Free radical DPPH (DPPH•) at the maximum wavelength (λmax 515 – 517 nm) can easily accept electrons or hydrogen atoms from antioxidant compounds into non-radical molecules (Soare et al., 2009). In determining antioxidant activity using the DPPH method, IC50 is used, i.e., the concentration of the sample required to capture DPPH radicals as much as 50%.

In this study, the results of the antioxidant activity test using the DPPH method show that the piperine compound was found to be inactive against the DPPH test, in contrast to Trolox as a comparison compound which had IC50 at a concentration of 2.59±0.03 µg/mL (Figure 1). This may be due to the inability of piperine compounds to interact with DPPH, which indicates the potential reduction of the free radical system (Rekka et al., 1996). Although the DPPH method is widely used to screen antioxidant activity in vitro in plant compounds, the DPPH method has limitations, especially in large molecules that are difficult to access the N-radical portion of DPPH due to steric inhibition of the compound molecule (Dayan, 2008; Dejian Huang et al., 2005).

FRAP assay

FRAP is a method used to measure antioxidant ability by reducing ferric-tripyridyl-triazine (Fe(III)TPTZ) (Fe^{3+}-TPTZ) to Ferro-tripyridyl-triazine (Fe(II)TPTZ) (Fe^{2+}-TPTZ) complex (Ou et al., 2002). Determination of the antioxidant capacity of the FRAP method was carried out by calculating the standard Trolox curve. Determination of antioxidant activity carried out by the FRAP method has an

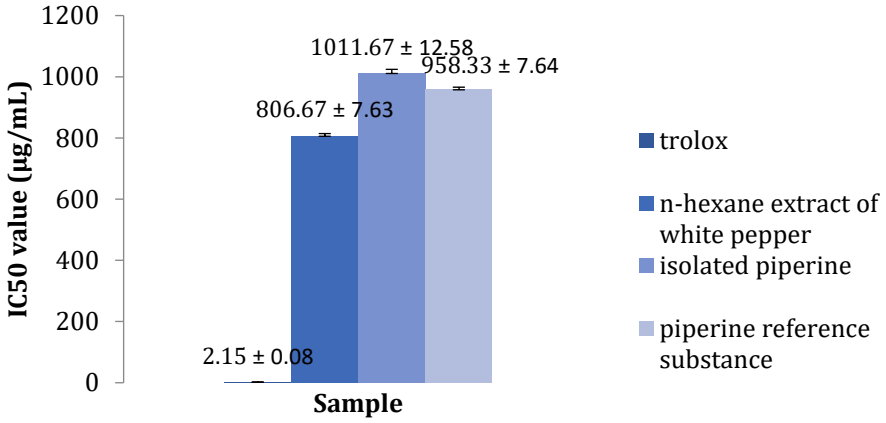

Figure 1. The result of IC50 value of isolated piperine, piperine reference substance, extract n-hexane, and Trolox in the DPPH test

antioxidant capacity which is expressed as equivalent to Trolox (TEAC) and is expressed in units of mol trolox/g sample (Widyastuti, 2010). TEAC coefficient (Trolox equivalent antioxidant capacity) is the concentration of Trolox, which has an antioxidant capacity equivalent to the sample analyzed.

The results show that piperine compounds had antioxidant activity. Based on the results of the FRAP analysis test, piperine compounds were able to reduce the complex (Fe(III)TPTZ) to (Fe(II)TPTZ) by 10.53 ± 0.06 mol TE/g sample and the n-hexane extract of white pepper were able to reduce the complex (Fe(III)TPTZ) to (Fe(II)TPTZ) by 6.86 ± 0.08 mol TE/g sample (Figure 2) (Table I). Piperine can inhibit oxidation or stop free radical chain reactions by releasing electrons to free radicals. Piperine contributes to antioxidant activity by binding to ferrous metal ions. Metal ions can catalyze reactions that eventually produce free radicals (Ou et al., 2002).

ABTS assay

The ABTS method's working principle of antioxidant activity is the reaction between antioxidant compounds and ABTS cations. ABTS is a radical with a nitrogen center with a characteristic bluish-green color that will turn colorless when reduced by antioxidant compounds (Floegel et al., 2011; Mareček et al., 2017). Before being used, the ABTS reagent is reacted with potassium persulfate ($K_2S_2O_8$) to form the cation radical compound ABTS•. It has absorption at a wavelength of 743 nm with a bluish-green color and will turn colorless when receiving electron donations or hydrogen atoms from antioxidant compounds (Pellegrini et al., 2003; Sun et al., 2011; Thaipong et al., 2006). The IC50 parameter was used to determine the antioxidant activity using the ABTS method.

The results of the antioxidant activity test using the ABTS method (Figure 3) show that n-hexane extract of white pepper had an IC50 (2.53 ± 0.08 mg/mL or 9.79 mM), isolated piperine (IC50 4.35 ± 0.004 mg/mL or 15.25 mM), and piperine reference substance (IC50 4.74 ± 0.07 mg/mL or 16.61 mM. Thus, the n-hexane extract of white pepper has the lowest IC50 value where the lower the IC50 value, the stronger the antioxidant power. However, the IC50 value of n-hexane extract of white pepper was bigger IC50 than that of Trolox (IC50 2.15 ± 0.084 µg/mL) (Figure 3) as a positive control of antioxidant activity test with ABTS. The high antioxidant activity content in the n-hexane extract of white pepper is possible due to the presence of certain compounds that have more dominant antioxidant activity than piperine compounds.

The ABTS test has a higher sensitivity than DPPH (Floegel et al., 2011). Based on the ability of antioxidant compounds, the DPPH and ABTS have different reaction mechanisms, wherein the DPPH, the ability of free radical scavenging of a compound is seen based on the compound's ability to donate hydrogen atoms. Meanwhile, in the ABTS test, the ability of antioxidant compounds is based on the ability of antioxidant compounds to stabilize free radical

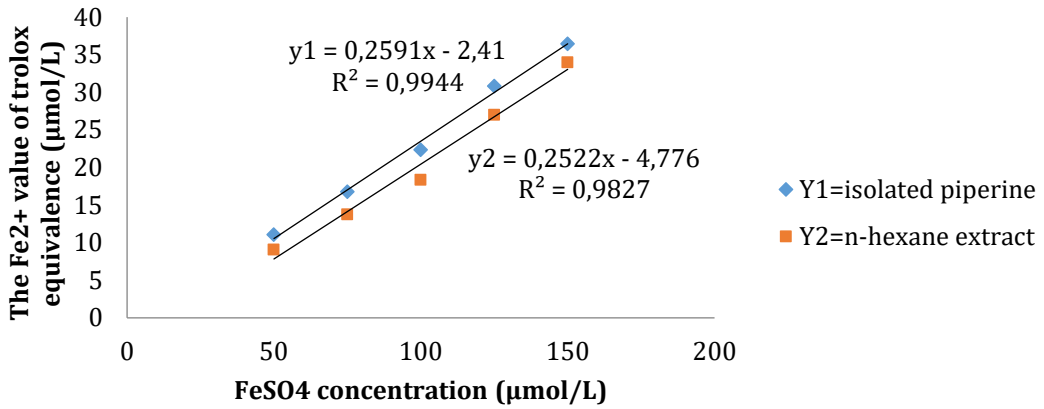

Figure 2. The relationship between Trolox concentration and FeSO4 in the FRAP test of isolated piperine and n-hexane extract of white pepper

Table I. The antioxidant capacity of isolated piperine and n-hexane extract of white pepper

Sample	The Fe^{2+} value of $FeSO_4$ equivalence in sample (mmol/L)	The Fe^{2+} value of trolox equivalence in sample (mmol/L)	Antioxidant capacity (mol TE/g sample)
1.5 ppm of isolated piperine	1.68 ± 0.03 mmol/L	15.79 ± 0.10 mmol/L	10.53 ± 0.06 mol TE/g sample
1.5 ppm of n-hexane extract of white pepper	1.23 ± 0.03 mmol/L	10.29 ± 0.12 mmol/L	6.86 ± 0.08 mol TE/g sample

compounds by donating proton radicals (Müller et al., 2011). Antiradical activity characterizes the ability of a compound to interact with free radicals (in a single free radical reaction), while antioxidant activity is its ability to inhibit the oxidation process. As a result, the DPPH and ABTS test system provides different information about free radical and antioxidant activity (Tirzitis and Bartosz, 2010).

Although many researchers state that piperine compounds have antioxidant activity, in fact, several previous studies (Nahak and Sahu, 2011; Zarai et al., 2013) showed that the ethanolic extract of white pepper (*Piper nigrum* L) has high antioxidant activity and high antioxidant activity of free radical inhibitory activity in the test using the DPPH method. This is due to the presence of polyphenols in the extract, which play an important role in the antioxidant activity of *Piper nigrum* L. The level of a single antioxidant in a food ingredient does not necessarily reflect the total antioxidant potential (Ramadan-Hassanien, 2008). The total amount of antioxidant potential depends on the synergistic and redox interactions between the different molecules present in the food.

The explanation of these data was based on the data of HPTLC (Figure 4). The detection under UV 254 nm showed several spots of cyclohexane fraction at Rf upper than the spot of piperine. It means that the cyclohexane fraction contained polar substances such as mono and sesquiterpene hydrocarbons composing the essential oil of white pepper. It was confirmed by the absence of fluorescence under UV 366 nm due to the lack of chromophores. According to the publication of Singh et al. (2013), the main component of white pepper essential oils are limonene, carene, sabinene, β-pinene, and α-pinene. All mentioned compounds belong to terpenoid hydrocarbons that do not have the capability to react as an antioxidant. The possible components of n-hexane extract having antioxidant activity are compounds with an Rf value lower than piperine. They have dull light blue fluorescence under UV 366 nm. These

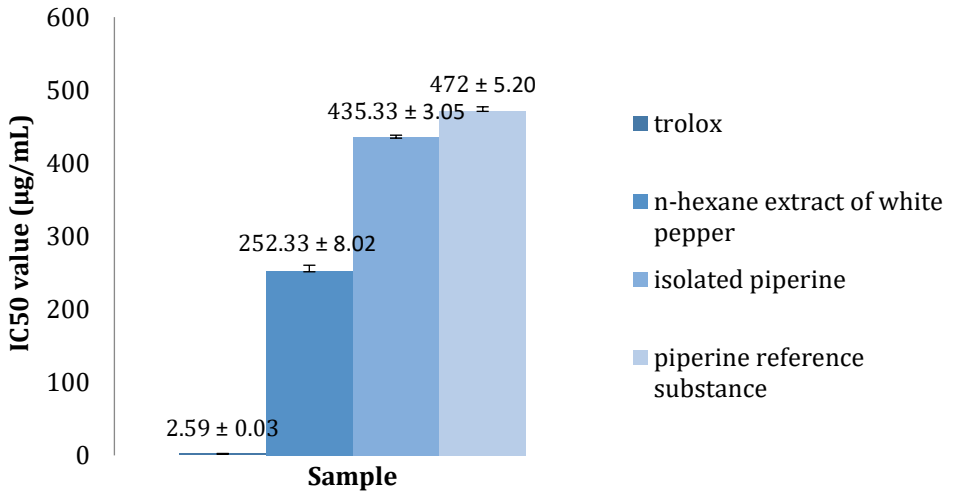

Figure 3. The result of IC50 value of isolated piperine, piperine reference substance, extract n-hexane, and Trolox in the ABTS test

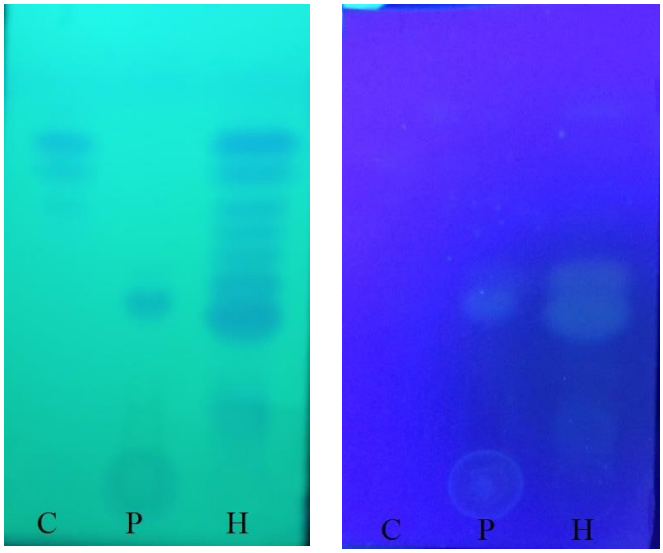

Figure 4. HPTLC profile of Cyclohexane fraction (C), Piperine reference substance (P) and Hexane extract of white pepper (H). Stationary phase: HPTLC Silica gel 60F254, mobile phase: n-Hexane-ethyl acetate (7:3). Detection: UV254 (A), UV366 (B).

compounds are different from these of ethanolic extract because they are lipophilic compounds so that they are subject to the next research.

CONCLUSION

Isolated piperine from white pepper n-hexane extract had weak antioxidant activity. The isolated piperine compound did not have free radical activity with DPPH reagent but had antioxidant activity in the ABTS test with the results of 4.35 ± 0.004 mg/mL and 2.53 ± 0.08 mg/mL, respectively and FRAP test was 10.53 ± 0.06 mol TE/g sample and 6.86 ± 0.08 mol TE/g sample, respectively. The high antioxidant activity of white pepper fruit extract is possible due to synergistic and redox interactions between other compounds and piperine compounds.

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