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DPPH RADICAL SCAVENGING ACTIVITY OF THE PURIFIED EXTRACT AND LIQUID-LIQUID EXTRACTION (LLE) FRACTION OF KAEMPFERIA ROTUNDA L.

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ABSTRACT

Kunci pepet (*Kaempferia rotunda* L.) is a plant with potential as a traditional medicine, containing various phytochemical compounds that contribute to pharmacological activities. This study aimed to determine the antioxidant activity of the purified extract and the Liquid Liquid Extraction (LLE) fractions of *Kaempferia rotunda* L. against DPPH. The rhizomes were extracted via maceration and re-maceration using 70% ethanol. The concentrated *K. rotunda* extract (KRE) was then purified with n-hexane. The purified extract was further fractionated using LLE with solvents of varying polarity (n-hexane and ethyl acetate). Phytochemical screening was perform qualitatively. The DPPH radical scavenging activity of the purified extract and LLE fractions was assessed using UV-vis spectrophotometry. The yield values obtained from the extraction, purification, and fractionation procedures were as follows: Crude K. rotunda Extract (8.58%), Purified Extract (24.8%), n-Hexane-soluble Fraction (3.81%), Ethyl Acetate-soluble Fraction (30.55%), and Water-soluble Fraction (55.60%) w/w. Phytochemical screening revealed that all samples tested positive for flavonoids. The DPPH scavenging assay showed that the purified extract sample exhibited the strongest activity with an IC_{50} value of 54.50 µg/mL. The purified extract contained a more varied profile of secondary metabolites compared to the other samples.

KEYWORDS: *Kaempferia rotunda*, LLE Fractions, Polarity level, Purified Extract.

INTRODUCTION

Indonesia, by virtue of its geographical and astronomical location, possesses a diverse tropical climate. This diversity fosters immense potential for biodiversity, including herbal plants for traditional medicine (jamu), whose development is currently accelerating.[1] Environmental factors such as temperature, humidity, and soil significantly influence the growth of this biodiversity.

Plant family with potential as an herbal medicine is Zingiberaceae, specifically *Kaempferia rotunda*. In Indonesia, *Kaempferia rotunda* is known as Kunci Pepet and Kunir Putih in Indonesia. The rhizome of Kunci Pepet is an easily cultivated plant, typically harvested at the end of the year. While not yet widely utilized as a formal medicine, the rhizome of this plant is traditionally believed by Indonesian communities to treat stomach ache, colds, wounds, and diarrhea.[2,3] Numerous researchers have reported that this plant possesses various biological activities, such as antioxidant, antimutagenic, and anticancer properties.

Medicinal plants generally exhibit higher free radical scavenging activity compared to fruits and vegetables. Free radical scavengers are compounds that can capture or inhibit the rate of oxidation and neutralize free radicals.[4] In the body, antioxidants play a role in protecting against damage from reactive oxygen species and inhibiting the onset of degenerative diseases. Free radicals are chemical compounds with one or more unpaired electrons, rendering them unstable.[5] They can be found in cigarette smoke, air pollution, toxic chemicals, pesticides, and UV radiation, resulting from excessive environmental metabolism, and can enter the body through the respiratory tract.[6]

The stable free radical commonly used as a reference for measuring free radical scavenging capacity is DPPH (2,2-diphenyl-1-picrylhydrazyl). The DPPH method is a technique for screening compounds capable of capturing and inhibiting free radicals and is used to assess antioxidant activity. When DPPH interacts with an antiradical compound, the free radical from DPPH is neutralized, forming reduced DPPH.[7]

Research by Malahayati et al.[8] reported an IC_{50} value of 67.95 ppm for the ethanol extract, classifying it as strong, meaning the ethanol extract of Kunci Pepet rhizome can inhibit 50% of free radicals. Furthermore, according to Dwira et al.[9], the ethanol extract of Kaempferia rotunda exhibits strong cytotoxic activity against cancer cells with an IC_{50} of 16.93 µg/ml. Methanol and chloroform extracts from Kunci Pepet (*Kaempferia rotunda*) rhizome possess antiradical activity.[10,11] The methanol extract of K. rotunda showed weak antiradical activity. However, when the extract was partitioned using chloroform-water, ethyl acetate-water, and then n-butanol-water, the chloroform-soluble extract demonstrated significant free radical scavenging activity. *K. rotunda* contains a flavanone compound, namely 5-hydroxy-7-methoxyflavanone. These compounds possess hydroxyl groups capable of inhibiting or capturing DPPH radicals.[2] Several studies have reported that the antioxidant compounds of *K. rotunda* are obtained from the separation of extracts (fractionation and isolation).[12]

Research to explore both the purified extract and the fractions resulting from liquid-liquid extraction of the ethanol extract of *Kaempferia rotunda* has not been reported in the last five years. There are no reports regarding the activity of fractions and purified extract products of *Kaempferia rotunda*. Previous studies have generally focused only on crude extracts and their separation methods. Therefore, the identification of bioactive compounds from the purified extract and liquid-liquid extraction fractions, along with their mechanisms of action, presents a highly promising knowledge gap worthy of exploration.

MATERIALS AND METHODS

Materials

The equipment used in this study consisted of an analytical balance (Toledo), maceration vessels, volumetric glassware, a rotary evaporator (Buchi), a micropipette (Dragonlab), an oven (Memmert), and a Biobase BK-UV1800 UV/Vis Spectrophotometer. The reagents used included organic solvents of analytical grade, such as n-hexane, ethyl acetate, 70% ethanol, and methanol. Other reagents used were DPPH (Sigma-Aldrich), ferric chloride, Mayer, Wagner, Bouchardat, and distilled water. The research material was Kaempferia Rotunda Rhizome, obtained from the collection of B2P2TOOT Tawangmangu, Karanganyar, Central Java.

Extraction and Fractionation

Five hundred grams of *Kaempferia rotunda* rhizome powder was dissolved in 70% ethanol and extracted using maceration-remaceration for 3 x 24 hours at room temperature.[13] The procedure involved periodic stirring every 6 hours. Solvent evaporation was conducted using a rotary evaporator until a concentrated *K. rotunda extract* (KRE) was obtained, after which the yield was determined.[14]

Purification of the extract was performed using a separatory funnel by dissolving 1 part of KRE in 2 parts of n-hexane.[15] After shaking, two layers were obtained. The n-hexane-insoluble part was evaporated to a concentrated state, subsequently referred to as the Purified Extract (PE), and its yield was determined.

Fractionation of the extract using the liquid-liquid extraction (LLE) method was carried out by dissolving 1 part of KRE in 20 parts of 50% ethanol, followed by the addition of n-hexane (1:1 v/v) and shaking until two layers formed. The n-hexane-soluble part was separated and evaporated to a concentrated state (FH - Hexane Fraction). The n-hexane-insoluble part was then treated with ethyl acetate (1:1 v/v) and shaken similarly to the previous step to obtain the ethyl acetate-soluble fraction (FEA - Ethyl Acetate Fraction) and the aqueous phase residue (FA - Aqueous Fraction).

Phytochemical Screening

Qualitative tests to determine the phytochemical content in the purified extract and its fractions were conducted using the following methods[16,17]

a. Alkaloids

A test solution was prepared by dissolving 50 mg of the extract in 2N HCl, followed by filtration. One portion served as a control, while two other portions were treated with 2 drops of Mayer's reagent and 3 drops of Wagner's reagent, respectively. A positive result was indicated by the formation of a precipitate: white for Mayer's and brown for Wagner's.

b. Flavonoids

The Willstätter method for flavonoid testing was by dissolving 50 mg of the sample in 5 mL of ethanol and heating it for five minutes, adding a few drops of concentrated HCl and 20 mg of Mg powder. A positive test was declared if a deep red or orange color formed within approximately three minutes.

c. Saponins

One mL of the extract was mixed with 2 mL of distilled water and shaken vigorously for 1 minute. After adding 2 drops of 1N HCl, the formation of stable foam 1-3 cm high persisting for ±7 minutes indicated the presence of saponins.

d. Tannins (Phenolic Compounds)

The test for phenolic compounds was performed by dissolving 500 mg of the sample in 20 mL of distilled water, boiling it, and then filtering it. To 0.5 mL of the resulting filtrate, 1 mL of 3% $FeCl_3$ was added. The formation of a dark green color indicated a positive test.

e. Terpenoids

Triterpenoid compounds were screened via a color reaction. Two mg of the extract dissolved in distilled water and chloroform was treated with concentrated H_2SO_4. A positive result was indicated by a color change to dark blue or greenish-black.

DPPH Radical Scavenging Activity Assay

a. Preparation of DPPH Solution

Based on the method by Muhafidzah, Dali, and Syarif[17], a 40 ppm DPPH solution was prepared by dissolving 4 mg of DPPH in 100 mL of analytical-grade methanol. Subsequently, a 16 ppm standard stock solution was prepared. Four mL of this solution was incubated under dark conditions for 30 minutes. The maximum wavelength (λmax) was determined by measuring its absorbance within the 500-525 nm range.

b. Activity Assay of Control Solutions (Ascorbic Acid and Quercetin) against DPPH

Standard solutions of ascorbic acid and quercetin at 100 ppm concentration were prepared by dissolving 10 mg of each compound in 100 mL of analytical-grade methanol.[18] From this stock solution, a concentration series of 20, 30, 40, 50, and 60 ppm was prepared. Subsequently, 1.5 mL from each standard solution was pipetted and reacted with 3.5 mL of the DPPH solution. The mixture was then incubated in a dark room for 30 minutes before its absorbance was measured at the predetermined λmax.

c. Radical DPPH Scavenging Activity of Purified Extract and Fractions of LLE

A sample stock solution with a concentration of 500 ppm was prepared by dissolving 50 mg of the LLE-purified kunci pepet rhizome extract in ethanol to a final volume of 100 mL. From this stock solution, a concentration series of 20, 30, 40, 50, and 60 ppm was then prepared. Sample treatment was performed identically to the control procedure. Final absorbance was measured at λmax, using analytical-grade methanol as the blank solution.

d. Determination of % Inhibition and IC_{50}

The obtained absorbance values were then used to calculate the percentage of inhibition. This inhibition percentage data was subsequently plotted against sample concentration to create a relationship curve. The IC_{50} value was determined from this curve using linear regression based on the equation $Y = ax + b$. All testing procedures were performed in triplicate.[19]

RESULTS AND DISCUSSION

Extraction and Fractionation

Extraction using the maceration method and subsequent fractionation via Liquid-Liquid Extraction (LLE) yielded the n-hexane soluble fraction (HF), the ethyl acetate soluble fraction (EAF), and the residue or aqueous phase fraction (AF). Furthermore, purification of the crude extract using n-hexane via the same method (LLE) yielded a Purified Extract (PE). The yield values for HF, EAF, AF, and PE are presented in Table 1 below:

Table 1: Yield of Purified Extract and LLE Fractions.

Sample	Weight of Crude Extract (g)	Solvent (mL)	Sample Weight (g)	Yield (%)
Purified Extract	10	200	2,48	24,80
N-Hexane Fraction (HF)			0.381	3.81
Ethyl Acetate Fractiom (EAF)			3.055	30.55
Aqueous Fraction (AF)			6.564	55.60

The purification process of the crude extract (10 g) using n-hexane yielded a Purified Extract (PE) with a yield of 24.80% w/w (Table 1). This indicates that nearly one-quarter of the crude extract consists of non-polar or semi-polar compounds soluble in n-hexane. Subsequently, this crude extract was systematically fractionated. The Aqueous Phase (AF) yielded the highest percentage (55.60% w/w), suggesting that the majority of components in the crude extract are polar and retained in the aqueous phase. The Ethyl Acetate Phase (EAF) showed a significant yield (30.55%), representing semi-polar compounds. The data in Table 1 reveals that the N-Hexane Phase (Hf) had the lowest yield (3.81% w/w), indicating that only a small amount of purely non-polar compounds were present in the initial crude extract. This yield data confirms the successful separation of extract components based on polarity through fractionation, with polar compounds constituting the dominant fraction.[20,21]

Phytochemical Screening

The results of the qualitative tests for secondary metabolites in the LLE fractions and the purified extract are shown in Table 2 below:

Table 2: Phytochemical Screening of Samples.

Secondary metabolite	Literature	Pustaka	HF	EAF	AF	PE
Alcaloids	Wagner, HCL 2N	Brown precipitate	-	+	-	-
	Mayer, HCL 2N	White/yellow precipitate	-	+	-	+
Flavonoids	Conc. HCL, Mg powder	Deep red/orange colour	+	+	+	+
Saponins	HCL 1N, Water	Stabil foam	-	-	+	-
Terpenoids	Conc. H2SO4, Kloroform, Water	Dark blue/blackish-green colour	+	-	-	+
Tannins	$FeCl_3$ 3%	Dark green or blue colour	-	-	+	+

Note: (+ : Positive) (- : Negative)

Phytochemical screening revealed the distribution of bioactive compounds in each fraction, correlating with the polarity of the solvents used. The N-Hexane Phase (FH) tested positive for flavonoids (non-polar/aglycones to semi-polar) and terpenoids/steroids. The Ethyl Acetate Phase (FEA) tested positive for alkaloids and flavonoids. The Aqueous Phase (FA) tested positive for flavonoids, saponins, and tannins. The Purified Extract (PE) tested positive for alkaloids, flavonoids, and terpenoids.

The results of the phytochemical screening presented in Table 2 are significant. The compounds positively identified in FH align with expectations, as these compounds are typically soluble in non-polar solvents like n-hexane. In the FEA, the properties of ethyl acetate are effective in extracting semi-polar compounds such as alkaloids and certain types of flavonoids (e.g., flavonoid glycosides). The profile in the aqueous fraction (FA) is highly consistent, as all three compound classes (flavonoids, saponins, tannins) are generally polar or exist as glycosides readily soluble in water.[22] The compound profile in PE is a combination of those found in FH and FEA, since PE was obtained by dissolving the

crude extract in n-hexane. This implies that PE is rich in semi-polar to non-polar compounds soluble in n-hexane but is devoid of highly polar compounds like saponins and tannins (which were not detected in PE).

DPPH Radical Scavenging Activity

The results of the spectrophotometric DPPH radical scavenging activity assay for the purified extract and LLE fractions, with vitamin C and quercetin as controls, are presented in Table 3 below:

Table 3: DPPH Radical Scavenging Activity Assay.

Sample	IC_{50} (ppm)	Category[23]
Purified Extract	54.60	Strong
N-Hexane Fraction (HF)	153.63	Weak
Ethyl Acetate Fractiom (EAF)	60.77	Strong
Aqueous Fraction (AF)	81.00	Strong
Ascorbic Acid	9.56	Very Strong
Quercetine	14.87	Very Strong

The controls (Ascorbic Acid & Quercetin) showed very low IC_{50} values (< 15 ppm). The Purified Extract (PE) and the Ethyl Acetate Phase (FEA) demonstrated strong activity with IC_{50} values of 54.60 ppm and 60.77 ppm, respectively. The Aqueous Phase (FA) was also categorized as strong, albeit with a higher IC_{50} (81.00 ppm). The N-Hexane Phase (FH) exhibited the weakest activity (IC_{50} 153.63 ppm).

The DPPH radical scavenging is widely used due to DPPH's relative stability as a radical and its commercial availability. Antioxidant activity is measured as the IC_{50} value (the concentration required to scavenge 50% of DPPH radicals). A lower IC_{50} value indicates stronger antioxidant activity.[24] The validity of the test method and the established activity baseline are strongly supported by the results for Vitamin C and quercetin controls, which showed very low IC_{50} values (<15 ppm). The FEA and PE extracts demonstrated strong potential. This potency is very likely attributed to the flavonoid and alkaloid content strongly detected in both samples, as flavonoids are well-known potent natural antioxidants. The Aqueous Phase (FA) was also categorized as strong, despite a higher IC_{50} (81.00 ppm). This activity may originate from its contained flavonoids, saponins, and tannins, although these polar compounds generally possess slightly lower antioxidant potential compared to semi-polar compounds like those in FEA. The weakest activity observed in FH suggests that, although it contains flavonoids and terpenoids, the type of flavonoid aglycones (non-polar) in this fraction may be less active in donating electrons/hydrogen to DPPH radicals compared to more polar flavonoid derivatives, or their concentration may be relatively low (consistent with the small yield).

The Ethyl Acetate Phase (FEA) is the Most Active Fraction from the Fractionation Process. Although PE had the best IC_{50}, it is not a direct fractionation product but a purification product. Among the three LLE fractions, FEA exhibited the strongest antioxidant activity (IC_{50} 60.77 ppm). This indicates that the potential antioxidant compounds in the sample tend to be semi-polar. The presence of flavonoids in all fractions (FH, FEA, FA, EP) and the strong activity in fractions containing them (FEA & EP) suggest that flavonoids are the primary contributors to the antioxidant activity in the studied sample.[25] There is a distinct difference in activity among FH, FEA, and FA. This proves that fractionation successfully concentrated certain active compounds into specific fractions. These results provide clear direction for subsequent research stages. The Ethyl Acetate Phase (FEA) is the most promising fraction for further isolation to obtain pure antioxidant compounds. Meanwhile, the Purified Extract (PE) also holds potential for development as a raw material for crude preparations with good activity.

CONCLUSION

This research successfully demonstrated that through a fractionation process, a semi-polar fraction (Ethyl Acetate Phase) containing alkaloids and flavonoids can be obtained, which was the fraction with the strongest DPPH radical scavenging activity among the separated fractions. These findings support the potential of the studied sample as a source of natural antioxidants, with flavonoid compounds from the semi-polar fraction suspected to be the primary contributors to this activity.

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