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Effects of Solvent Combinations of Phenolics and Antioxidants Extraction from *Kaempferia rotunda* Rhizomes

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Abstract

Kaempferia rotunda is a widely known herbal plant that can be used as a cooking ingredient in traditional medicine. This plant contains many polyphenolic compounds that act as natural antioxidants. This plant can be optimized by selecting an appropriate solvent to extract the polyphenol content and antioxidant capacity. This study aimed to determine the best solvents (water, ethanol, acetone, and their combination) for extracting the total phenolic content (TPC) and antioxidant capacity of *K. rotunda* rhizomes. TPC was calculated using the Folin-Ciocalteu method, whereas the antioxidant capacity was calculated using the ferrous ion-lowering antioxidant potential (FRAP) and 2,2'-azino-bis (3-ethylbenzothiazoline-6- sulfonic acid) (ABTS) methods. The results showed that the highest TPC and FRAP antioxidant capacity were found in the water-ethanol-acetone solvent extract with a TPC value of 0.9971 mg GAE/g DW and an antioxidant capacity of 2.9120 µmol TE/g DW. The highest ABTS antioxidant capacity was found in the water-acetone solvent, with an antioxidant concentration of 10.4456 µmol TE/g DW. Furthermore, the correlation of TPC with FRAP antioxidants was stronger than that with ABTS antioxidants, as determined by Pearson's correlation coefficient.

Keywords: *Kaempferia rotunda*, Total Phenolic, Antioxidants, FRAP, ABTS

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1. Introduction

Medicinal plants have long been known for various purposes, including research. Recently, the popularity of medicinal plants as an international source of medicines has increased rapidly because of their natural origin, community availability, cheaper purchase, ease of administration, and low potential for problems. Although it requires further research and development, herbal medicine can be a safer alternative to synthetic drugs [1]. One type of plant known as a medicinal plant is *Kaempferia rotunda* L. This plant belongs to the Zingiberaceae family and is widely used in food cooking and traditional medicine. This plant extract has been found to contain a range of secondary metabolites, including flavonols, flavonoids, stigmasterol, quercetin, chalcones, β-sitosterol, protocatechuic acid, and syringic acid. *K. rotunda* has many pharmacological benefits, such as antibacterial, antimutagenic and antioxidant activities [2]. Plant rhizomes have been found to contain syringic acid, ferulic acid, gallic acid, p-coumaric acid, caffeic acid, myricetin, kaempferol, and quercetin [3].

K. rotunda is also known to have antioxidant activity that benefits human health. Antioxidants play an important role in human health by boosting immunity and by reducing the risk of diseases caused by free radicals. Oxidative stress, caused by high levels of free radicals, is a vital factor that can trigger several degenerative diseases. These degenerative diseases include cancer, rheumatoid arthritis, cardiovascular diseases, autoimmune disorders, aging, neurodegenerative disorders, and cataracts [4]. Antioxidants are “redox drugs” that interact with free radicals through redox signalling [5]. An important aspect of antioxidant therapy is the absorption, distribution, metabolism, and excretion of specific antioxidants, which requires detailed analysis of many compounds, especially nutraceuticals [6].

Phenolic and flavonoid compounds act as antioxidants. Phenolic compounds are a varied group of phytochemicals that encompass multiple families of aromatic secondary metabolites in plants and possess the ability to neutralize multiple free radicals [7]. Testing antioxidants derived from phenolic and flavonoid groups involves various mechanisms of action. The mechanism of this reaction involves either the transfer of a hydrogen atom or a single electron. Multiple electron and proton transfers, such as hydrogen atom transfer combined with single-electron transfers, stepwise electron-proton transfers, shared electron-proton transfers, or electron transfers with overlapping proton losses, can occur in several situations. There are various methods for assessing antioxidant activity through chemical-based assays, including those that rely on stable free radical scavenging activity (ABTS (2,2′-azino-bis (3-ethylbenzene thiazoline-6-sulfonic acid))), or metal ion reduction (FRAP (ferrous ion-lowering antioxidant potential)) [8].

The extraction of phenolic compounds can be optimized by selecting an appropriate solvent to maximize phenolic compounds. It is necessary to select the appropriate solvent to optimize extraction from *K. rotunda* rhizomes to obtain the optimal compound content. In addition, this study aimed to measure the total phenolic content and antioxidant capacity of the *K. rotunda* rhizome using various solvents (water, ethanol, and acetone) and their combinations, and to find the best solvent to optimize the extracts obtained.

2. Materials and methods

This study was conducted at the Biochemistry Laboratory, IPB University, Bogor, Indonesia, from February to May 2023. The samples used in this study were collected from the Biopharmaca Collection Garden (-6.5470915, 106.711514), Tropical Biopharmaca Research Center, IPB University, Bogor, Indonesia. The rhizome was the part of the plant used as the sample. The solvent consisted of three types of solvents (water, ethanol, and acetone) in seven solvent combinations (Table 1). The test was performed in triplicate for each solvent combination.

Table 1: The combination of solvents used in this study

Combination Code	Water	Ethanol	Acetone
w	1	0	0
we	0.5	0.5	0
wa	0.5	0	0.5
e	0	1	0
ea	0	0.5	0.5
a	0	0	1
wae	0.33	0.33	0.33

Note: w, water; e, ethanol; a, acetone; we, water-ethanol; wa, water-acetone; ea, ethanol-acetone; and wae, water-acetone-ethanol.

2.1 Sample Preparation and Extraction

The rhizomes of the samples were dried at a temperature of 45°C for a period of 48 h. The dried material was then ground to produce an 80-mesh powder. To obtain the plant extract, a method called ultrasound-assisted extraction was used, with some modifications [4]. Specifically, 4 g of the sample powder were placed in a 100-milliliter vial, and 20 mL of each solvent composition were added (as shown in Table 1). The flasks were then covered with aluminum foil and placed in a sonicator bath. The samples were sonicated for 30 min at room temperature. Finally, the filtrate was filtered using filter paper (Whatman No. 42).

2.2 Total Phenolics Analysis

The method for total phenolic analysis was modified from the procedure described by Pinto et al. [9]. A 20 µL sample extract was added to 120 µL of Folin-Ciocalteu (10%) and placed in a microplate, which was then incubated in the dark for 5 min. Next, 80 µL of a 10% Na_2CO_3 (w/v) solution was added to the microplate and incubated in the dark for 30 min. The absorbance of the solution was measured using a spectrophotometer (SPECTROstarNano BMG LABTECH) at 750 nm. Gallic acid (0–300 ppm) was used as a standard. TPC was reported as mg/g gallic acid equivalent (GAE) based on the gallic acid calibration curve, and then converted to a mg GAE/g dry weight (DW).

2.3 FRAP Antioxidant Activity Measurement

Antioxidant activity was determined using the FRAP method, as described by Husni et al. [10] with modifications. To prepare the FRAP reagent, 10 mM TPTZ was dissolved in 40 mM HCl, 20 mM FeCl3.6H2O in distilled water, and 10 mM acetate buffer at pH 3.6. The reagent was stored at 37°C for 30 min before use. Extracts were mixed with the FRAP reagent (1:1:10 ratio) and incubated for 30 min at 37°C in the dark. The absorbance was measured at 593 nm using a spectrophotometer (SPECTROstarNano BMG LABTECH) set to read at 734 nm. Each sample's antioxidant activity according to the FRAP method was reported as its Trolox equivalents per grams of dry weight (µmol TE/g DW).

2.4 ABTS Antioxidant Activity Measurement

The antioxidant activity of *K. rotunda* extract was measured using the ABTS method described by Liwanda et al. [11], with some modifications. To perform the assay, 20 µL of the extract was added to 280 µL of ABTS reagent, and the mixture was incubated for 6 min. The absorbance was then measured at a wavelength of 734 nm using a SPECTROstarNano BMG LABTECH nano spectrophotometer. Trolox equivalents per grams dry weight (µmol TE/g DW) were used to quantify the antioxidant activity of each sample.

2.5 Data analysis

The data were analyzed using the one-way ANOVA method in the data average test and IBM SPSS Statistics 25 with Tukey's follow-up test. Microsoft Office Excel 2019 was employed for data calculations, and the results were presented as graphs using GraphPad Prism 8 software.

3. Results and Discussions

3.1 Total Phenolic Content

The results for the TPC of various solutions and their combinations are presented in Figure 1a. The measurements depended on the solvent used for extraction, and the extraction efficiency was affected by the solvent's polarity.

To evaluate the effect of pure or mixed solvents on the extraction of phenolic compounds, it is important to study the appropriate polarity [12]. In this study, the highest TPC was extracted through a combination of dissolving water-ethanol-acetone (0.9971 mg GAE/g DW), while the lowest was obtained through pure water (0.3074 mg GAE/g DW). Chan et al. [13] carried out a TPC using a methanol extract of K. *rotunda* leaves, resulting in a concentration of 140 ± 48 GAE/100 g. Rachkeeree et al. [14] conducted n-hexane, ethyl acetate, and ethanol extractions to obtain TPC concentrations of 4.86, 154.28, and 14.54 mg GAE/g extract, respectively. Differences in the research results can be caused by several factors, such as variation in plant matrices, polarity of solvents used in extraction, and the composition and antioxidant activity of the extracts. Extracts with the highest phenolic content and hydroxyl groups tend to exhibit higher antioxidant activity [7].

3.2 Antioxidant Activity

Antioxidant content in plants typically varies with plant type [4]. In this study, the antioxidant was measured in two methods, namely FRAP and ABTS. The FRAP test is the only test that can directly determine the level of antioxidants in a sample. The FRAP method is based on the principle of reduction of ferroin analogues [15]. The Ferric Reducing Antioxidant Power assay (FRAP) is based on reducing the colourless Fe^{3+}-TPTZ complex to the dark blue Fe^{2+}-TPTZ upon interaction with a potent antioxidant. This method proved useful for screening antioxidant capacity and comparing the potency of different compounds. The FRAP method was used to measure the antioxidant capacity of selected phenolic acids [16]. The ABTS method was used to measure the antioxidant activity by measuring antioxidant submersion to free radicals. ABTS expresses the blue-green color, the color of the solution will be reduced by the reaction of the antioxidant reaction in the sample, and then color intensity can be measured using a nano spectrophotometer [17].

The first method used to measure the antioxidant activity of *K. rotunda* is FRAP, which involves different solvents, as shown in Figure 1b. The results show that there is no significant difference in the antioxidant activity between the various solvents. The solvent with the highest antioxidant activity on FRAP measurement is the water-ethanol-acetone extract, with an activity value of 2.9120 µmol TE/g DW, while the lowest is the ethanol-acetone extract, with an activity value of 1.5043 µmol TE/g DW. Although there is a difference in the value produced, the FRAP method's antioxidant capacity is not affected by the combination of solvents. These results indicate that the Tukey test did not significantly differ in the mixture of these three solvents: water, ethanol, and acetone.

The antioxidant activity of *K. rotunda* with the different solvents in the ABTS method is shown in Figure 1c. The water-ethanol (1:1) and water-acetone (1:1) solvents were used to extract the antioxidant sample that has the best antioxidant activity with the highest antioxidant activity at water-acetone solvent in 10.4456 µmol TE/g DW. At the same time, the lowest antioxidant activity shown is found in acetone solvent with the value of 0.3683 µmol TE/g DW. The results showed a significant difference in the antioxidant activity of the extraction solvent. Based on the Tukey test, the highest ABTS antioxidant capacity was produced by combining water-acetone, water-ethanol, and water-ethanol-acetone, respectively. These results indicate that water plays a large role when combined with organic solvents. However, using water alone as a solvent does not give the highest results due to the presence of phenolic group compounds that do not react with the ABTS reagent [4].

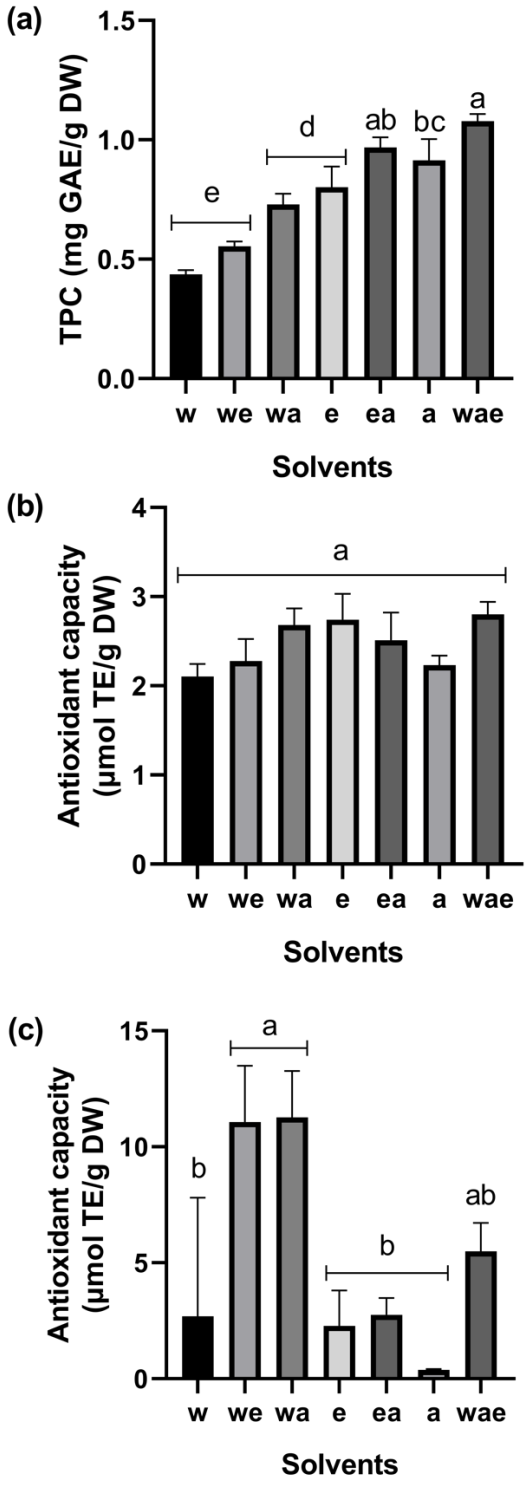

Figure 1. Total phenolic content (TPC) (a), FRAP antioxidant (b), and ABTS antioxidant capacities (c) in extracts of *K. rotunda* rhizome. Each value is presented as the mean of three replicates ± standard deviation (SD). The mean

value marked with different letters differs significantly at $p < 0.05$ usingTukey test. w = water; e = ethanol; a = acetone.

3.3 Correlation between total phenolic content and antioxidant capacity

To identify the correlation of antioxidant activity of *K. rotunda* by the various solvent to extract, simple linear correlation coefficients of Pearson's between the total phenolic content (TPC) and its antioxidant capacity have been analyzed for any extracts (Figure 2). The Pearson correlation coefficients were calculated to identify the relationship between the total phenolic contents and antioxidant activity [18].

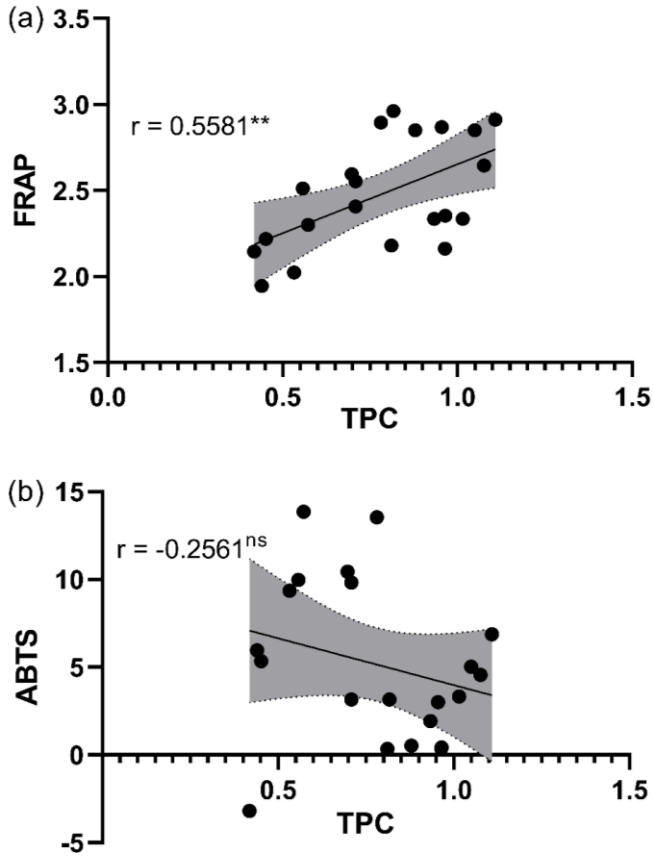

Figure 2. Simple linear correlation of total phenolic content (TPC) with antioxidant FRAP (a) and ABTS (b) in the obtained extract. r, the value of Pearson's correlation coefficients; ** and ns represent significance at $P < 0.01$ and insignificance, respectively.

The correlation between the FRAP and TPC methods was determined to be positive, with a coefficient of 0.5581**, as shown in Figure 2a. This suggests that a high antioxidant activity in a plant extract is associated with a high phenolic content. TPC is often utilized alongside the FRAP to expand the information database of a particular plant extract [19].

An analysis of the correlation between TPC and ABTS revealed negative correlations, which were considered to be none or insignificant with a value of r = -0.2561 (Figure 2b). This was in contrast to the previous research by Samaniego et al. [20], who found a significant correlation between TPC and AA/ABTS with a value of r = 0.86.

4. Conclusions

The measurements of the total phenolic content and antioxidant capacity of *K. rotunda* rhizome using a combination solvent of water, ethanol, and acetone yielded various values. The results showed that the highest total phenolic content and FRAP antioxidant was found in the water-ethanol-acetone solvent extract (0.9971 mg GAE/g DW for TPC and 2.9120 µmol TE/g DW for FRAP). Meanwhile, the highest antioxidant capacity of ABTS was found in the water-acetone solvent with an antioxidant concentration of 10.4456 µmol TE/g DW. Additionally, the TPC was found to be more towards the FRAP antioxidant than the ABTS antioxidant based on the Pearson correlation test.

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