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Determination of Total Phenolic, Flavonoid, and DPPH Scavenging Activity of Ruku-ruku (*Ocimum tenuiflorum* L.) Leaf

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ABSTRACT

Ruku-ruku (*Ocimum tenuiflorum* L.) is a plant that contains essential oils as well as phenolic and flavonoid compounds that act as antioxidants. This study aims to determine the total phenolic (TPC) and flavonoid content (TFC) and the DPPH scavenging activity of ruku-ruku leaves. Ruku-ruku leaf extraction was carried out using the maceration method using methanol. Measurement of TPC by Folin Ciocalteu method, TFC by $AlCl_3$ complex formation method, and antioxidant activity by DPPH method. The test results using a microplate reader showed that the methanol extract of ruku-ruku leaves had a total phenolic content of 300.71±2.86 mgGAE/g extract and total flavonoid content of 66.42±10.17 mgQE/g extract as well as very weak antioxidant activity (IC_{50} = 2501.07±1.2 µg/mL).

Keywords: Antioxidant, total phenolic content, total flavonoid content, ruku-ruku (*Ocimum tenuiflorum* L.)

Introduction

According to the WHO, the use of traditional medicine in developing countries is in demand by about 3.4 billion people (80% of the total population in the world), where traditional medicine is the main approach in primary health care (WHO, 2019). Medicinal plants are plants in one or more organs containing substances that can be used for therapy and treating diseases. Medicinal plants also source semi-synthetic chemo pharmaceuticals because they contain medically active chemical components (Salim, 2017).

Medicinal plants in the modern era are still an option for some people to be used as medicine or in health care. According to research by Aggarwal & Mali (2015), many people return to using natural medicines. One of them is the basil plant, where the plant has been proven as a treatment. Ruku-ruku (*Ocimum tenuiflorum* L.) leaves treat fever, cough, gout, canker sores, tinea versicolor, nausea, and vomiting. Holy basil seeds treat gonorrhoea and eye diseases, and the roots treat skin diseases (Sudarsono *et al.*, 2002).

The people of Rimbo Tarok use Ruku-ruku as a toothache medicine for children. Some people also use crushed Ruku-ruku flowers for early treatment of toothache, especially for children who do not want to take medicine (Wahyuni *et al.*, 2017; Fadhli *et al.*, 2022).

The research by Raina *et al* (2013), Ruku-ruku leaves contain caryophyllene (7.3 – 8.4%), methyl eugenol (0.2%), eugenol (67.4 – 72.8%), and germacrene D (2.4 – 2.2%). In addition, there are also methyl chavicol (68 – 64.9%), linalool (21.9 – 25.6%), and terpineol (1.2 – 0.1%). Meanwhile, based on research by . (2015), several phenolic acid compounds, such as caffeic acid derivatives, were identified. In addition, based on research by Divisha *et al*, (2018), rosmarinic acid, propanoic acid, apigenin, and cirsimaritin were also identified. Meanwhile, the identified flavonoids are orientin and vicenin.

Flavonoids are secondary metabolites derived from phenolic compounds found in plants (Banjarnahor & Artanti, 2014). The antioxidant effects of flavonoids and phenolic compounds occur by donating electrons to reduce free radicals (Treml & Šmejkal, 2016).

The basis of this study is the relationship between the presence of flavonoids and phenolic compounds and the presence of antioxidant activity in the Ruku-ruku plant. This study aims to determine the total phenolic content, flavonoids content and antioxidant activity with DPPH method from ruku-ruku leaves.

Research methods

Materials

Ruku-ruku leaves**,** ethanol (Emsure®), aquadest, methanol (Emsure®), chloroform (Emsure®), HCl (Emsure®), magnesium metal (Emsure®), $FeCl_3$ (Emsure®), norite, $(CH_3CO)_2O$ (Emsure®), H_2SO_4 (Emsure®), chloroform-ammonia 0,05 N, Daragendorff's reagent, DPPH reagent (Sigma-Aldrich®), Vit. C (Merck®), gallic acid, Folin-Ciocalteu's reagent 0,25 N, Quercetin, Sodium nitrite ($NaNO_2$ 5%), $AlCl_3$ 10%, NaOH 1 M.

Experiments

1. Preparation of methanol extract and phytochemical profile of extract

   Ruku-ruku leaves were taken on Jl. Pemuda, Perawang Village, Siak Regency, Riau Province. Plants identified in the Botanical Laboratory, Department of Biology, FMIPA, Riau University (No. 671/UN.19.5.1.1.3.4.1./EP/2021). 1 kg of Ruku-ruku leaves was sorted wet and washed with

running water. Then it is dried for a week, sorted dry, crushed, and weighed. Two hundred fifty grams of dried Ruku-ruku leaf were macerated with 4 liters of methanol in a well-closed container and protected from light for five days with three repetitions. The methanol extract was then concentrated on a rotary evaporator (40° C., 227 mbar).

2. Phytochemical test.

   Phytochemical tests were carried out according to the Julianto (2019) method covering alkaloids, flavonoids, phenolics, saponins, terpenoids, and steroids.

   a. Test for alkaloids, to 1 mL of extract, a few drops of Mayer's reagent were added. Alkaloids are present, as evidenced by the white precipitate that formed.
   b. Tests for flavonoids, one milliliter of the extract is treated with a few drops of HCl and a piece of magnesium; the resulting pink color indicates the presence of flavonoids.
   c. Test for phenolic compound, one milliliter of extract was mixed with two milliliters of a 5% ferric chloride solution, and dark blue staining indicated the presence of phenolic compounds.
   d. Saponin test, a total of 5 mL of extract in the tube was shaken vigorously and then allowed to stand for five minutes. Foam formation indicated the presence of saponins.
   e. Test for steroids/terpenoids. The test tube contained three drops of sample extract and three drops of glacial acetic acid. Five drops of concentrated H_2SO_4 were added to the mixture through the tube wall. Samples with terpenoids have a purple or red solution, while those with steroids have a blue or green solution.

3. Determination of total phenolic content of ruku-ruku extract

   Five mg (0.005 g) of methanol extract was weighed into a 5 ml volumetric flask (Pyrex ®), and ethanol was added to the limit (1000 µg/ml). Then the sample solution was diluted to a concentration of 250 µg/mL. The concentration was pipetted 100 L into a 96 wells microplate, 20 µL Na_2CO_3 7.5%, and then allowed to stand for 5 min in the dark. Then, 10 µL of Folin-Ciocalteau 0.25 N reagent was added and left in the dark for 30 min. The absorbance of the solution was measured at 760 nm with microplate reader (Epoch Biotek®). Sample measurement is repeated three times (Cicco *et al.*, 2009). Perform blank measurements in the same way, without adding the test solution. The standard was gallic acid at 10, 20, 30, 40 and 50 µg/mL concentrations. Create a calibration curve and calculate the concentration of each sample. Analyze the data using the absorbance of samples within the absorbance range on the calibration curve. TPC was calculated using the linear regression equation obtained from the standard curve of gallic acid (formula 1).

$$Y = ax \pm b$$

In the standard curve equation, the value of y is the absorbance obtained, while the value of x is the concentration of phenolic in the extract (µg/mL). Then the concentration was changed to mg/ml. Then the total phenolic content is calculated using the formula (2):

$$TFC = \frac{v\ (mL) x\ X\ \left(\frac{mg}{mL}\right) x\ FP}{(g)}$$

Where TFC is the total phenolic content (mg GAE/g extract) or the total flavonoid content (mg QE/g extract), X is the phenolic content (mg/mL), FP is the dilution factor (mL), V is the Volume (mL), and g is the sample weight (g).

4. Determination of total flavonoid content of methanol extract

   Five milligrams of methanol extract and 5 mL of ethanol were placed into a volumetric flask (1000 g/mL). Then the solution was pipetted 100 L into a 96 wells microplate then added 60 L of 5% sodium nitrite, covered with microplate cover, and allowed to stand for 5 minutes. Next, add 50 µL of 10% $AlCl_3$ and 30 µL of 1 M NaOH, then the mixture is allowed to stand for 30 minutes at room temperature and protected from sunlight, then the absorbance is measured at a wavelength of 430 nm. The absorbance data are means of three measurements (Xu & Chang, 2007). Perform blank measurements in the same way, without adding the test solution. The standard was quercetin with 20, 40, 60, 80, and 100 µg/mL concentrations. Create a calibration curve and calculate the concentration of each sample. Data analysis used absorbance of samples that entered the absorbance range on the calibration curve. TPC was calculated using the linear regression (y= ax ± b), equation obtained from the standard curve of the quercetin standard.

   The absorbance of the sample was entered into the standard curve equation as the y value, and the x value obtained was the phenolic concentration in the extract in ppm (µg/mL). Then the concentration was changed to mg/ml. Then calculate the total phenolic content using the formula (2).

5. Test on antioxidant activity of methanol extract

Antioxidant activity assay with DPPH assay was performed according to the procedure of Kedare & Singh (2011). Samples were diluted by double dilution from a concentration of 1000-31.25 µg/mL. 50 µL of the extract was added to 80 µL of DPPH solution at 80 µg/mL. Blank using methanol. The test solution was left for 30 min at room temperature under dark conditions. The absorbance of the assay was measured at 517 nm using a microplate reader. The standard is Ascorbic acid in concentrations 6.125, 12.5, 25, 50 and 100 µg/mL. The antioxidant activity of the sample is determined by the amount of DPPH radical scavenging by calculating the % inhibition with formula 3:

$$\% \text{ inhibition} = \frac{CA - SA}{CA} \times 100\%$$

Where CA is the Absorbance of DPPH solution, and SA is the Absorbance of DPPH solution in the sample solution.

After obtaining the percentage of inhibition from each concentration, a curve is made by plotting ln concentrations (x-axis) with the percentage of inhibition (y-axis) to obtain a linear regression equation (formula 4)

$$y = ax \pm b$$

All measurements were performed in triplicate, and the final results are reported as the mean with standard deviation (mean ± SD).

Result and Discussion

Phytochemical Profile

Table 1 presents the phytochemical test. Bioactive compounds are present in the methanol extract of the sample. The Dragendorff test's positive results are characterized by the formation of a brown/yellow precipitate. The estimate is that the precipitate is a potassium-alkaloid complex (Harborne, 2016). The saponin test's foam formation indicates the presence of glycosides that can form foam in water that is hydrolyzed into glucose and other compounds.

The reaction of steroids with anhydrous acetic acid and a drop of concentrated sulfuric acid will result in a green or blue color. The acetylation reaction of the -OH group on steroids is the result of the interaction between steroids and anhydrous acetic acid. The color difference between triterpenoids and steroids is due to the difference in groups on the C-4 atom (Marliana & Suryanti, 2005).

The methanol extract showed positive phenol content, and a significant blackish-blue color change was observed as a result of $FeCl_3$ reacting with the aromatic –OH group (Erdityo *et al.*, 2014).

Table 1. Phytochemical screening results of methanol extracts.

Chemical constituent	Test	Conclusion
Alkaloids	Dragendorff's test	+
Flavonoids	Shinoda's test	+
Phenolics	1% $FeCl_3$ test	+
Saponins	Foam test	+
Terpenoids	Liebermann-Burchard's test	+
Steroids	Liebermann-Burchard's test	+

Table 2. TPC of methanol extract at concentration 250 µg/mL.

Absorbance	KTFe (mgGAE/g)	KTFE (mgGAE/g) ± SD
0.644	300.71	300.71 ± 2.86

Table 3. TFC of methanol extract at concentration 1000 µg/mL.

Absorbance	TFC (mgQE/g)	TFC (mgQE/g) ± SD
0.709	66.42	66.42 ± 10.17

The phytochemical testing of the methanol extract identified flavonoids with a change in color to orange or red. Flavonoids typically dissolve in methanol because they are polar. Flavonoids can be removed from their salt form by using methanol. Flavonoid salts can be formed by adding concentrated chloride to protonated flavonoids. Flavonoids create an orange or red color when magnesium powder is added. It is due to the reduction of HCl and magnesium (Rahayu *et al.*, 2015). Based on phytochemical testing, the results are the same as the research results conducted by Alain & Manullang (2023), in which the ethanolic extract of *Ocimum tenuiflorum* contains alkaloid, flavonoids, tannins, saponins, steroids, and terpenoids compounds.

Total Phenolic Compound

Determination of total phenolic content (TPC) was determined by the Folin-Ciocalteu method. Phenolic compounds will be oxidized to form phenolic ions. Folin-ciocalteu reagent will be reduced to form a phosphotungstate-phosphomolybdate complex. Then form a blue molybdenum blue complex. The darker the blue colour formed, the more the phosphotungstate-phosphomolybdate complex was reduced. The whole reaction occurs in an alkaline condition obtained by adding $NaNO_2$ (Cicco *et al.*, 2009). Before measuring the absorbance, the mixture was incubated for 1 hour to ensure that the reaction was carried out thoroughly to maximize the intensity of the color produced (Aminah *et al.*, 2016).

The reference standard used is gallic acid. Gallic acid is a phenolic compound with three phenolic hydroxy groups known to have antioxidant activity (Nayeem *et al.*, 2016). This compound is a

derivative of hydroxybenzoic acid, a simple phenolic acid (Rawel *et al.*, 2002). The results of making the standard curve obtained a linear regression equation y = 0.0077x + 0.0258 with a value of R2 = 0.9925. This equation is obtained by plotting variations in the concentration of gallic acid concentration (x-axis) and the absorbance value of gallic acid (y-axis).

Based on this equation, the TPC of the methanol extract of Ruku-ruku at a concentration of 250 µg/mL was 300.17 mgGAE/g of the extract. The concentration levels used to TPC test were 62.5 to 1000 µg/mL (Table 2). A concentration of 250 µg/mL was chosen because it has an absorbance value of 0.2 to 0.8 and a correlation value of 0.999. The concentration is excellent because the good correlation value is close to 1 (Asuero *et al.*, 2006). In addition, data replication (repetition) is more accurate and precise. The TPC in the extract is expressed in GAE (Gallic Acid Equivalent), which means the number of mg of gallic acid equivalent in 1 g of extract (Lee et al., 2003). The higher the concentration of phenolic compounds in the sample, the more intense the blue color and the higher the absorbance (Aleixandre-Tudo *et al.*, 2017). The results of the measurement of total flavonoid content in the methanol extract of ruku-ruku leaves are in Table 2. The table shows that the total phenolic content of the methanol extract of ruku-ruku leaves is 300.71 ± 2.86 mgGAE/g of the extract.

Based on the data in Table 3, phenolic compounds are found in methanol extract, this indicates that phenolic compounds contained in ruku-ruku leaf extract are mostly polar molecules that dissolve in methanol solvents. The principle of like dissolves like states that a compound can dissolve in solvents with the same polarity properties (Barchan *et al.*, 2014).

Total Flavonoid Content

TFC was determined using the colorimetric method with an $AlCl_3$ reagent. This determination was based on forming a complex between $AlCl_3$ and flavonoid compounds in the ortho hydroxy ketone group. The standard for comparison is quercetin. Quercetin was chosen because it is the largest in different plants. Quercetin and its glycosides account for about 60-75% of flavonoids. Furthermore, quercetin is one of the compounds from the flavonoid group that can react with $AlCl_3$ to form complexes (Kelly, 2011).

The standard quercetin curve representation results obtained the linear regression equation y = 0.0086x + 0.0428 with the value R2 = 0.9898. This equation is obtained by plotting the concentration of quercetin (x-axis) against its absorbance (y-axis). Based on this equation, the total flavonoid content of Ruku-ruku leaf extract was 66.42 mgEQ/g extract (Table 3). The total content of flavonoids in the extract is expressed in units of QE (Quercetin Equivalent), which means the equivalent number of mg of quercetin in 1 g of extract.

This study had a higher amount of total phenolics and flavonoids than (Upadhyay *et al.*, 2015). (Upadhyay *et al.*, 2015) found that the ruku-ruku leaf extract had a total phenol content of 9.41 mg GAE/g extract and a total flavonoid concentration of 6.69 mgQE/g extract. The plants in the study were grown in Indonesia, while Upadhyay et al.'s (2015) research involved plants grown in India. This disparity in plant development environments accounts for the variance in total phenol and total flavonoid levels. The content of phenolic compounds, including flavonoids, can be impacted by environmental factors such as soil composition, temperature, rainfall, and UV light (Borges *et al.*, 2013). The solvent is crucial in extracting flavonoids and phenolic compounds. Flavonoids are widely dispersed throughout plant tissues through polar glycosides (Khoddami *et al.*, 2013). Methanol, which is more polar than ethanol, is better able to extract flavonoid components (Zuraida *et al.*, 2017).

DPPH Scavenging Activity

The antioxidant activity of the sample was determined by the degree of free radical inhibition of DPPH. The higher the inhibition level of the sample, the higher the antioxidant activity (Koleva *et al.*, 2002). The test solution was left for 30 min to allow a complete reaction between the compounds in the extract and the DPPH free radicals (Marinova & Batchvarov, 2011). Absorbance was measured at 517 nm because the DPPH solution could optimally absorb UV rays at this wavelength. In addition, the % inhibition value is calculated for each concentration of solution tested (Table 4, Figure 1).

Then the regression equation y = 11.041x – 36.39 with R2 = 0.8741 was obtained by plotting the Ln value of the test concentration (x-axis) against the % inhibition value (y-axis) (Figure 1).

Based on the IC_{50} value calculation, the methanol extract of Ruku-ruku leaves showed an IC_{50} value of 2501.07 µg/mL. At the same time, vitamin C as a comparative antioxidant had an IC_{50} value of 14,572 µg/mL. Based on the IC_{50} value, the antioxidant activity of the methanolic extract of Ruku-ruku leaves was classified as low. In contrast, the antioxidant activity of ascorbic acid (<50 ppm)was classified as very powerful antioxidant (Setha *et al.*, 2013). These results show that the antioxidant activity of the extracts tested in this study is very low compared to the antioxidant activity of ascorbic acid.

Antioxidant activity in plants is thought to be due to flavonoid and phenolic compounds. Flavonoid and phenolic compounds are known to have the ability to donate hydrogen atoms and chelate metal ions (Muchtadi, 2013).

Table 4. Antioxidant activity of methanol extract

Concentration (µg/mL)	Ln concentration	∑ Absorbance	% inhibition	IC_{50} (µg/mL)
1000	6.908	0.163±0.012	46.31	
500	6.215	0.196±0.003	31.08	
250	5.521	0.221±0.004	19.38	2501.07±1.2
125	4.828	0.237±0.005	12.00	
62.5	4.135	0.248±0.010	7.08	
31.25	3.442	0.244±0.005	8.62	

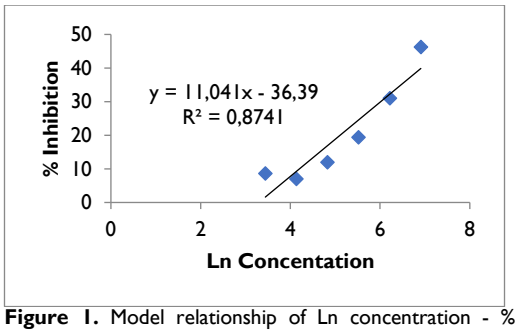

Figure 1. Model relationship of Ln concentration - % inhibition of methanol extract

The antioxidant activity of flavonoid compounds is affected by the pattern of hydroxylation and the presence of sugar groups. In addition, flavonoids have the strongest antioxidant activity due to their chemical structure containing o-diphenol groups and -OH groups at positions 3 and 5 (Halbwirth, 2010). The hydroxy group in the aromatic ring of phenolic compounds will donate hydrogen to free radicals, which will cause the species to become non-reactive and prevent tissue damage (Engwa, 2018).

However, based on this study, the methanolic extract of Ruku-ruku leaves did not have antioxidant activity (IC_{50} = 2501.07 µg/mL). Ruku-ruku leaves contain eugenol and linolool (Raina *et al.*, 2013). Dabire *et al.*, (2011), reported that the decrease in eugenol content in the essential oils, in presence of linalool, gave rise to a significant decrease in its antioxidant power (reduction in antioxidant potency of ±87%).

Conclusion

The methanol extract of Ruku-ruku leaves a total phenolic content value of 300.71 ± 2.86 mgGAE/g extracts and total flavonoid content of 66.42 ± 10.17 mgQE/g extract and had very low antioxidant activity (IC_{50} = 2501.07 µg/mL).

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