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Phytochemical content and antioxidant activity of Komba-komba (*Eupatorium odoratum* L)

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Abstract

Komba-komba (*Eupatorium odoratum* L) is a shrub, which is native to America. Traditionally, komba-komba is used to treat burns, wounds, skin infections, postpartum wounds, malaria, coughs and colds, and has anti-gonorrhoea, anti-inflammatory, anthelmintic, analgesic and antioxidant properties. This study aimed to investigate the phytochemicals from Komba-komba roots and *in vitro* antioxidant activity. Komba-komba root powder was macerated using methanol as solvent. Then fractionated with n-hexane, chloroform, and ethyl acetate. The antioxidant power was measured using the DPPH radical inhibition method and reducing agents using the FRAP method. Komba-komba root exhibited the strongest antioxidant activity. The ethyl acetate fraction showed the strongest antioxidant activity using both the DPPH method and the FRAP method with IC_{50} values, 10.0598±0.0665 g/mL, and 16.4782±0.3558 g/mL, respectively. The antioxidant activity of komba-komba root was correlated with its phenolic and flavonoid content. Komba-komba root showed very high phenolic and flavonoid contents to be developed as a source of natural antioxidants.

1. Introduction

Komba-komba (*Eupatorium odoratum* L) is a shrub, which is a native of America, present throughout the tropical forests of Asia, Australia, and West Africa and grows wild in the forest. The height of this plant reaches three to eight meters (Chakraborty *et al*., 2010; Amatya and Tuladhar, 2011; Bhargava *et al*., 2013). Traditionally, komba-komba is used to treat burns, wounds, skin infections, postpartum wounds, malaria, stomachache, and heartburn (Nwachukwu *et al*., 2016). It also exhibits anti-gonorrhoea, anti-inflammatory, anthelmintic, analgesic, and antifungal properties (Omokhua *et al*., 2017). In addition, it is also used in the treatment of catarrh, nasal congestion, diabetes, diarrhoea, fever, pertussis, rheumatism, and as a vermifuge (Vijayaraghavan *et al*., 2017). Komba-komba plants contain secondary metabolites of flavones, flavonoids, tannins, alkaloids, sesquiterpenes, phenolic compounds, and saponins (Omokhua *et al*., 2017). Several studies of Komba-komba (*E. odoratum*), especially the leaves, have shown bioactivity as antifungal, anticancer, antioxidant, anti-inflammatory, antiplasmodial, antidiabetic, and antibacterial (Putri and Fatmawati, 2019).

Antioxidants are substances found in food that can inhibit cell damage caused by free radicals by neutralizing them. Normal biochemical reactions in the body with increased exposure to nature and total levels of dietary xenobiotics result in the reproduction of reactive oxygen species (ROS) and reactive nitrogen species (RNS). ROS and RNS produce oxidative stress under various pathophysiological conditions. Oxidative stress is closely related to systemic inflammatory processes, endothelial cell proliferation, apoptosis, and vasoconstriction. Oxidative stress has a role in the occurrence of various diseases, primarily degenerative diseases such as cancer, diabetes mellitus, and atherosclerosis which is the cause of coronary heart disease or heart failure. Most natural antioxidants are obtained from plants. This is because plants contain many antioxidant compounds, mainly phenolic and polyphenolic compounds, including carotenoids and vitamins (Berawi and Agverianti, 2017; Rahman *et al*., 2020). This study aimed to investigate the phytochemical content of Komba-komba roots and *in vitro* antioxidant activity tests.

2. Materials and methods

2.1 Plant materials

Komba-komba roots were obtained from the forest in the village of Lepo-lepo, Kendari City, Southeast Sulawesi Province, Indonesia. Chemicals used were

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methanol, n-hexane, chloroform, gallic acid (Sigma-Aldrich®), quercetin (Sigma-Aldrich®), DPPH (Sigma-Aldrich®), FRAP (Sigma-Aldrich®), Folin-Ciocalteau reagent (Sigma-Aldrich®), aluminium chloride, $K_3Fe(CN)_6$, trichloroacetic acid.

2.2 Extraction

A total of 1 kg of komba-komba root powder was macerated using methanol solution 3 times, each for 24 hrs. Then, the filtrate obtained was concentrated using a rotary evaporator at 50°C to obtain methanol extract. A total of 90 g of methanol extract was partitioned using the liquid-liquid fractionation method based on the polarity of the solvent. Firstly, it was partitioned with n-hexane solvent, then chloroform, and finally using ethyl acetate. Then it was concentrated with a rotary evaporator to obtain the n-hexane fraction, chloroform fraction, ethyl acetate fraction, and water fraction. The extraction process is shown in Figure 1.

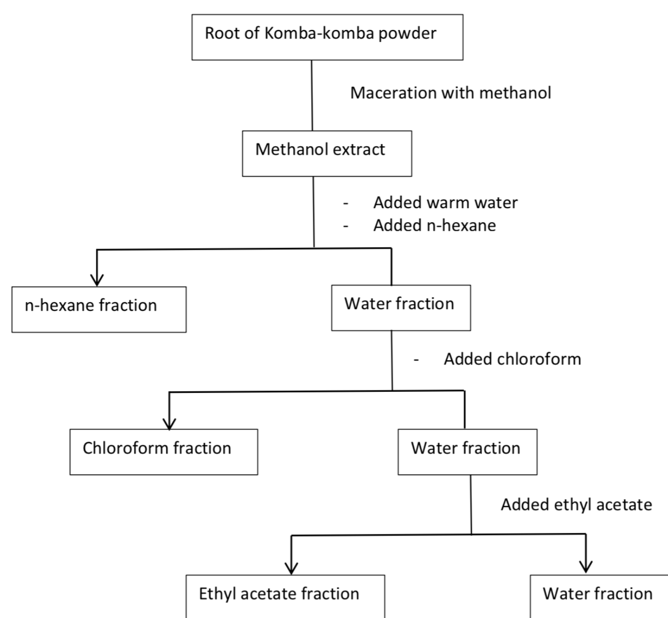

Figure 1. Schematic of extraction and fractionation of Komba-komba root

2.3 Phytochemical screening

Phytochemical screening of extracts and fractions of komba-komba root was done according to the method of Ngibad (2014) and Sabarudin *et al.* (2021).

2.4 Determination of Total Phenolic Content

The total phenolic content of komba-komba roots using the Folin-ciocalteu method was conducted according to Noreen *et al.* (2017). The Folin-Ciocalteu method is an electron transfer-based assay and provides a reduction capacity expressed as phenolic content. The total phenolic content of plant extracts and yields depend on the solvent selected for extraction. External calibration was carried out using different concentrations of gallic acid, namely, 10 µg/mL, 20 µg/mL, 30 µg/mL, 40 µg/mL, 50 µg/mL. Briefly, 1 mL of the sample was placed in a test tube, then 0.4 mL of Folin-Ciocalteau reagent was added, and allowed to stand for 5-8 mins. Then, 4 mL of 7% Na_2CO_3 solution was added and shaken until homogeneous. Then it was allowed to stand for 30 mins at room temperature then the absorbance was measured at 750 nm. The total phenolic content was calculated as milligrams gallic acid equivalent (mg GAE)/g sample using a gallic acid calibration curve.

2.5 Determination of total flavonoid content

Measurement of flavonoid content was carried out using the aluminium chloride method. The principle of the aluminium chloride method is the reaction of $AlCl_3$ to form a stable acid complex with a C-4 keto group and a C-3 or C-5 hydroxyl group of flavones and flavonols. In addition, it also includes labile acid complexes with ortho-dihydroxyl groups on rings A or B of flavonoids. Measurement of flavonoid content in komba-komba roots was conducted according to Bag *et al.* (2015) and Sembiring *et al.* (2018), with few modifications. External calibration using quercetin concentration series, 20 µg/mL, 40 µg/mL, 60 µg/mL, 80 µg/mL and 100 µg/mL. Briefly, 1 mL of the sample was added to 3 mL of methanol p.a, then 0.2 mL of 10% $AlCl_3$ and 0.2 mL of 1 M potassium acetate were added. Then complete the mixture to 10 mL with distilled water. It was shaken until homogeneous and incubated at room temperature for 30 mins. Then the absorbance was measured at 417 nm. The total flavonoid content was calculated as milligrams of quercetin equivalent (mg QE)/g sample using a quercetin calibration curve.

2.6 Measurement of antioxidant activity using DPPH method

The radical scavenging activity of komba-komba roots was measured against DPPH radicals following the method by Yamin, Ruslin, Mistriyani, Fitrawan *et al.* (2021) with a few modifications. Briefly, 3 mL of 0.6 mM DPPH solution was mixed with 2 mL of sample solution (concentration series 1 µg/mL, 2 µg/mL, 3 µg/mL, 4 µg/mL and 5 µg/mL), Then, 3 mL of methanol p.a was added, then incubated for 30 mins, and the absorbance was measured at 517 nm. Ascorbic acid was utilized as a positive control. The formula used to calculate radical scavenging activity was as followed:

$$\%\,Inhibition = \frac{A_{blank} - A_{sample}}{A_{blank}} \times 100\%$$

The IC_{50} value is obtained by replacing y with 50 in the linear regression equation y = bx + a, to obtain the value of x. The value of x is the IC_{50} value of the sample.

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2.7 Determination of Ferric Reducing Antioxidant Power

The measurement of antioxidant activity with the Ferric Reducing Antioxidant Power (FRAP) method was carried out according to Kusumawati and Haryoto (2014) with few modifications. Briefly, 1 mL of sample was added to 1 mL of 0.2 M phosphate buffer (pH 6.6), and 1 mL of 1% $K_3Fe(CN)_6$ and incubated. Then, 1 mL of 10% TCA was added to the potassium ferricyanide complex, precipitated then centrifuged at 3000 rpm for 10 mins to speed up the precipitation process. After centrifugation, 1 mL of the top layer was pipetted into a test tube with 1 mL of distilled water and 0.5 mL of 0.1% $FeCl_3$. The solution was allowed to stand for 10 mins and the absorbance was measured at 724 nm.

2.8 Data analysis

All data were analysed using SPSS 22 software. The data displayed presented as mean ± SD.

3. Results and discussion

3.1 Phytochemical screening

The phytochemical screening results showed that the extract and fraction of Komba-komba root contained flavonoids, tannins, alkaloids, terpenoids, and saponins as shown in Table 1.

3.2 Antiradical activity test using the DPPH method

The antioxidants mechanism consists of 5 mechanisms, namely radical scavenging, chelating agents, reducing power, inhibition of lipid peroxides, and synergists. The free radical scavenging mechanism is widely reported in evaluating the antioxidant capacity of plant sources because the activity of the DPPH radical is a stable radical, it can be used to evaluate antioxidant activity, which requires a relatively short time. The 2,2-diphenyl-1-picrylhydrazyl is a radical that is soluble in non-polar solvents (Permatasari and Rohman, 2016). Sharma and Bhat (2009) optimized the use of solvents on DPPH radicals, the results showed that the most sensitive solvents for measuring DPPH radical activity were methanol and methanol buffer, indicated by their ability to provide high absorbance values. Thus, in this study, methanol was used as a solvent.

DPPH has been widely used as a radical to evaluate reducing agents and reagents with antiradical activity. The occurrence of antiradical activity is characterized by a decrease in colour intensity caused by the event of the DPPH-H pair. The fading of DPPH colour depends on the stoichiometry of the number of electrons attached (Fatiha and Abdelkader, 2019).

The results showed that the extract and fraction of Komba-komba root reduced DPPH radicals, seen by the change in colour of DPPH when given a sample solution changing from purple to yellow indicates the formation of the DPPH-H bond (Raghavendra and Prashith Kekuda, 2018; Yamin, Sabarudin, Zubaydah *et al.*, 2021).

The reaction mechanism between DPPH radicals and antioxidants as shown in Figure 2.

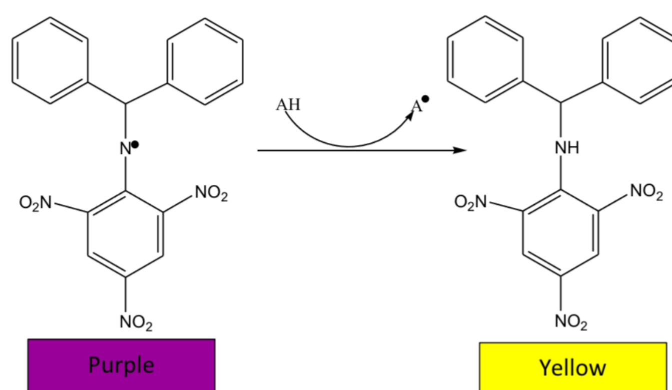

Figure 2. DPPH-H formation reaction by antioxidants

The antiradical activity of extracts and fractions of Komba-komba root are shown in Table 2, with ascorbic acid as the positive control. The results showed that the extract and fraction of komba-komba root showed the strongest antiradical activity against DPPH radicals. A sample is declared to have the strongest antiradical activity if the IC_{50} value is IC_{50} < 100 µg/mL (Cane *et al.*, 2020).

Table 2 shows that the ethyl acetate fraction has the strongest antiradical activity compared to the chloroform fraction, methanol extract, n-hexane fraction, and water fraction. Shown by the IC_{50} values 10.0598±0.0665 µg/mL, 13.6388±0.00334 µg/mL, 14.7384±0.2552 µg/mL, and 24.3504±0.2261 µg/mL, respectively. As the standard ascorbic acid used. This is in line with previous studies, including the ethyl acetate fraction of the Red Fruit (*Pandanus conoideus* Lam) by Rohman *et al.* (2010), ethyl acetate fraction of *Hagenia abyssinical* root

Table 1. Results of phytochemical screening of extracts and fractions of komba-komba root

Sample	Test				
	Flavonoid	Tannin	Alkaloid	Terpenoid	Saponins
Methanol extract	+	+	+	+	+
n-hexane fraction	+	+	+	+	+
Chloroform fraction	+	+	+	+	+
Ethyl acetate fraction	+	+	+	+	+
Water fraction	+	+	+	+	+

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by Fan *et al.* (2020), ethyl acetate fraction of *Clerodendrum cyrtophyllum* Turcz by Zhou *et al.* (2020), ethyl acetate fraction of jackfruit peel by Yamin, Ruslin, Mistriyani, Sabarudin *et al.* (2021), and the ethyl acetate fraction of pumpkin leaf by Sabarudin *et al.* (2021).

3.3 Ferric Reducing Antioxidant Power

The addition of TCA solution serves to precipitate the potassium ferrocyanide complex. Meanwhile, the addition of ferric chloride aims to form a green to blue complex. Antioxidant potential using the FRAP method was measured by the antioxidant ability of the compound to reduce Fe^{3+} compounds to Fe^{2+}. The process of reducing Fe^{3+} to Fe^{2+} is marked by a change in the colour of the Fe^{3+} solution (yellow) to Fe^{2+} (green) caused by Fe^{3+} radicals receiving electrons from antioxidant compounds (Kusumawati and Haryoto, 2014; Mbaye *et al.*, 2017; Maesaroh *et al.*, 2018) as shown in the following chemical reaction (Figure 3):

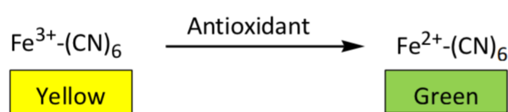

Figure 3. The process of reducing Fe^{3+} to Fe^{2+} by antioxidants

The data in Table 2 shows that the ethyl acetate fraction has a very strong antioxidant activity using the FRAP method compared to the chloroform fraction, methanol extract, n-hexane fraction, and water fraction, with IC_{50} values, 16.4782±0.3558 µg/mL, 19.1196±0.2416 µg/mL, 24.6635±0.6203 µg/mL, 25.0248±0.4413 µg/mL, and 39.1505±0.3997 µg/mL, respectively. The positive control used in this research was ascorbic acid. These results are in line with previous studies, including the ethyl acetate fraction of citrus fruits from Korea (Assefa *et al.*, 2016), ethyl acetate fraction of Rambutan peel (*Nephelium lappaceum* L) (Mistriyani *et al.*, 2018), and ethyl acetate fraction of *Clinacanthus nutans* (Burm F.) Lindau leaves extracts (Murugesu *et al.*, 2019).

Table 2. The antioxidant activity of komba-komba root using DPPH and FRAP methods

Sample	IC_{50} value (µg/mL)	
	DPPH	FRAP
Methanol extract	14.7384±0.2552	25.0248±0.4413
Hexane fraction	15.6888±0.0532	24.6635±0.6203
Chloroform fraction	13.6388±0.00334	19.1196±0.2416
Ethyl acetate fraction	10.0598±0.0665	16.4782±0.3558
Water fraction	24.3504±0.2261	39.1505±0.3997
Ascorbic acid	3.6841±0.0453	4.7507±0.0636

3.4 Total phenolic and flavonoid contents

Antioxidant activity correlates with the phenolic and flavonoid content contained in a sample. The antioxidant activity of phenolic and flavonoid compounds is determined by the presence of free hydroxy groups present in the phenolic molecule. The antioxidant activity of phenolics increases when there are free hydroxy groups in the molecule. Meanwhile, the effectiveness of free radical scavenging by flavonoid compounds is the hydroxy position in ring B. The hydroxy position that provides higher stability against free radical scavenging is at the 3', 4'-O-dihydroxy position on ring B because at this position, the electrons in the form of radicals in the flavonoid structure will undergo a delocalization process (Mistriyani *et al.*, 2018; Fan *et al.*, 2020).

Table 3 shows the phenolic and flavonoid content of Komba-komba root (*Eupatorium odoratum* L). The ethyl acetate fraction has a high total phenolic content compared to the chloroform fraction, n-hexane fraction, methanol extract, and water fraction, with values 28.0526±1.8007 mg/g, 27±1.0526 mg/g, 24.0175±1.8039 mg/g, 23.1404±1.0956 mg/g, and 10.6842±1.0526 mg/g, respectively. Meanwhile, the flavonoid content of Komba-komba root showed that the ethyl acetate fraction had a higher flavonoid content than the chloroform fraction, n-hexane fraction, methanol extract, and water fraction, with values of 54.5298±3.9237 mg QE/g, 47.4464±1.1318 mg QE/g, 39.5893±3.0788 mg QE/g, 34.5298±2.1114 mg QE/g, and 31.7441±1.3447 mg QE/g. The data on phenolic and flavonoid content in Table 3 and the IC_{50} value in Table 2 show that the more phenolic content of a sample, the stronger its potential as an antioxidant. Therefore, the antioxidant activity correlates with the phenolic and flavonoid content in the sample (Rohman *et al.*, 2006). The correlation of phenolic and flavonoid content to antioxidant activity (IC_{50} value) in Komba-komba roots is shown in Figure 4 and Figure 5.

Table 3. Phenolic and flavonoid content of komba-komba root (*Eupatorium odoratum* L)

	Total phenolic content (mg GAE/g sample)	Total flavonoid content (mg QE/g sample)
Water fraction	1.6842±1.0526	31.7441±1.3447
Ethyl acetate fraction	28.0526±1.8977	54.5298±3.9237
Methanol extract	23.1404±1.0956	34.5298±2.1114
Hexane fraction	24.0175±1.8039	39.5893±3.0788
Chloroform fraction	27±1.0526	47.4464±1.1318

Figure 4A shows the correlation of phenolic content in scavenging DPPH radicals of R^2 = 0.9515. This shows that phenolic compounds in Komba-komba contribute 95.15% to inhibiting DPPH radicals, while Figure 4B shows the correlation of flavonoid content in inhibiting DPPH radicals of R^2 = 0.7179. This shows that phenolic compounds in Komba-komba contribute 71.79% to

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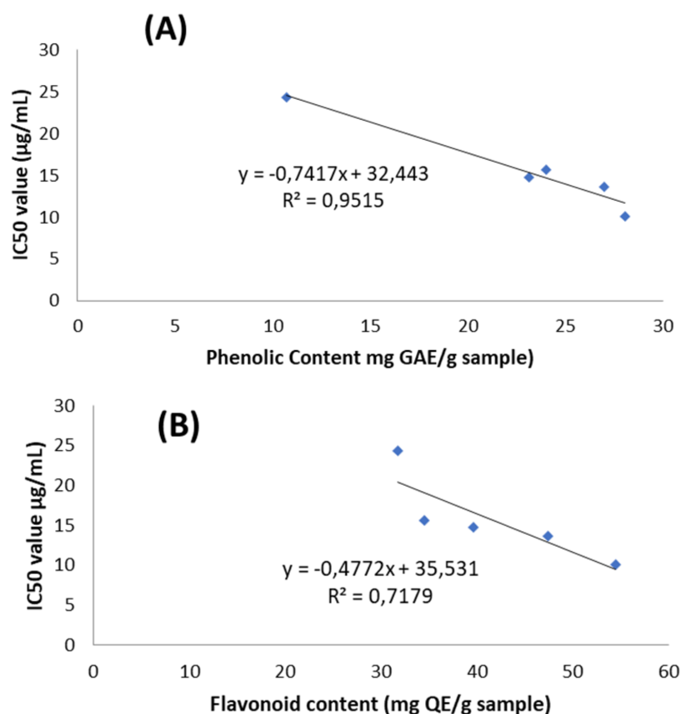

Figure 4. Correlation between (A) phenolic content and (B) flavonoid content with IC_{50} DPPH value

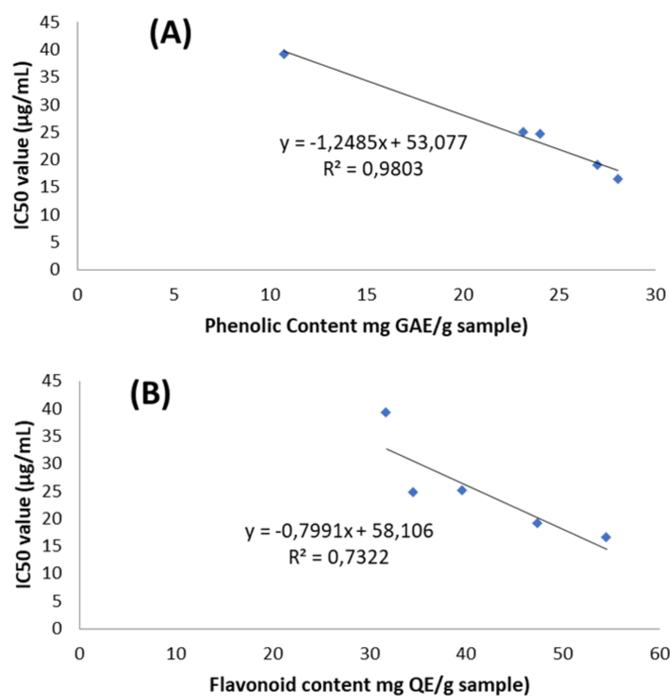

Figure 5. Correlation between (A) phenolic content and (B) flavonoid content with the antioxidant activity using the FRAP method

inhibiting DPPH radicals. Figure 5A shows the correlation of phenolic content in reducing Fe^{3+} to Fe^{2+} of R^2 = 0.9808. This shows the ability of phenolic compounds to reduce Fe^{3+} by 98.08%. Figure 4B shows the correlation between the content of flavonoid compounds and reducing agents of R^2 = 0.7322. This shows the ability of flavonoid compounds to reduce Fe^{3+} by 73.22%.

4. Conclusion

The root of Komba-komba can scavenge DPPH radicals and act as a reducing agent by using the FRAP method. The ethyl acetate fraction has the strongest antioxidant properties through the DPPH method and the FRAP method. The phenolic and flavonoid content is strongly related to its antioxidants. Therefore, Komba-komba root can be developed as a natural source of antioxidants.

Conflict of interest

The authors declare no conflict of interest.

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