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FULL PAPER

Determination of total phenolic and flavonoid content of Ghontoghe (*Kleinhovia hospita* L.) leaves: *in vitro* antioxidant study

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Abstract

The Ghontohge plant (*Kleinhovia hospita* L.) has been used as a traditional medicine to treat scurvy, lice, eye pain, jaundice and hepatitis, cholesterol, diabetes, hypertension and treat pneumonia. Therefore, it is possible that plants can be used as antioxidants and redox signalling sourced from natural materials. The purpose of this study was to investigate the antioxidant potential and evaluate the total phenolic and flavonoid content of Ghontoghe leaves and investigate the most potent fraction as an antioxidant from the extract and fractions of Ghontoghe leaves. Ghontoghe leaf powder was extracted using the maceration method. Antioxidant activity was evaluated using 2,2-diphenyl-1-picryhidrazyl (DPPH) and 2,2-Azinobis 3-ethyl benzothiazoline 6-sulfonic acid radicals (ABTS). The total phenolic content was determined using the Folin-Ciocalteau method. Meanwhile, the total flavonoid content was determined using the aluminium chloride complex colourimetric method. The ethyl acetate fraction showed the strongest antioxidant activity on both DPPH radicals and ABTS radicals with IC_{50} values of 6.253±0.008 g/mL and 6.486±0.023 g/mL, respectively. The ethyl acetate fraction also showed high phenolic and flavonoid content with values of 26.92±0.10 mg GAE/g sample and 95±0.69 mg QE/g sample, respectively. The ethyl acetate fraction has the potential to be used as a source of natural antioxidants and has the potential to be used as a functional food.

1. Introduction

Ghontoghe plant (*Kleinhovia hospita* L.) is a plant that has been used as traditional medicine in several countries such as Malaysia, Indonesia, and Papua New Guinea. Traditionally used to treat scurvy, lice, eye pain medication, jaundice and hepatitis (Arung *et al*., 2009; Djabir *et al*., 2019). In addition, this plant has benefits such as lowering cholesterol, diabetes, hypertension and treating pneumonia (Rizky *et al*., 2018). Therefore, plants are used as antioxidants and redox signalling, and most of the literature reports that plants have potential as antioxidant drugs. Antioxidants from natural ingredients become an alternative to synthetic antioxidants (Lukitaningsih *et al*., 2020).

Natural antioxidants are defined as chemical compounds that can counteract the damaging effects of oxidative physiological processes in tissues. Natural antioxidants are also described as compounds that act as the body's biological defence against the harmful effects of excessive oxidation processes. (Lukitaningsih *et al*., 2020; Rohman *et al*., 2020). In addition, antioxidants are also defined as compounds that play a role in reducing the formation of free radicals, namely by inhibiting the autoxidation of lipids or other molecules by inhibiting the initiation or propagation of oxidative chain reactions, so that these compounds can prevent cell damage (Ningsiha *et al.,* 2016). Thus, these compounds may benefit from restoring normal functions and diseases that occur due to exposure to free radicals. Therefore, antioxidant compounds have an essential role in the body's defence against damage caused by free radicals (Ibrahim *et al*., 2020; Yamin, Ruslin, Sartinah *et al*., 2020). Among all the metabolites obtained from plants, the most promising metabolites for research both *in vitro* and *in vivo* as antioxidants are phenolic compounds. Phenolics from plants are grouped into five main groups, phenolic acids, flavonoids, lignans, stilbenes and tannins (Kowa *et al.*, 2018). Flavonoids are the main antioxidant compounds. Therefore, flavonoids are compounds that have the potential to be developed as candidates for antioxidant drugs. (Sagandykova *et al*., 2020). Therefore, the purpose of this study was to investigate the antioxidant potential and to evaluate the total phenolic and flavonoid content of Ghontoghe leaves. In addition, another objective of this study was to investigate the

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most potent fraction as an antioxidant from the extract and fraction of Ghontoghe leaves.

2. Materials and methods

2.1 Materials

Ghontoghe fresh leaves from Halu Oleo Botanical garden, Kendari City, Southeast Sulawesi Province, in April – May 2019. Authenticated leaves material at the Laboratory of Biology, MIPA, University of Halu Oleo. The fresh leaves (10 kg) were washed under tap water to remove sand and debris and was air-dried under shade for ten days. The final weight after drying was 750 g and powdered using a mechanical grinder. 2,2-diphenyl-1-picrihidrazyl (DPPH) (Sigma-Aldrich®, USA), methanol (E. Merck, Germany), 2-2-Azinobis 3-ethyl benzothiazoline 6-sulfonic acid (ABTS) (Sigma-Aldrich®, USA), Galic Acid (Sigma-Aldrich®, USA), ethyl acetate, Quercetin (Sigma-Aldrich®, USA), and n-hexane.

2.2 Extraction

A total of 750 g of Ghontoghe leaf powder were macerated with 15 L methanol for 72 hrs. The extract obtained was concentrated using a rotary evaporator to obtain crude extract (125.8 g). The crude extract (90 g) was partitioned with the aid of a separating funnel into hexane-soluble (29.3 g), chloroform soluble (10.4 g), ethyl acetate soluble (3.9 g), and water fractions (47.6 g).

2.3 Determination of total flavonoid content

The flavonoid content of Ghontoghe leaves was determined using the aluminium chloride colourimeter method as carried out by Zou *et al.* (2004) with modification. Briefly, 1 mL of sample was then added with 0.2 mL of 10% $AlCl_3$ and 0.2 mL of potassium acetate, then added distilled water until 10 mL. Then incubated at room temperature for 30 mins, then the absorbance was measured at 409 nm. The total flavonoid content was expressed as quercetin equivalent (QE) in mg/g samples

2.4 Determination of total phenolic content

The phenolic content of Ghontoghe leaves was determined using Folin-Ciocalteau reagent, as was the procedure performed by Permatasari and Rahman (2016), with slight modification. Briefly, 1 ml of the sample was added with 0.4 mL of Folin-Ciocalteau reagent and 4.0 mL of 7% sodium carbonate. Then, made up the volume was to 10 mL by adding distilled water, then incubated for 30 mins. The absorbance was measured at 750 nm

2.5 Evaluation of antioxidant activity

2.5.1 DPPH

The measurement principle of the DPPH method is based on electron transfer, and the process is carried out in a dark room without exposure to light at room temperature, which is indicated by a constant violet colour. In this study, the antioxidant activity of Ghontoghe leaves was measured using a spectrophotometer at 515 nm, as described by Yamin, Ruslin, Mistriyani *et al*. (2021). The following equation can calculate the scavenging activity of Ghontoghe leaves on DPPH radical:

$$\% \text{ inhibition} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100$$

The IC_{50} value was obtained by plotting the percentage of antioxidant activity against the sample concentration (µg/mL), defined as the concentration of the sample necessary to cause 50% scavenging of DPPH radical calculated by linear regression curve (Rohman *et al*., 2006).

2.5.2 ABTS

The antiradical activity of the sample against ABTS was measured using UV-Vis spectrophotometer (Smith *et al*., 2018; Yamin, Sabarudin, Zubaydah*, et al.,* 2021). Briefly, 7 mM mixed ABTS solution with 2.45 mM potassium persulfate in a ratio of 1: 1 and then left in the dark for 12-16 hrs to produce a radical cation stock solution. This solution was then measured using a spectrophotometer at an absorbance between 0.6 to 0.8. Next, 3 mL of ABTS solution was mixed with 30 L of blank, sample or standard solutions. The absorbance was measured at 745 nm. . The ability of sample to scavenge ABTS cation radical is calculated using the equation:

$$\% \text{ inhibition} = \frac{\text{Absorbance blank} - \text{Absorbance sample}}{\text{Absorbance blank}} \times 100$$

The IC_{50} value was obtained by plotting the percentage of antioxidant activity against the sample concentration (µg/mL), defined as the concentration of the sample necessary to cause 50% scavenging of ABTS cation calculated by linear regression curve (Rohman *et al*., 2006)

2.6 Data analysis

All data were analysed using SPSS version 24 using mean±standard deviation (SD).

3. Results and discussion

Interest in medicinal plants has recently become very popular in the community. This is because the costs used are minimal, and the side effects are relatively small. In

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addition, secondary metabolites contained in plant parts provide an excellent therapeutic effect. Phytochemical compounds found in plants are reported to have anticancer, antimicrobial, antiplasmodic, anti-inflammatory, antidiabetic and antihypertensive activities (Smith *et al.*, 2018).

3.1 Antioxidant activity test by DPPH

The DPPH method is a method that is widely applied to estimate antioxidant activity in recent years. DPPH is a stable free radical. When reacting with antioxidant compounds, the colour of the DPPH radical will change from purple to yellow. The IC_{50} value determined the radical scavenging strength of the DPPH radical. The lower the IC_{50} value of a sample, the stronger the antioxidant activity (Parchin *et al.,* 2015; Enengedi *et al.,* 2019; Sabarudin *et al.,* 2021). The DPPH radical is a radical that is very sensitive to polar solvents, such as methanol and ethanol (Yamin, Ruslin, Mistriyani *et al*., 2021). Therefore, in this study, the solvent used was methanol.

Figure 1 shows the IC_{50} value of Ghontoghe leaf extract and fraction using the DPPH radical, which has a powerful antioxidant activity. This is as shown by Cane *et al*. (2020) where the IC_{50} value is <50 ppm, it is included in the very strong category.

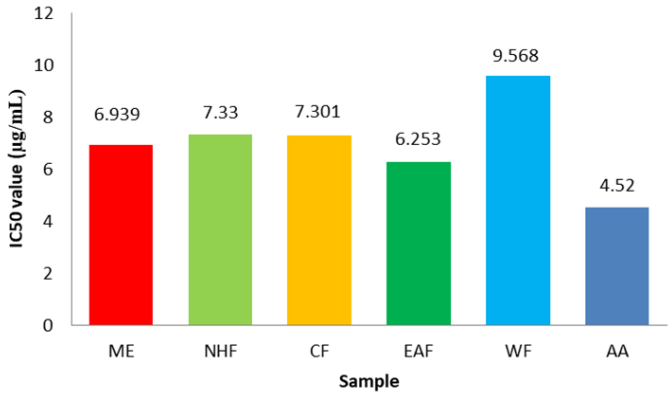

Figure 1. The IC_{50} value DPPH methods of the extract and the variation of the fraction

This study used ascorbic acid as a standard, the data shown in Figure 1 shows that the ethyl acetate fraction showed a very strong antioxidant activity compared to the methanol extract, chloroform fraction, n-hexane fraction and water fraction with IC_{50} values of 6.253±0.008 µg/mL, 6.939±0.002 µg/mL, 7.301±0.07 µg/mL, 7.330 ±0.026 µg/mL, and 9.568±0.708 µg/mL, with ascorbic acid standards.

This is in line with previous studies, which showed that the ethyl acetate fraction showed stronger antioxidant activity than the extract, n-hexane fraction, chloroform fraction and water fraction. These studies include the ethyl acetate fraction of the Raghu bark (Yamin, Ruslin, Sartinah *et al*., 2020), Pumpkin (*Cucurbita moshata* Duch) leaf ethyl acetate fraction (Sabarudin *et al*., 2021), ethyl acetate fraction of langsat seeds (Yamin, Ruslin, Sabarudin *et al*., 2020) and ethyl acetate fraction of jackfruit seeds (Zubaydah *et al*., 2021) and the ethyl acetate fraction of the bark of *Oroxilum indicum* Linn (Trang *et al*., 2018). This shows that polar and semi-polar compounds such as flavonoids, tannins, sterols, and anthraquinones can scavenge free radicals or act as antioxidants. (Vifta *et al*., 2019).

3.2 Antioxidant activity test with ABTS

Antioxidant testing using the ABTS method was carried out to determine antioxidant compounds' ability to reduce ABTS free radicals. Testing with ABTS is based on a decrease in the intensity of the blue/green colour of $ABTS^{*+}$, due to reduced antioxidants. The antioxidant compounds contained in the sample are in the form of phenolic compounds or flavonoids. Flavonoids react with ABTS+ cations to form ABTS, which is a more stable radical or non-radical compound. The decrease in absorbance indicates the ability of antioxidant compounds to reduce $ABTS^{*+}$ (Vifta *et al.*, 2019).

The antioxidant activity using ABTS methods is the best method compared to other methods because the ABTS method can determine the antiradical capacity of hydrophilic and lipophilic antioxidants because it can be used in organic and aqueous solvent systems (Labiad *et al.*, 2017)

ABTS is often used to measure antioxidant capacity in the food industry and agricultural research. The ABTS assay was also used to measure the relative ability of antioxidants to scavenge ABTS radicals compared to standard Trolox (Yamin, Sabarudin, Zubaydah *et al*., 2021).

Table 1 shows that the extract and fraction of Ghontoghe leaves have very strong antioxidant power, this is as shown by Cane *et al*. (2020), if the IC_{50} value is <50 ppm, it is included in the very strong category. The results in Table 1 showed ethyl acetate fraction has a very strong antioxidant activity compared to the methanol extract, n-hexane fraction, chloroform fraction, and water fraction with IC_{50} values of 6.486±0.023 µg/mL, 6.542±0.032 µg/mL, 7. 430±0.009 µg/mL, 8. 454±0.043 µg/mL, and 9. 877±0.032 µg/mL respectively, ascorbic acid was used as the standard. This result is in line with previous studies, which showed that the ethyl acetate fraction was stronger as an antioxidant than the other fractions. These studies include the ethyl acetate fraction from rambutan fruit peels (Mistriyani *et al.,* 2018), and the ethyl acetate fraction of Kamena-

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mena leaves (Yamin, Sabarudin, Zubaydah *et al*., 2021).

These results indicated that the strongest antioxidant compounds' capacity to reduce ABTS may be readily soluble in semi-polar solvents such as ethyl acetate. However, soluble compounds in other solvents from polar to non-polar solvents such as water-ethanol and n-hexane can provide antioxidant capacity to block ABTS radicals (Nur *et al*., 2019).

Table 1. IC_{50} values of extracts and fractions of Ghontoghe leaves using the ABTS method

Sample	IC_{50} value (µg/mL)
Methanol extract	6.542±0.032
n-hexane fraction	7.430± 0.009
Chloroform fraction	8.454± 0.043
Ethyl acetate fraction	6.486± 0.023
Water fraction	9.877±0.032
Ascorbic Acid	4.481± 0.021

3.3 Total phenolic and flavonoid content

The antioxidant power of the ethyl acetate fraction of Ghontoghe leaves may be related to the phenolic and flavonoid content contained in the extract and fraction (Rusmana *et al*., 2017). Table 2 data showed that the ethyl acetate fraction has a higher total phenolic and flavonoid content than the extract, n-hexane fraction, chloroform fraction and water fraction with values of 95±0.69 mg/g sample, respectively. 93.27±0.61 mg QE/g sample, 76.67±0.52 mg QE/g sample, 87.47±0.57 mg QE/g sample, and 59.0.57 mg QE/g sample, respectively for flavonoids. Meanwhile, phenolic values showed 26.92±0.10 mg GAE/g sample, 23.52±0.09 mg GAE/g sample, 13.66±0.199 mg GAE/g sample, 21.60±0.006 mg GAE/g sample, and 13.28±0.07 mg. GAE/g sample, respectively.

The antiradical activity of natural compounds is usually correlated with the presence of phenolic compounds and flavonoid compounds. Phenolic compounds contribute to radical scavenging activity mainly due to their redox properties and the presence of high hydrogen mobility in their molecular structure (Arina and Rohman, 2013; Lewoyehu and Amare, 2019). This is because phenolic compounds have hydroxyl groups, o-hydroxy groups, which significantly affect free radical scavenging. In addition, polyphenol compounds such as flavonoids indicate an ortho-dihydroxy structure in ring B, 2,3 double bonds in conjugation with a 4-oxo function in ring C, hydroxy groups at positions 3 and 5 on ring A, or the angle between the inner circle. The structure of the compound causes an antioxidant effect. A decrease indicated antiradical in extract and fractions in the absorbance value in the sample (Aadesariya *et al*., 2017; Rusmana *et al*., 2017).

Table 2. Total flavonoid and phenolic content of *Ghontoghe* leaves

Sample	Flavonoid (mg/g sample)	Phenolic (mg/g sample)
Methanol extract	93.27±0.61	23.52±0.09
n-hexane fraction	76.67±0.52	13.66±0.199
Chloroform fraction	87.47±0.57	21.60±0.006
Ethyl acetate fraction	95±0.69	26.92±0.10
Water fraction	59.5±0.57	13.28±0.07

The ability of phenolic compounds and flavonoids to scavenge free radicals is indicated by the correlation value (R^2), which is the relationship between phenolic or flavonoid levels on the x-axis and the IC_{50} value y-axis. Based on Figure 2 and Figure 3 shows the correlation between phenolic compounds on DPPH and ABTS radical scavenging R^2 = 0.6143 or (61.43%) and R^2 = 0.4838 (48.38%), respectively. Meanwhile, the contribution of flavonoids scavenging DPPH and ABTS radicals was R2 = 0.8965 (89.65%), and R^2 = 0.7446 (74.46%), respectively.

4. Conclusion

Extracts and fractions of Ghontoghe leaves showed antiradical activity using the DPPH and ABTS radical methods. Among the samples evaluated, the ethyl acetate fraction of Ghontoghe leaves showed very strong antioxidant activity and very high phenolic and flavonoid content. Ghontoghe leaves can be used as a source of natural antioxidants and can be used as functional food ingredients.

Conflict of interest

The authors declare no conflict of interest.

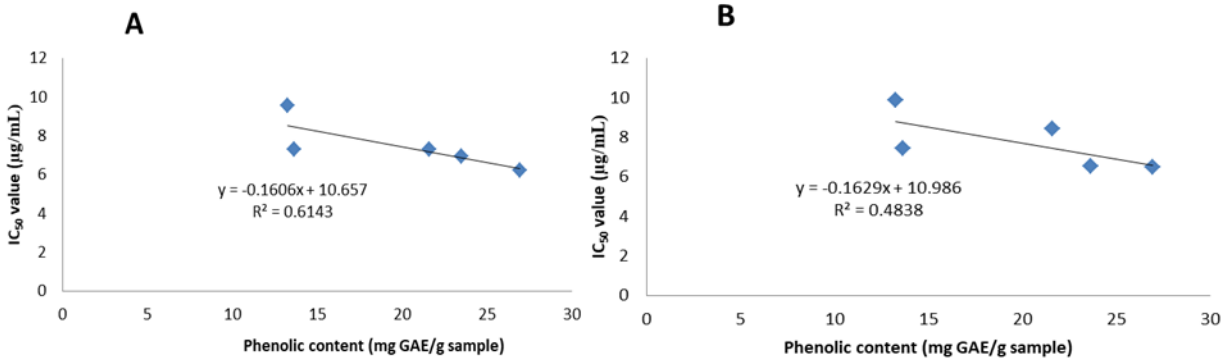

Figure 2. Correlation of phenolic content with IC_{50} values radical DPPH (A) and ABTS (B)

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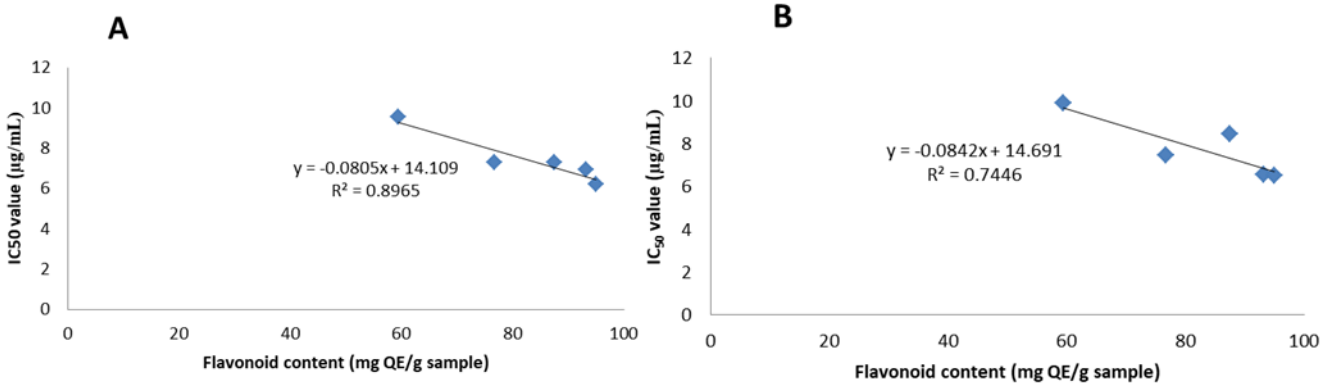

Figure 3. Correlation of flavonoid content with IC_{50} values radical DPPH (A) and ABTS (B)

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