Available online: http://journal.ipb.ac.id/index.php/jgizipangan
Accredited based on DGHE, Republic of Indonesia No.12/M/Kp/II/2015
ISSN 1978-1059 EISSN 2407-0920

J. Gizi Pangan, July 2019, 14(2):61-68
DOI: 10.25182/jgp.2019.14.2.61-68

Total Phenolic Content, Quercetin, and Antioxidant Activity of Gandaria (*Bouea Macrophylla* Griff.) Leaf Extract at Two Stages of Maturity

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ABSTRACT

The aim of this study was to determine the effect of leaf maturity stage and extraction solvents on Total Phenolic Content (TPC), quercetin, and antioxidant activity of gandaria leaves as well as the relationship of TPC and quercetin content to antioxidant activity. In this study, leaves were extracted using maceration method with three different solvents (96% ethanol, ethyl acetate, and hexane) followed by evaporation using rotary evaporator to obtain Crude Extracts (CE). Tender and mature gandaria leaves from Indonesian Institute of Science were examined for TPC and quercetin content. Measurement of antioxidant activity were performed by Ferric Reducing Antioxidant Power (FRAP) method. The antioxidant compound analysis indicated that hexane extract of mature gandaria leaves contained the highest TPC and quercetin content (30.84±0.54 mg GAE/g and 4.36±0.23 mg QE/g, respectively). Meanwhile, the ethanol extract of mature gandaria leaves demonstrated the highest reducing power (5.62±0.38 mg FeSO4 equivalent/g). These findings showed positive correlation between TPC and reducing power as well as quercetin and reducing power from gandaria leaf extracts depending on their maturity stage and extraction solvents. In addition, mature gandaria leaves had better extraction yield, making it potential for development of functional food.

Keywords: antioxidant, extraction solvent, gandaria leaf, quercetin, total phenolic content

INTRODUCTION

Gandaria (*Bouea macrophylla* Griffith) is a group of tropical plants emanating from the Anacardiaceae family. Gandaria originated from the area of West Java and North Sumatra and spread to in the ASEAN region including Indonesia, Malaysia, and Thailand. The edible parts of the plant are leaves and fruit. Ripe fruits (sweet to sour in taste) can be consumed directly or processed into various beverages, jellies, and dried snacks, while unripe fruits (sour) are usually pickled and/or made into salad (Rajan *et al*. 2014). Tender gandaria leaves are purplish green and they become dark green following maturation. According to Lim (2012), tender gandaria leaves can be eaten traditionally as "lalapan" in West Java.

The stage of maturity of a plant affects composition of its bioactive components. Several studies have reported the maturity stage of leaf influences its antioxidant compounds thus its antioxidant potentials (Felicia *et al*. 2016; Andriyani *et al*. 2010; Bhuvaneshwari *et al*. 2014). The active compounds in Gandaria plant mostly from phenolic and flavonoid groups and often studied in the fruit. (Rajan and Bhat 2017; Lolaen *et al*. 2013). Therefore, it is also important to investigate the leaf extract. Since it is more abundant, less expensive, and easier to harvest without seasonal limitation. Various active compounds in mapraang/gandaria have been confirmed by Thummajitsakul and Silprasit (2017). In Indonesia, Andina and Musfirah (2017) have found several active compounds of ramania/gandaria leaf cortex extract from South Kalimantan.

Extraction solvents such as water, ethanol, methanol, dichloromethane, ethyl acetate, and hexana are widely used to extract antioxidant compounds (Bakhouche *et al*. 2015; Hoon *et al*. 2015). The type of extraction solvents affect the TPC and antioxidant activity due to differences in solvent polarity. In addition, Sun *et al*. (2012) reported that the effect of solvent on extraction methods were based on their properties. The use of solvent in the analysis of antioxidant activity affects the extraction of secondary metabolites. Rajan and Bhat (2016) found that methanol and ethanol are the most effective solvent in extracting

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antioxidant compounds in Gandaria. The results also mentioned that the active components of unripe gandaria fruits were higher than those in ripe gandaria fruits. There has been no scientific reports available on antioxidant composition or activities exhibited by tender and mature gandaria leaves. Therefore, the purpose of this study was to determine the effect of leaf maturity stage and extraction solvents on TPC, quercetin, and antioxidant activity of gandaria leaves, as well as the relationship between TPC and quercetin with antioxidant activity.

METHODS

Design, location, and time

The study used a nested design where maturity of leaf (tender and mature) and three different solvents (ethanol, ethyl acetate, and hexane) were defined as factors. Thus there were 6 treatments and each treatment was replicated three times. Gandaria leaves were obtained from Indonesian Institute of Science, Cibinong, Bogor. The antioxidant extraction and analysis was performed in the Nutritional and Biochemistry Laboratory, Department of Community Nutrition, Faculty of Human Ecology, IPB University. This research was conducted from October to December 2018.

Materials and tools

The standards for gallic acid, quercetin, and iron (II) sulfate heptahydrate were purchased from Merck (Merck KGaA, Darmstadt Germany). Reagents such as Folin-Ciocalteu, sodium carbonate, sodium nitrite, aluminium chloride hexahydrate, iron (III) chloride were obtained from Merck (Merck KGaA, Darmstadt Germany). The 2,4,6-tris2-pyridyl-5-triazine (TPTZ) was purchased from Sigma-Aldrich (St. Louis, MO, USA). All spectrophotometric readings were done using a UV-visible spectrophotometer (UV-1800 Shimadzu, Kyoto, Japan).

Procedures

Extracts preparation. Tender and mature gandaria leaves were collected and cleaned to remove dust and other materials. They were air dried and ground using a blender at 20000 rpm for 10 second per batch. The extraction of tender and mature gandaria leaves were done by maceration method by Andina & Musfirah (2017) that that has been modified. Samples were extracted with solvents (1:3, w:v) such as 96% ethanol, ethyl acetate, and hexane, separately at room temperature for 48 h. Afterward, the infusions were filtered with Whatman No. 41 (CAT No. 1441-090) filter paper and leaf extracts were re-extracted with an equal volume of solvents for 48 h. The supernatants collected were evaporated using a rotary evaporator (IKA RV.8) at 40^{o}C, 60-80 rpm to obtain crude extracts. Extract was kept in light-protected eppendorf tube at 4^{o}C while preparing all analysis. Percentage of extraction yield was obtained from weight of crude extract (g) weight of sample (g) and times with 100.

Measurement of total phenolic content. Total phenolic content was determined by the Singleton and Rossi method (1965). As much as 1 ml extract (1 mg/ml) was mixed with 1 ml Folin-Ciocalteu reagent then added with 1 ml of Na_2CO3 solution (0.1 mg/ml distilled water). After 90 minutes incubation in the dark at room temperature, the absorbance was measured with a spectrophotometer at 765 nm. The standard used was gallic acid and results were determined as mg of GAE/g of CE.

Quercetin analysis. Quercetin analysis refered to Sanghavi *et al*. (2014). Two grams of gandaria leaf extract was extracted consecutively with 50 ml hexane (fraction I), 50 ml diethyl ether (fraction II), and 50 ml ethyl acetate (fraction III) with the help of a separating funnel. Fraction I and II still contained free fatty acids and flavonoids while fraction III was used for further processing because it contained quercetin in the form of glycoside bonds (Sanghavi *et al*. 2014). Next, fraction III was concentrated and hydrolyzed with 7% sulfuric acid (10 ml/g extract) for 5 h, then filtered and extracted with ethyl acetate (1:1) by using a separating funnel. After that, it was concentrated to obtain coarse quercetin and crystallized with 10% ethanol to get pure quercetin.

A quercetin stock solution was prepared 100 ppm concentration and was used to prepare standard curve at 0.01, 0.05, 0.1, 0.15, and 0.2 ml respectively, diluted with ethanol pure analysis to 1 ml. Each 200 µl of concentration was taken into a tube which contained 800 µl of distilled water, then added 60 µl of NaNO2 solution (0.5 mg/10 ml distilled water). After 5 minutes, 60 µl of AlCl3 (1 mg/10 ml of distilled water) was added and mixture was allowed to stand for 6 minutes. Prior to recording the absorbance at 510 nm with a UV-Vis spectrophotometer, the mixture was reacted with 400 µl of 10% NaOH and adjusted with distilled water up to 2 ml. Measuring the sample was carried out the same steps as standard, results was expressed as mg of QE/g CE.

Measurement of antioxidant reducing capacity. The measurement of antioxidant activity was determined using a modified Ferric Reducing Antioxidant Power (FRAP) assay (Benzie and Strain 1999). FRAP reagent was prepared, it contained 100 ml of acetate buffer (300 mM, pH 3.6), 10 ml of 10 mM TPTZ solution in 40 mM HCl, and 10 ml of 20 mM FeCl3 solution. For analysis, 200 µl of extract (1mg/ml) was mixed with 1800 µl of FRAP reagent and incubated for 30 minutes at room temperature. The absorbance readings were carried out at 595 nm using a spectrophotometer. The standard curve is made with several concentrations of FeSO4·7H2O (2; 1; 0.5; 0.25; 0.125; 0.0625; and 0.03125 mM). FRAP activity was determined as miligram FeSO4 equivalent per milligram of sample. The control contains FRAP solution and its solvent while the sample blank contains the extract and its solvent.

Data analysis

Data processing and analysis was done with Microsoft Excel 2013 and SPSS version 16 for Windows. The statistical analysis performed were a Two-way Analysis of Variance (ANOVA) and Duncan's new multiple range test to identify the individual effect of maturity level of leaf and the different of solvents on TPC, quercetin, and antioxidant activity. Statistical significance was determined at $p<0.01$. All data were presented in averages and Standard Error of Mean (SEM).

RESULTS AND DISCUSSION

Extraction result

Gandaria leaves in this study were different in their maturity stage, as can be seen in Figure 1. Tender leaves are purplish light green, picked from the first three segments of the tip, while mature leaves were dark green, picked from below the third first segments of the tip. Tender and mature gandaria leaves had a different texture. Tender leaf had more moisture content and the cell matrix was not tightly bound so the texture was flexible and brittle, while mature leaf had less moisture content and its matrix was tightly bound so the texture was stiff. The extraction process applied to plants was expected to open matrix bound so that it run effectively and efficiently as well to obtain target compounds (Sun *et al*. 2012). The selection of solvents represented polar (ethanol), semipolar (ethyl acetate), and nonpolar (hexane) properties, based on solvents commonly used for extraction. The extraction was aimed to determine the potential of antioxidant compounds found in tender and mature gandaria leaves.

Xu *et al*. (2017) described that antioxidant compounds based on their polarity (water-soluble such as phenolic and flavonoid, and lipid-soluble such as β-carotene and lycopene) have biological effects. The extraction yield of gandaria leaves corrected by moisture content can be seen in Figure 2. The ethanol extract of tender leaves showed the highest extraction yield and ethyl acetate extract of tender leaves yield the lowest. Hexane yield in both types of leaves demonstrated the same rate. In tender leaves, extraction yield was increase followed by raising polarity of solvents. Different solvents will dissolve different compounds depending on their level of polarity. Nakamura *et al*. (2017) mentioned in their study on extraction yield of *Sasa quelpaertensis* Nakai leaf with different solvents resulted in different yield namely with ethanol was 80% (9.26%), ethyl acetate (6.11%), and with hexane (24.20%). The interaction between type of leaf and type of solvent affected the concentrated extraction yield of gandaria leaves ($p<0.01$).

Total phenolic content

Polyphenolic compounds are the largest bioactive components found in plants and played an important role in determining antioxidant activity. Total phenolic content of mature gandaria leaf extracts were higher than tender gandaria leaf extracts (Figure 3). The highest total phenolic content of gandaria leaf was extracted by using

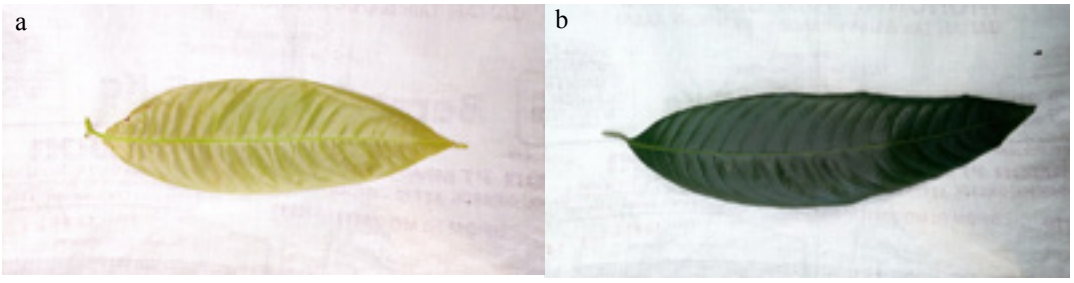


Figure 1. Tender (a) and mature (b) gandaria leaf

hexane followed by 96% ethanol. Our results found that the polarity of solvents in extracting active compounds is also influenced by the type of leaf. There was significant differences in interaction between leaf type and solvents on TPC ($p<0.01$). More nonpolar active compounds were extracted from mature leaves than tender leaves. Similar findings were confirmed by Nantitanon *et al*. (2010), in which different solvents have different potential in extracting phenolic content from guava leaves. In contrast, M'rabet *et al*. (2017) found that TPC of methanol extract from tender berry leaves (*Melia azedarach* L.) (85.4 mg GAE/g extract) was higher than in mature leaves (59.1 mg GAE/g extract).

TPC of blueberry leaves extracted with acetone, ethanol, and methanol significantly increased along with increased in maturity stage (Deng *et al*. 2014). Whereas the maturity stage of purple basil leaves had a significant effect on the TPC ranged from 3.30-20.08 mg GAE/g (McCance *et al*. 2016). These variations in results were probably due to plant species, maturity stage, solvents concentration and type, also extraction methods.

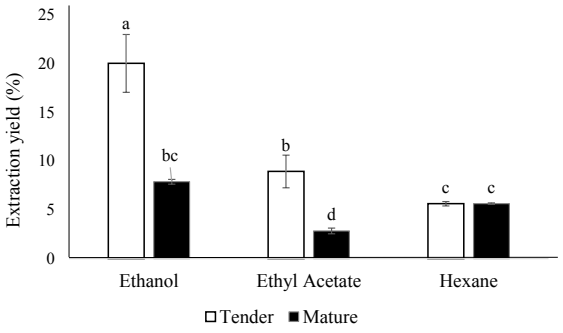
Quercetin

Quercetin was a flavonol bioactive compound, a class of flavonoid that had an antioxidant function. Quercetin can be an antioxidant and alter Reactive Oxygen Species (ROS) metabolism directly or indirectly by inhibition of enzymes associated with antioxidant activities such as tioredoxin reductase and glutathione S-transferase (Gibellini *et al.* 2015). Pan *et al*. (2018) examined that the main active component of phenolic group is found in mango leaf extract such as mangiferin (7.43%) followed by quercetin (0.86%) which is from the same family as gandaria plant, the Anacardiaceae family. In this study, it was assumed that active component which acts as an antioxidant in gandaria leaf extract was also similar to the mango leaf, so quercetin analysis was conducted. Quercetin content of tender and mature gandaria leaf extracts varied from 2.76-4.36 mg QE/g. Quercetin of mature leaf extracts were higher than tender leaf extracts except in ethyl acetate (Figure 4). Extract with increase TPC also contained high of quercetin which was associated with increase in antioxidant activity. Quercetin of tender leaf extracts increased with the increasing polarity of solvent and followed the order of: hexana < ethyl acetate < ethanol. Although ethyl acetate extract was higher than ethanol for mature leaf, it was not statistically different. The study confirmed that hexane extract of mature leaf had the highest quercetin according to TPC. The interaction between maturity stage of gandaria leaves and solvents had a significant effect on quercetin content ($p<0.01$).

As previously reported by Vongsak *et al*. (2013), *Moringa oleifera l*eaf extract macerated with ethanol, contains high TPC, isoquecertin compounds, and antioxidant activity. Quercetin compound from mature *Moringa oleifera* leaf extracts identified with UHPLC were 985±4 µg/g (Prabakaran *et al*. 2018). The differences depend on differences of plant species, solvents and their concentrations, and extraction methods.

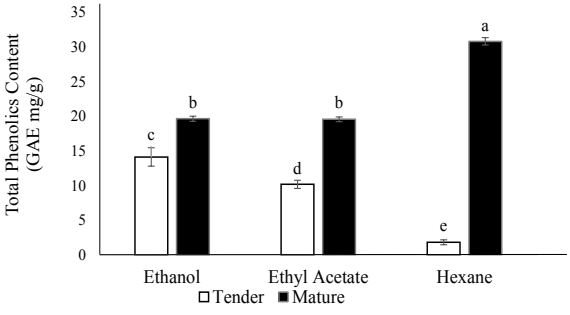
FRAP assay

Antioxidant measurements in vitro are usually consistent with the assessment of TPC to evaluate antioxidant properties in phenolic-rich extracts as described by Granato *et al*. (2018).



*Vertical line above each bars stand for standard deviation. Bars with similar letter were not significantly different ($p<0.01$)

Figure 2. Extraction yield of gandaria leaf extracts using different extraction solvents and at different maturity stages



*Vertical line above each bars stand for standard deviation. Bars with similar letter were not significantly different ($p<0.01$)

Figure 3. Total phenolic content of gandaria leaf extracts using different extraction solvents and at different maturity stages

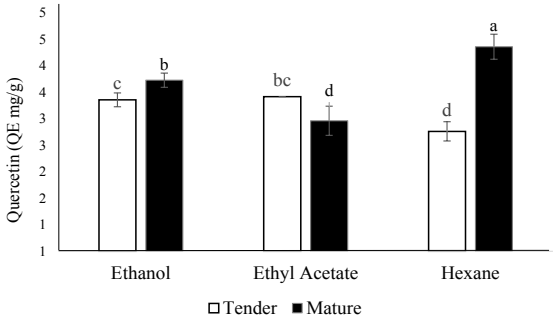
In this study, antioxidant activity was conducted based on the ability to reduce plasma ferric ions, the method was chosen because FRAP assay showed a fast and easy reaction (Huang *et al*. 2005). FRAP analysis results ranged from 1.05-5.63 mg FeSO4 equivalent/mg extract (Figure 5). Ethanol extract of tender and mature leaves had the highest value compared to the others. FRAP antioxidant capacity was the lowest at hexane extract of tender leaves. There was no difference in antioxidant reducing capacity between ethyl acetate and ethanol extract of tender leaves.

According to FRAP method by Benzie and Strain (1999), ferry ions (Fe^{3+}) were reduced to ferrous (Fe^{2+}). The reaction represented that an electron exchange so that it binds metal. The most of strong iron chelators can affect the results as excess of free Fe^{3+} in the FRAP reagents. Free iron has a low redox potential so that it provided more oxidizing Fe^{3+}-TPTZ (2,4,6-tripyridyl-s-triazine). Thereby, when there is a large amount of iron chelator, then increasing Fe^{3+} in the reagent might prevent chelation interference. The antioxidant FRAP of *Moringa oleifera* leaf ethanol extract was evaluated by Wang *et al*. (2017), each mg contains strong antioxidants (0.95-1.35 mmol FeSO4). These study was similar with Hasim *et al*. (2017). There was an interaction between type of gandaria leaves and solvents against FRAP antioxidant activity ($p<0.01$).

The correlation between TPC against reducing power as well as quercetin and reducing power showed positive correlation, respectively. The R2 of quercetin versus FRAP was 0.33 which was greater than TPC versus FRAP (R2=0.1899). It was provided that more content of antioxidant compounds (phenolic and quercetin) will produce higher FRAP antioxidant activity. The highest content of phenolic and quercetin found in hexane extracts of mature gandaria leaf but the highest FRAP antioxidant activity in ethanol extract, remained in the same leaf. The raising of antioxidant activity in ethanol extract was due to the role of other antioxidant compounds in addition to phenolic group e.g. alkaloid, steroid, and carotenoid. Nevertheless, the relationship still showed linear correlation. The results of other studies also showed a positive correlation (McCance *et al*. 2016; Sati *et al*. 2013).

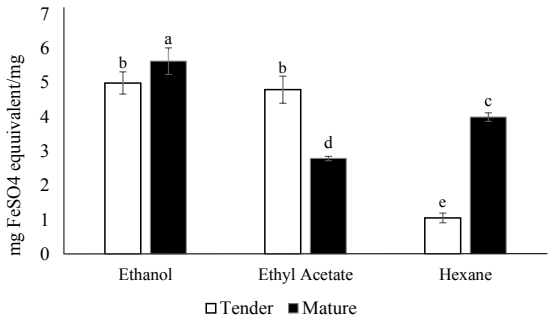
Phenolic and quercetin acted as antioxidant compounds because they had hydroxyl group which could release H^{+}. The differences of polarity level of solvents determined chemical structure of extracted phenolic compound. The mechanism of phenolic and flavonoid compounds as antioxidant agent by counteracting free radicals, stimulating antioxidant enzymes, or inhibiting prooxidant enzyme. The mechanism of the body's antioxidant defense was not only through enzymes such as Superoxide Dismutase (SOD), catalase, and glutathione peroxidase, but also non-enzyme such as glutathione, ascorbic acid, and vitamin E (Panche *et al*. 2016). Ascorbic acid and vitamin E were natural antioxidants and both of them had different solubility. Granato *et al*. (2018) explained antioxidants counteract free radicals in cell membranes through three mechanisms, transfer hydrogen atoms, transfer electron, and their ability for chelating metals.

Quercetin was a bioactive compound that has health benefits other than antioxidants. Recent studies reported that quercetin can function as an antidiabetic agent through several mechanisms, such as by increasing the glucose uptake, controlling glycemia, protecting the pancreatic β cells against oxidative damage



*Vertical line above each bars stand for standard deviation. Bars with similar letter were not significantly different ($p<0.01$)

Figure 4. Quercetin content of gandaria leaf extracts using different extraction solvents and at different maturity stages



*Vertical line above each bars stand for standard deviation. Bars with similar letter were not significantly different ($p<0.01$)

Figure 5. Antioxidant activity of gandaria leaf extracts using different extraction solvents and at different maturity stages

and inhibits β cells apoptosis. Quercetin activates silent information regulator 1 (SIRT1) to improve insulin sensitivity, stimulates glucose transporter 4 (GLUT4) translocation by increasing AMP-activated protein kinase (AMPK) and phosphatidylinositide-3-kinase (PI3K) phosphorylation (Heger *et al*. 2019; Shi *et al*. 2019). In addition, quercetin activates the peroxisome Proliferator-activated receptor Gamma, Coactivator 1 alpha (PGC-1α). Its function as coactivator for nuclear receptor and regulates energy metabolism which is activated by SIRT1.

Based on FRAP analysis, more concentration of Fe^{3+}-TPTZ reduced, indicated higher antioxidant activity (Pisoschi & Negulescu 2011). Fe^{3+}-TPTZ represents oxidator compound in human body that caused cells damage. Antioxidant compounds including phenolic and quercetin could reduce Fe^{3+}-TPTZ to Fe^{2+}-TPTZ. Abundant antioxidant compounds in the body produce more ferrous ion is implicated in the antioxidant activity.

CONCLUSION

The highest TPC and quercetin content were found in hexane extract of mature leaf while FRAP activity was the highest in ethanol extract of mature leaf. There were interaction effect between type of gandaria leaves and solvents on TPC, quercetin, and FRAP antioxidant activity. There was a positive correlation between TPC and quercetin content og Gandaria leaf with its antioxidant activity, as shown by the result of FRAP assay. This study showed that there was antioxidant potential of both tender and mature gandaria leaves with mature gandaria leaves having better antioxidant extraction yield, making it suitable for development of functional food. Therefore, further studies are needed to explore the health benefits of gandaria leaves as functional food.

ACKNOWLEDGEMENT

The authors would like to thank The Ministry of Research, Technology, and Higher Education through Superior Basic Research Program of University which has funded this research according to Contract Number: 1750/1T3.11/PN/2018. We wish to thank Indonesian Institute of Science, Cibinong, Bogor who providing gandaria leaves for this research. The authors have no conflict of interest.

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