

Radical scavenging activity of ethanolic extract of green and red leaves of Cantigi (*Vaccinium varingiaefolium* Bl. Miq.)

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Abstract

Indonesia's Cantigi (*Vaccinium varingiaefolium* Bl. Miq.) is a member of the blueberry family that grows abundantly around volcanoes. It has two distinct leaf colors, red color leaf (RCL) and green color leaf (GCL), which contain secondary metabolites including anthocyanins. This study aimed to characterize the antioxidant properties of Cantigi leaf using various methods. DPPH (2,2'-diphenyl-1-picrylhydrazyl), BHA (butylhydroxyanisole), and BHT (butyl hydroxytoluene) were used to check the antioxidant activity of 96% ethanolic Cantigi extract. The phytochemical screening showed positive results for flavonoids, steroids, tannins, and saponins. The antioxidant activity of RCL and GCL leaves as determined by DPPH radical scavenging assay was expressed using IC₅₀, a concentration capable of scavenging 50% DPPH radicals. The IC₅₀ values of DPPH radical scavenging assay for red leaf and green were 29.87±3.2, and 24.65±3.3 mg/kg, respectively. Based on the DPPH radical assay, there was no significant difference between RCL and GCL; however, the BHA/BHT assay using a similar method with DPPH shows significant differences between RCL and GCL. From this study, Cantigi leaf could potentially be used as an ingredient in a food supplement due to its strong activity as radical scavenging.

1. Introduction

As a good source of bioactive compounds, medicinal plants or herbals have long been used in preventing and treating certain diseases, either in modern or traditional medicines. Besides, herbals are widely used as nutraceuticals which provide some bioactive compounds or phytochemicals capable of promoting human health status (Ghasemzadeh *et al.*, 2016). Furthermore, some active compounds are responsible for biological activities, including antioxidants, mainly from phenolics and flavonoid classes in the herbals (Amir *et al.*, 2011). An antioxidant is capable of delaying and preventing the oxidation reactions in the human body by blocking or scavenging free radicals (Lim *et al.*, 2011).

Two synthetic antioxidants, namely butylated hydroxytoluene (BHT) and butylated hydroxyanisole

(BHA) are frequently employed to slow down the oxidation processes. However, these antioxidants have the problem of being flammable and quickly decomposing at high temperatures (Martínez-Tomé *et al.*, 2001). With the campaign of Back to Nature, scientists are motivated to explore natural antioxidants since consumers are becoming more concerned about the number of chemicals in their food. In recent years, there has been an increased awareness among societies to use natural antioxidants rather than synthetic ones to stabilize foods from oxidative deterioration. Therefore, it is not surprising that the demand for natural antioxidants originating from plants, fruits and vegetables has increased as a result of these observations (Kumar *et al.*, 2015).

There are numerous endemic plants in Indonesia,

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which are found all around the country, containing some phytochemicals that have beneficial effects on human health, including antioxidants (Rohman *et al.*, 2020). A member of the Ericaceae family, *Vaccinium varingaefolium* Bl. Miq. is one of them. The name of this plant in local dialects is Cantigi, and in some Indonesian areas, such as West Java, it is known as purple Cantigi. This indigenous shrub resembles blueberries (*Vaccinium corymbosum*) and bilberries (*Vaccinium myrtillus*) (Yulyana *et al.*, 2016). This Cantigi deserves the moniker *Bilberry of Java*. The blueberry and bilberry families are full of antioxidants. One variant of bilberry extract called Cantigi may have antioxidant properties. This plant thrives in Java's proximity to sulfur vents or volcano regions. On the island of Java, a plant known as Cantigi usually grows in high agricultural areas more than 1,000 feet above sea level. This plant is renowned for withstanding a variety of grips, the majority of which is knowledge regarding the origin of conventional on-land craters. The blueberry is a member of the *Vaccinium* and *Cyanococcus* subgenera. Extracts of Cantigi have been reported to provide antioxidant and anti-cancer activities (Syahputra *et al.*, 2016; Yulyana *et al.*, 2016).

As a result of their high bioactive chemical content, particularly flavones and other polyphenolic compounds, blueberries are considered a functional food because of their potent antioxidant effects. Cantigi is reported to contain anthocyanins which are reported to have antioxidant activities. Anthocyanins, the most abundant subclass of flavonoids and the majority of total phenolic compounds are found in almost all fruits and vegetables worldwide (Hussain *et al.*, 2020). Furthermore, various parts of a plant source may have diverse bioactivities. According to a paper by Yuan *et al.* (2014), they discovered that the antioxidant activity of hawk tea depends on its age.

The antioxidant activity of the blueberry family has been studied in the past ten years, however, the antioxidant study for *V. varingaefolium* is limited. The antioxidant activities of herbals and plant-based functional groups are typically correlated with phenolics and flavonoid contents (Widodo *et al.*, 2019). Therefore, this study aimed to compare the antioxidant activity profile between red and green Cantigi leaf obtained by 96% ethanolic extract.

2. Materials and methods

2.1 Plant material

Red and green leaves of Cantigi were obtained from the Papandayan Mountain in Indonesia. The leaves were collected in July 2020 during a dry season. The Cantigi

leaves were harvested from naturally grown trees and then subjected to some processing after four-day harvesting. The authenticity of plant samples was carried out at the Indonesian Institute of Sciences.

2.2 Preparation of Cantigi extract

Red and green leaves of Cantigi (RCL and GCL, respectively) were extracted by maceration according to Mistriyani *et al.* (2018) using 96% ethanol solvent at a ratio of 1:3 wt/volume (or until the simplicia was submerged). The mixture was stirred for 5 mins, soaked for 15 mins, and then filtered to obtain a liquid extract. It was then concentrated with a vacuum rotary evaporator. The standardization procedure was carried out before the subsequent chemical and formulation evaluation.

2.3 Total phenolics content

The total phenolic content (TPC) of Cantigi leaf extracts was evaluated using Folin-Ciocalteu's reagent (FCR) according to Mansouri *et al.* (2005) with a minor adjustment. The extract was added with methanol, FCR, and 5% CaCO₃. The absorbance of reaction mixtures was read at 725 nm (Hitachi U-2000 spectrophotometer 1210002, Tokyo, Japan). TPC was quantified in mg (+)-gallic acid equivalents (GAE) per gram of extract.

2.4 Total flavonoids content

The total flavonoid concentration (TFC) of Cantigi extracts was assessed using the method reported by Samirana *et al.* (2017). The extract (250 L, concentration 1-10 mg/mL depending on solvent) was combined with distilled water (1.25 mL) and a 5% sodium nitrite solution (75 L). After 6 mins, aluminium chloride (10%, 150 L) was added to the mixture, followed by sodium hydroxide (1 M, 500 L). Distilled water was used to dilute the samples rapidly. At 510 nm, the absorbance was measured. TFC was calculated as mg (+)-catechin equivalents (CE) per gram of extract.

2.5 Determination of radical scavenging activity

The 2,2-diphenyl-1-picrylhydrazyl radical (DPPH•) scavenging activity of Cantigi extracts was measured using the method developed by Brand-Williams *et al.* (1995) technique (Molyneux, 2004). In this study, methanol (2 mL) and a methanolic solution of 1 mM DPPH radicals (0.25 mL) were combined. The extracts (0.1 mL) at various concentrations (0.4-2.0 mg/assay) were then added. The absorbance was measured at 517 nm after a 20-min reaction in the dark. The IC₅₀ value (the half-maximal effective concentration), the required concentration to inhibit 50% of DPPH radicals, was calculated using an absorbance value against the concentration of extracts through linear regression.

Similar steps were taken when using BHA/BHT as the positive control.

2.6 Chemical compounds characterization

To elucidate the chemical properties of Cantigi extract, Fourier transformed infrared (FTIR) spectrophotometer (Shimadzu, Japan) was used to see the differences between RCL and GCL. Gas chromatography-tandem with mass spectroscopy (GC-MS) was used to identify the chemical constituents of both extracts (Agilent technologies 7890 gas chromatograph with autosampler and 5975 mass selective detector). Experimental conditions of the GC-MS system were as follows: HP Ultra 2 capillary column Length (m) 30×0.20 (mm) I.D $\times 0.11$ (μm) film thickness. The flow rate of the mobile phase (carrier gas: He) was set at 1.2 ml/min. In the gas chromatography part, the initial temperature was at 80°C , then rising at $3^\circ\text{C}/\text{min}$ to 150°C (1 min) and finally rising $20^\circ\text{C}/\text{min}$ to 280°C held for 26 mins. Samples dissolved in ethanol and the results were compared by using the Chem-station library search program.

2.7 Statistical analysis

All the measurements were replicated three times, and the data are presented as mean \pm standard deviation (SD). To determine the significance ($p < 0.05$) between treatment means, the effects of adding natural antioxidant extracts were examined, and the results were then subjected to a statistical analysis using SPSS software (version 16.0 for Windows, SPSS Inc., USA).

3. Results and discussion

3.1 Phytochemical properties of red and green color leaves

In order to identify the compounds, phytochemical screening was done to assess the secondary metabolite profiles present in red and green Cantigi leaves. The results were shown in Table 1 indicating that the phytochemical compositions of both Cantigi leaf extracts were comparable. This result was in agreement with that reported by Yulyana *et al.* (2016).

Table 1. Phytochemical screening of Cantigi extract.

Phytochemical constituents	Red leaves	Green leaves
Alkaloids	+	+
Phenolics	+	+
Flavonoid	+	+
Saponins	+	+
Tanin	+	+
Steroids	+	+
Terpenoid	-	-

3.2 Total phenolic and flavonoid content

The total phenolic content of the extract was measured using the Folin-Ciocalteu's phenol reagent. A millilitre of the sample was dissolved in 1.5 mL of distilled water, 2.5 mL of 10% Folin-Ciocalteu's phenol reagent, and 7.5 mL of 20% Na_2CO_3 at various concentrations ranging from 1.0 to 0.1 mg mL^{-1} . The completed combination was shaken and left to sit for two hours in the dark. At 750 nm, the mixture's absorbance was measured. Gallic acid equivalent (g GAE g^{-1} dry weight of extract) was used to express the results. Three copies of each test were run on each sample.

Despite frequently being oxidized themselves, the plant extracts have significantly reduced oxidation. Plant phenolic chemicals are thought to be responsible for this inhibitory action. Their total phenolic content indicates the antioxidant capacity of plant extracts. As a result, the number of total phenols in the ethanolic extract of Cantigi was determined as shown in Table 2. The standard curve of gallic acid was defined as $y = 0.00536x + 0.0959$. The urgency of phenolic contents is based on the fact that phenolic compounds are responsible for the antioxidant activities of natural products due to their capability to donate hydrogen radicals (Herlina *et al.*, 2018). Phenolic compounds are believed to contribute to the antioxidant activities especially radical scavengers of natural products, mainly due to their redox properties. Generally, the mechanisms of phenolic compounds for antioxidant activity are neutralizing lipid free radicals and preventing the decomposition of hydroperoxides into free radicals (Lukitaningsih *et al.*, 2020).

Flavonoids are the largest class of phenolic compounds found in nature. Flavonoids are composed of a C15 skeleton with an aromatic ring in position 2, 3, or 4 on the chroman ring. Most flavonoid compounds are known as glycosides, whereas aglycones are comparatively uncommon. The total flavonoid content in Cantigi leaves is demonstrated in Table 2. The standard curve of catechin was $y = 0.00093 + 5.9288x$. Flavonoids are phenolic compounds acting as antioxidants through reducing power, chelating agents, and lipid peroxidation inhibition. Flavonoids donating their radicals become radicals. However, due to resonance stabilization, the flavonoid radicals are stable (Sukweenadhi *et al.*, 2020).

3.3 Antioxidant activity

One of the most used techniques for assessing antioxidants is DPPH (2,2-Diphenyl-1-picrylhydrazyl). DPPH radicals are stable in organic solvents (Rohman *et al.*, 2010). When a sample is added, the DPPH solution's

Table 2. Antioxidant content and antioxidant activity of RCL and GCL.

Sample	TPC (mg GAE/g of dried extract)	TFC (mg QE/g of dried extract)	DPPH ($\mu\text{g/g}$ of dried extract)	BHA ($\mu\text{g/g}$)	BHT ($\mu\text{g/g}$)
RCL	79.6 \pm 2.7	47.7 \pm 1.9	29.87 \pm 3.2	17.59 \pm 2.4*	23.79 \pm 1.8*
GCL	81.1 \pm 2.3	45.6 \pm 1.2	24.65 \pm 3.3	114.46 \pm 1.6*	94.94 \pm 2.1*

*significantly different at $p < 0.05$.

purple colour declines and turns from light purple to yellow, indicating the presence of antioxidant action. The stronger the antioxidant power, the more yellow the solution is in colour. IC_{50} values were obtained after an examination of antioxidants in a 96% ethanol extract of red, and green Cantigi leaves. BHA/BHT was also tested, which was demonstrated in Table 2. This result indicated that Cantigi could potentially be used as a food supplement or herbal medicine due to its strong activity as an antioxidant.

Numerous spices and herbs, typically used to season food, are a great source of phenolic compounds, which have been shown to have significant antioxidant activity (Zheng and Wang, 2001). Antioxidants can stop lipid peroxidation by employing the following mechanisms: scavenging chain-initiating radicals to prevent chain inhibition; interrupting chain reactions; decomposing peroxides; lowering localized oxygen concentrations; and binding chain-initiating catalysts, such as metal ions (Dorman *et al.*, 2003). The results of this study open the

possibility of Cantigi extract as a BHA/BHT substitute, although other assays should be conducted to support the finding, like CUPRAC (cupric ion reducing antioxidant capacity), FRAP, ABTS/TEAC, and other methods. FDA guidelines state that an oxidant scavenger is no longer necessary for extracts with BHA/BHT contents of more than 20 ppm. Only RCL was under 20 ppm in the data (Table 2).

3.4 Characterizing chemical constituents of red and green color leaves

Gas chromatography-mass spectrometry (GC-MS) analysis of RCL and GCL methanolic extract resulted in the volatile compounds present in both samples. GC-MS confirmed the presence of multiple compounds with varied retention times, as shown in Figures 1A and 1B. Table 3 displays the numerous compounds present in the extract analyzed. The major component of RCL was 1-cyclopentylethanone (CPE), while the main component present in GCL was 1,3,4,5-

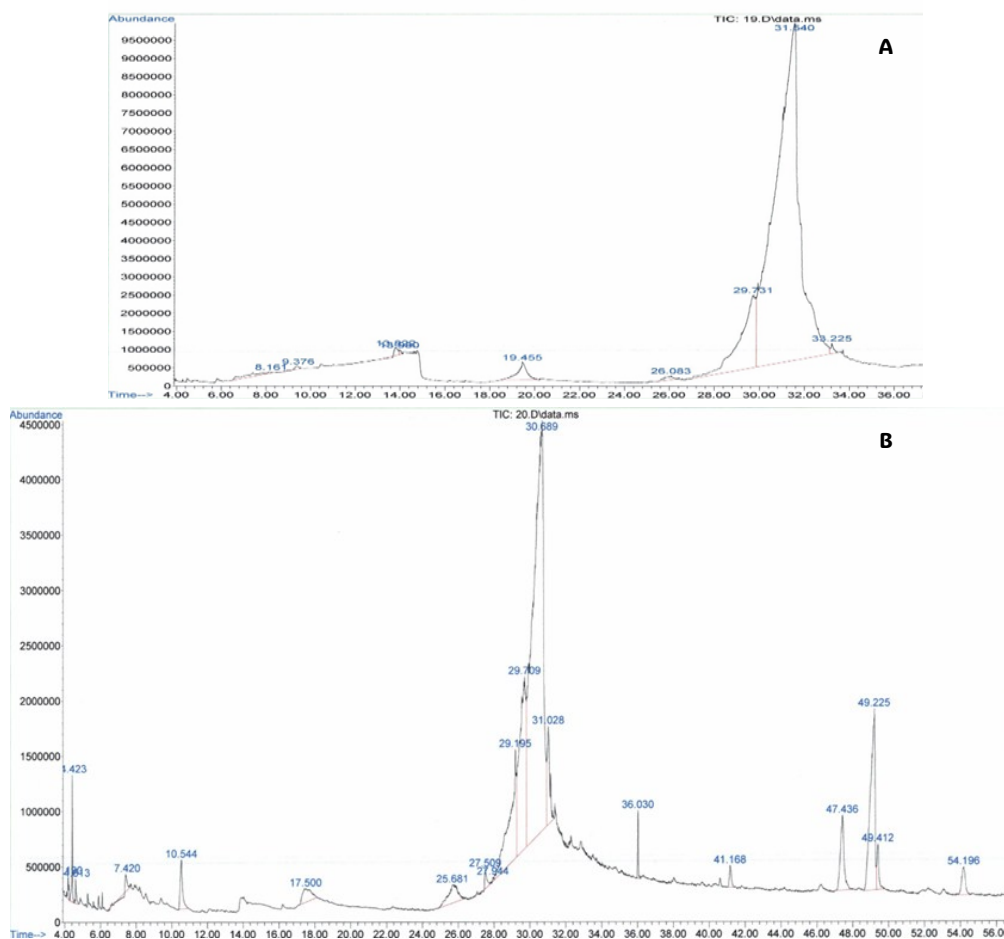


Figure 1. GC-MS chromatogram of ethanolic extract of RCL (A) and GCL (B).

Table 3. Compounds identified in the ethanolic extract of RCL and GCL In GC-MS.

RT	Molecular formula	IUPAC Name	MW	Peak Area %	PubChem ID
RCL					
19.457	C ₆ H ₄ (OH) ₂	1,4-benzenediol	110	1.73	785
29.731	C ₇ H ₁₂ O ₆	1,3,4,5-Tetrahydroxycyclohexanecarboxylic acid	192	11.40	1064
31.537	C ₇ H ₁₂ O	1-cyclopentylethanone	112	85.50	22326
GCL					
10.541	C ₆ H ₈ O ₄	4h-pyran-4-one,2,3-dihydro-roxy -6-methyl-	144	1.40	119838
17.498	C ₆ H ₄ (OH) ₂	1,4-benzenediol	110	1.39	785
25.683	C ₆ H ₁₂ O ₆	beta-d-glucopyranose	180	2.09	64689
30.689	C ₇ H ₁₂ O ₆	1,3,4,5-tetrahydroxycyclohexanecarboxylic acid	192	65.11	1064
31.027	C ₂₄ H ₅₀ O ₂	1-(octyloxy) octane	370	3.20	110471
47.438	C ₃₀ H ₅₀ O	olean-12-en-3-ol	426	3.29	225689
49.223	C ₃₀ H ₅₀ O	urs-12-en-3-ol	426	9.83	225688

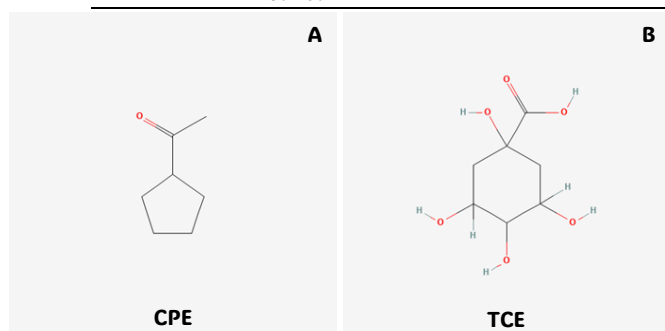


Figure 2. Chemical structure of 1-cyclopentylethanone (A) and 1,3,4,5-tetrahydroxycyclohexanecarboxylic acid (B).

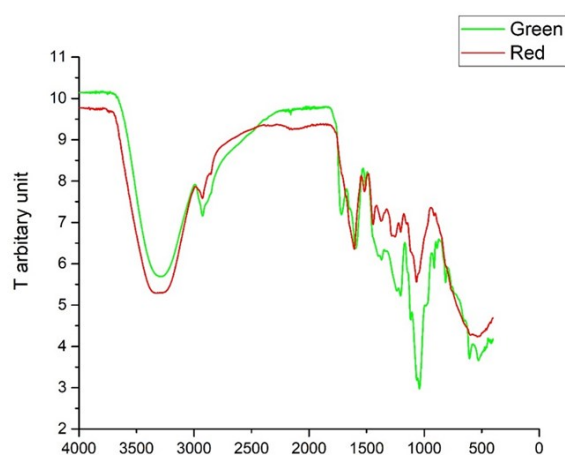


Figure 3. The comparison of FT-IR spectrum of RCL and GCL. Red indicates RCL, while green indicates GCL.

tetrahydroxycyclohexanecarboxylic acid (TCC). Interestingly, both chemical structures (CPE and TCC) have similar core chemical structures (cyclohexane), indicating that both compounds are derived from similar precursors (Figure 2A and B). Secondary metabolites, such as flavonoids, are produced by the biosynthesis of primary metabolites (Yadav *et al.*, 2021).

The key distinction between TCC and CPE is that the first contains a hydrolyzed group, whereas the other does not. FT-IR data in Figure 3, stretching the OH band in GCL, confirmed this finding. Additionally, the

hydroxyl group contributes to antioxidant action by directly neutralizing free radicals through two mechanisms: contributing a hydrogen-free radical (H.) or donating an electron (e-) (Al-Mamary and Moussa, 2021). Although TCC, which belongs to GCL, contains more hydroxylated groups than CPE (which belongs to RCL), the antioxidant activity of RCL and GCL was not significantly different (Table 2). This result was due to the higher CPE content in RCL compared to TCC in GCL. Furthermore, the BHA/BHT value of GCL was significantly different from RCL (Table 2). This discrepancy was reportedly caused by TCC's stronger antioxidant than RCL.

4. Conclusion

There are no significant differences between Cantigi red leaf and green leaf Cantigi in terms of antioxidant activities and bioactive compounds responsible for these activities. Green Cantigi contain total catechin content with more potent antioxidant activities than red Cantigi as an antioxidant. From this study, Cantigi leaf has the potential to be used as an ingredient in a food supplement due to its strong activity as radical scavenging.

Conflict of interest

The authors declare no conflict of interest.

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