Pharmacogn J. 2019; 11(4):771-776
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
www.phcogj.com

Original Article

Antioxidant Activity of Ethanolic Extract and Various Fractions from Green Tea (Camellia sinensis L.) Leaves

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ABSTRACT

Background: Free radicals are one of the causes that can cause premature aging and degenerative disease. To overcome this problem, the body needs antioxidant intake. Green tea (Camellia sinensis L.) leaves are one of the plants known as antioxidant agent due to its flavonoids and phenolic compounds or better known as catechin compounds. Catechin is polar flavonoid compounds so it is necessary to separate it from non-polar compounds so their antioxidant activity becomes effective. **Objective**: This study aims to determine antioxidant activity of ethanolic extract of green tea leaves and its fractions namely ethyl acetate and water fraction, and measure the total flavonoid content, total phenolic content and catechin content. **Materials and Methods**: Green tea leaves extracted using maceration method with 96% ethanol. Fractionation was conducted using liquid-liquid extraction using a solvent of n-hexane, ethyl acetate and water. Screening of flavonoid and phenolic and antioxidant activity was performed against the ethanolic extract, ethyl acetate fraction and water fraction. Antioxidant activity was determined by 2,2-diphenyl-1-picrylhydrazyl method using ultraviolet-visible spectrophotometry with ascorbic acid as standard. **Results**: The ethanolic extract, ethyl acetate fraction and water fraction contains flavonoids and phenolic compounds. The IC_{50} value of ethanolic extract, ethyl acetate fraction and water fraction were 9.017; 3.926 and 7.408 µg/mL consecutively. The ethyl acetate fraction also showed better antioxidant activity than ascorbic acid (4.855 µg/mL). **Conclusion**: The ethanolic extract, ethyl acetate fraction and water fraction showed very powerful antioxidant activity but ethyl acetate fraction has the best antioxidant activity.

Keywords: Antioxidant activity, DPPH method, Ethanolic extract, Ethyl acetate fraction, Green tea leaves.

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History

- Submission Date: 22-03-2019;
- Review completed: 12-04-2019;
- Accepted Date: 06-05-2019.

DOI : 10.5530/pj.2019.11.122

Article Available online

http://www.phcogj.com/v11/i4

INTRODUCTION

Free radicals, known as reactive oxygen species (ROS), are the products of normal cellular metabolism. A free radical can be defined as an atom or molecule containing one or more unpaired electrons. The odd number of electron(s) of a free radical makes it unstable, short lived and highly reactive.[1-3] Free radicals are compounds that trigger the aging process and degenerative diseases. Antioxidants are compounds that prevent the formation of free radicals. Antioxidants are divided into two types, natural and synthetic antioxidant. Natural antioxidant commonly found in plants such as fruits, grains, and vegetables. Some natural antioxidant compounds are derivatives of flavonoids, phenol, ascorbic acid, coumarin, hydroxycinnamic, dihydroflavone, tocopherol, and catechin. Synthetic antioxidants include butyl hydroxylanisol, butyl hidroxytoluen, propyl gallate, ethoxyquin.[4] Synthetic antioxidants such as butylated hydroxy aniline (BHA) and butylated hydroxy toluene (BHT) have been known to have large side effects including causing liver damage.[5] This encourages the development of natural product to be a source of antioxidants.

Natural product that can be used as antioxidants is green tea leaves (Camellia sinensis L). The antioxidant activity of green tea is caused by polyphenolic compounds, especially flavonoid groups.[6,7] Catechin is flavonoids compound that contained in green tea leaves. The types of catechins that contained in green tea are epigallocatechin (EGC), epigallocatechin gallate (EGCG), epicatechin gallate (ECG), and gallate epicatechin (GEC).[8-10]

Components in plants can be taken by extraction methods. The choice of solvent must consider several factors including selectivity, ability to extract, toxicity, ease of evaporation and also prices.[11] The extraction solution used was adjusted to the polarity of the desired compound. According to the principle of like dissolves like, a solvent will dissolve compounds that have the same level of polarity. Flavonoids are polyphenol compounds that are widely distributed in plants. Flavonoid divided into two groups, namely aglycon flavonoids and glycoside flavonoids. Aglycon flavonoid are flavonoids which do not bind sugar to their structure while glycosides flavonoid are flavonoids that bind sugar to their structure. The presence of sugar in the flavonoid structure causes the glycosides flavonoid become polar compounds while the aglycons flavonoid are less polar. Catechin

Cite this article: Indarti K, Apriani EF, Wibowo AE, Simanjuntak P. Antioxidant Activity of Ethanolic Extract and Various Fractions from Green Tea (Camellia sinensis L.) Leaves. Pharmacog J. 2019; 11(4):771-6.

in green tea leaves are the polar flavonoid compound. Polar solvents commonly used for extraction of flavonoids are methanol, acetone, ethanol, ethyl acetate, water and isopropanol. Therefore, this research was conducted to separate the flavonoids contained in green tea leaves from other compounds to obtain a fraction with high levels of flavonoids which have strong antioxidants activity and to see the effect of polarity solvent used. Green tea leaves are macerated using 96% ethanol. Fractination was performed by liquid-liquid partition method using a solvent of n-hexane, ethyl acetate and water to obtain water fraction and ethyl acetate fraction. Ethanolic extract, water fraction and ethyl acetate fraction were measured for total flavonoid content and total phenolic content and then tested for antioxidant activity using DPPH method and ascorbic acid as standard. Substances that had the highest antioxidant activity will be measured for catechin levels.

MATERIALS AND METHODS

Plant materials

Green tea leaves (Camellia sinensis L.) obtained from Gunung Mas Plantation, Bogor, West Java, Indonesia and determined in Herbarium Bogoriense, Botanical Field, Biology Research Center-LIPI, Cibinong, West Java, Indonesia.

Preparation of green tea leaves powder

Green tea leaves were collected, sorted and then dried by oven at temperature 40°C – 50°C for seven days. The green tea leaves powder are made by grinding the dried green tea leaves using a grinder. The green tea leaves powder is kept in a brown bottle tightly closed, protected from sunlight and humid air.

Preparation of ethanolic extract of green tea leaves

The green tea leaves powder was used 1000 grams. One part of green tea leaves powder was added 7 parts of 96% ethanol. The powder is soaked in 96% ethanol for one day and stirred every 6 hours. This process was repeated six times and maceration time for three days. All the macerates were collected and concentrated with a vacuum evaporator until the solvent evaporated perfectly or viscous ectract was obtained.

Preparation of ethyl acetate and water fraction

Fractionation of ethanolic extract was carried out by liquid-liquid extraction method (ECC). An amount of ethanolic extract was dissolved in 200 mL of distilled water and then put into a separating funnel and added n-hexane at an amount equal to the first solvent. Then, it was shaken with occasional air inside the funnel issued. The n-hexane fraction and water fractions were separated. Into the water fraction was added the same amount of ethyl acetate and shaken as above to obtain ethyl acetate fraction.

Qualitative analysis of flavonoids

Qualitative analysis was carried out for identification of flavonoids. Identification of the presence of flavonoids was carried out according to the procedures in WHO guidelines and Harborne.[11,12]

Determination of total flavonoids content

The flavonoids content was determined by aluminium trichloride colorimetric method using quercetin as reference compound. This method was modified from the procedure reported by Chang *et al.*[13] Quercetin was used to make the calibration curve. One milliliter of quercetin was dissolved in 96% ethanol and then diluted to 2.0; 4.0; 6.0, 8.0 and 10.0 µg/mL. The diluted standard solutions were separately mixed with 3.0 mL of 96% ethanol, 0.2 mL of aluminum trichloride, 0.2 mL of 1 M potassium acetate and 5.6 mL of distilled water. After incubation at room temperature for 30 min, the absorbance of the reaction mixture was measured at 440 nm with a UV-Vis spectrophotometer. Similarly, 2.5 mL of ethanolic extracts, water fraction and ethyl acetate fraction (100 µg/mL) were reacted with aluminum trichloride for determination of flavonoid content as described above. The sample solution was carried out three replications.

Determination of total phenolic content

The phenolics content was determined by colorimetric method using gallic acid as reference compound. This method was modified from the procedure reported by Singleton *et al.*[14] Gallic acid was used to make the calibration curve. Ten milligrams of gallic acid was dissolved in methanolic solution and then diluted to 0.5; 1.0; 5.0; 10.0 dan 25.0 µg/mL. The diluted standard solutions were introduced into the test tubes, 0.4 mL of Folin-Ciocalteu's reagent and 4.0 mL of 7% sodium carbonate were added. The tubes were mixed with distilled water and allowed to stand for 2 hours. The absorbance of the reaction mixture was measured at 744.8 nm with a UV-Vis spectrophotometer. Similarly, 1.0 mL of ethanolic extracts, water fraction and ethyl acetate fraction were reacted as described above. The sample solution was carried out three replications.

Determination of antioxidant activity

Antioxidant activity was carried out based on the modification of standard DPPH method[15,16] in which DPPH solution was prepared with a concentration of 0.4 mM. The antioxidant activity of green tea leaves was assessed in comparison to standard antioxidant ascorbic acid. Ascorbic acid as a comparison was made with a concentration of 1.0; 3.0; 5.0; 7.0 and 9.0 µg/mL using methanolic solution as a solvent. The ethanolic extract, water fraction and ethyl acetate fraction were made at a concentration of 1.0; 5.0; 10.0; 15.0 and 20 µg/mL using methanolic solution as a solvent. Each test solution and reference mixed with 600 µL of 0.4 mM DPPH and methanol solution to 3.0 mL and homogenized. The test solution with several concentrations was incubated at 37°C in waterbath for 30 minutes. Then the mixture was measured at 517 nm with a UV-Vis spectrophotometer. From the absorbance obtained, percentage inhibition and regression curves were made, and the linear equation was applied to calculate the IC50.

$$Scavenging\ activity\ (\%) = \left(\frac{A-B}{A}\right) X\ 100$$

where A is absorbance of control (DPPH solution without the sample), B is the absorbance of DPPH solution in the presence of the sample (test solution and ascorbic acid). The scavenging activity (%) or % inhibition was then plotted against log concentration and from the graph IC50 (Inhibition concentration 50) value was calculated by linear regression analysis.

Determination of catechin

Determination of cathecin was carried out in a test solution that has the best antioxidant activity. This study used High Performance Liquid Chromatography (HPLC) system. This method was modified from the procedure reported by Saito *et al.*[17] COSMOSIL packed column SC_{18}-MS-II 4.6ID × 150 mm was used as column in this study. Photometric diode array (PDA) was used for both the operation of the detector and for data processing. Detection was carried out by measurement of UV absorbance at 278 nm. The mobile phase was composed of water/acetonitrile/methanol/o-phosphoric acid/ethyl acetate (77.5:18:2:0.5:12 v/v). The mobile phase flow rate started at 1.0 mL/min and inject volume in this study was 10.0 µL.

Catechin used as standard for this determination. Catechin as a comparison was made with a concentration of 62.5; 125.0; 250.0; 500.0 and 1000.0 µg/mL using methanolic solution as a solvent. Ten

milligrams of samples dissolved in methanolic solution. Then 10.0 µL of catechin solution and sample solution was injected to HPLC system.

RESULTS

The collection of green tea leaves (Camellia sinensis L) was obtained from the Gunung Mas Plantation, Bogor, West Java, Indonesia. The macroscopic characteristics of green tea leaves can be seen in Figure 1. Green tea leaves are light green to dark green with an average length of no more than 5 cm, short stalked, coriaceous, alternate, lanceolate and serrate margin. Green tea leaves's smell is aromatic and taste is little tight.

Green tea leaves was collected, sorted and dried so green tea leaves simplicia was obtained. The green tea leaves powder are made by grinding the dried green tea leaves using a grinder. The green tea leaves produced is green. The morphology of simplicia and green tea leaves powder can be seen in Figures 2A and 2B. Green tea leaves powder was macerated using 96% ethanol and concentrated using a vacuum evaporator to obtain a viscous ethanolic extract. The ethanolic extract produced was viscous concentrated brown extract, weighing 291.57 g (29.15% yield) (Figure 2C). Furthermore, the ethanolic extract was fractionated by the liquid-liquid partition method using n-hexane, ethyl acetate and water solvents to obtain the n-hexane fraction, ethyl acetate fraction and water fraction. The yields of n-hexane, ethyl acetate and water fractions were 44.43 g (4.44% yield), 83.77 g (8.38% yield) and 21.16 g (2.12% yields). consecutively.

Ethanolic extract, n-hexane fraction, ethyl acetate fraction and water fraction were carried out qualitative test of flavonoid. The results of the qualitative test of flavonoid showed that in ethanolic extract, ethyl acetate fraction and water fraction contained flavonoids. However, the n-hexane fraction did not contain flavonoids. This result can be seen in Table 1.

From the results of qualitative tests of flavonoids, the n-hexane fraction was not continued as a test material in subsequent studies because it did not contain flavonoids.

Ethanolic extract, ethyl acetate fraction and water fraction were measured for total flavonoids content using UV-Vis spectrophotometer.

Figure 1: Field view of green tea (Camellia sinensis L.) plant.

Table 1: The result of qualitative test of flavonoids.

Compound	Ethanolic Extract	n-Hexane Fraction	Ethyl Acetate Fraction	Water Fraction
Flavonoids	+	-	+	+

Description: (+)means it contains flavonoids, (-) means it does not contain flavonoids.

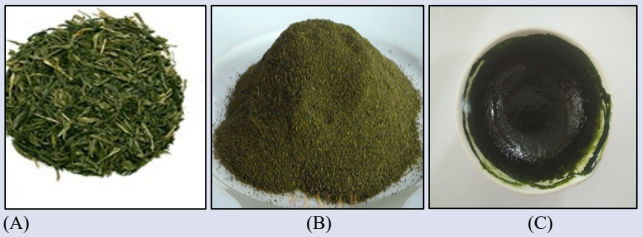

Figure 2: A. Simplicia of green tea leaves; B. Powder of green tea leaves; C. Ethanolic extract of green tea leaves.

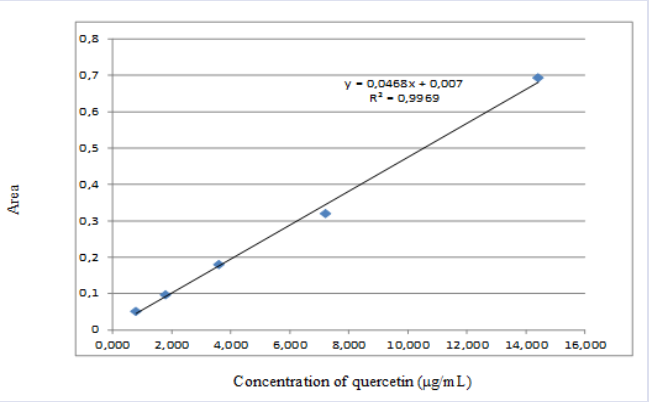

Figure 3: Calibraton curve of quercetin standard.

Determination of total flavonoids content using the colorimetric method which refers to the procedure of Chang *et al.*[13] Quercetin was used as the standard. The regression equation obtained from the calibration curve against the standard quercetin is y = 0.0468x + 0.007 with R2 of 0.9969 which indicates that the curve is linear (Figure 3).

From the regression equation, the total flavonoids content in ethanolic extract, ethyl acetate fraction and water fraction can be calculated. The results of total flavonoids content can be seen in Figure 4.

Based on the results, the three samples have been shown to contain flavonoids according to the qualitative tests previously conducted but the highest total flavonoid content were found in ethyl acetate fractions (0.54%) and the lowest total flavonoids content were found in the water fraction (0.14%).

In this study also measured total phenolic content. Total phenolic content was determined using the Folin-Ciocalteu method using UV-Vis spectrophotometry. Gallic acid was used as a standard in this test and produces a linear regression equation, y = 0.0216x - 0.0011 with R2 of 0.9941 (Figure 5).

The total phenolic content of ethanol extract, ethyl acetate fraction and water fraction can be calculated using the regression equation obtained. The results of total phenol levels can be seen in Figure 6.

From the results above, it can be seen that the largest total phenolic content of green tea leaves is in the ethyl acetate fraction (42.34%), then followed by ethanolic extract (31.16%) and the lowest is in the water fraction (23.78%). These results are linear with the results of total flavonoids content.

Based on the three tests that have been carried out, it is proven that ethanolic extract, ethyl acetate fraction and water fraction contain flavonoids and phenolic compounds which acts as antioxidant compounds, although with different levels. So the three samples were continued to antioxidant activity test using DPPH method. In this test, ascorbic acid was used as a standard. The results obtained from this

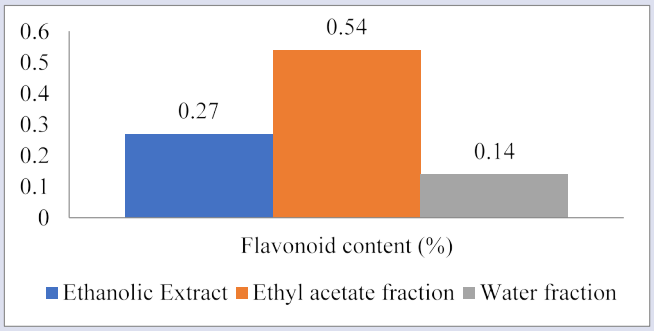

Figure 4: The result of total flavonoids content.

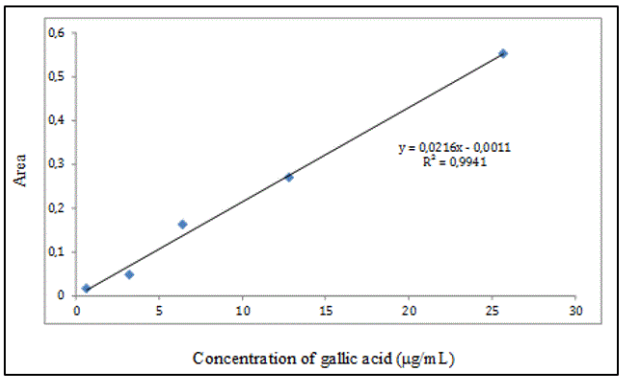

Figure 5: Calibration curve of gallic acid standard.

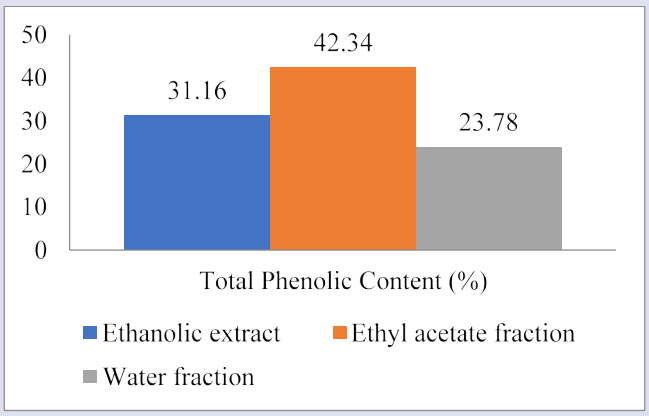

Figure 6: The result of total phenolic content.

study are IC_{50} values (Inhibition Concetration 50). IC_{50} value states the antioxidant concentration (µg/mL) which can inhibit 50% of free radicals. The smaller the IC_{50} value means the antioxidant activity become stronger. The IC_{50} value results from the antioxidant activity test can be seen in Figure 7.

Based on Figure 7, ethyl acetate fraction has the smallest IC_{50} value of 3.926 µg/mL which means that ethyl acetate fraction has strong antioxidant activity. While the largest IC_{50} value is in ethanolic extraxt (9,017 µg/mL). From the above results it is also seen that the IC_{50} ethyl acetate fraction is smaller than the positive control (ascorbic acid). This results indicate that antioxidant activity of ethyl acetate fraction is better than ascorbic acid.

Ethyl acetate fraction has proven have the best antioxidant activity so in this study ethyl acetate fraction was measured for catechin levels. Catechins are the main polyphenol content in green tea leaves which act as antioxidants. Catechins was used as a standard in this test and produce a linear regression equation that is y = 7453.9x - 154172 with R2 of 0.9958 (Figure 8).

The regression equation is used to calculate the level of catechin in ethyl acetate fractions. Ethyl acetate fraction contains catechin of 309.77 µg/mL. The results of the chromatogram and the level of catechin in ethyl acetate fraction can be seen in Figure 9 and Table 2.

DISCUSSION

Green tea leaves can act as antioxidants due to its polyphenol compounds. *Polyphenols* are a *group* of plant-based chemicals that have at least one phenol *group.* Flavonoids are included in a large group of polyphenols contained in green tea leaves. Flavonoids are also known act as antioxidants. Flavonoids are divided into several groups such as flavones, flavonols, isoflavones, catechins, flavanones, leukoantocyanins, aurons, chalcons and dihydroflavonols. Catechins are polyphenol or flavonoids compounds contained in green tea in high concentration around 45-90%.[18,19]

Ethanolic extract, ethyl acetate fraction and water fraction proved to contain flavonoids. While the n-hexane fraction does not contain flavonoids. This is because the ability of the solvent to dissolve flavonoids depending on the level of polarity of the solvent and the extracted

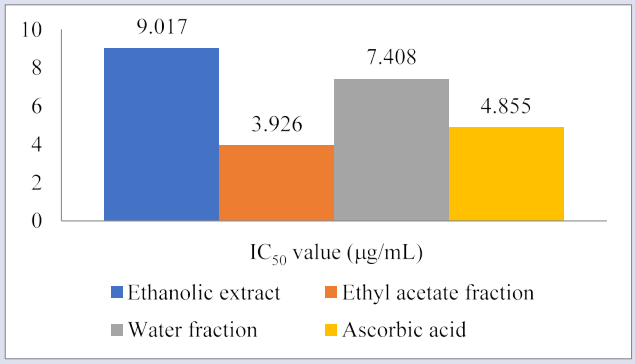

Figure 7: IC_{50} value of antioxidant activity.

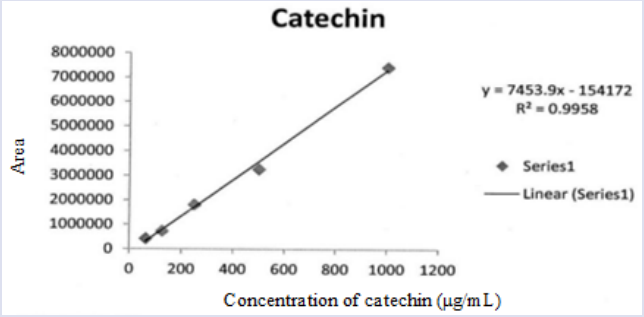

Figure 8: Calibration curve of catechin standard.

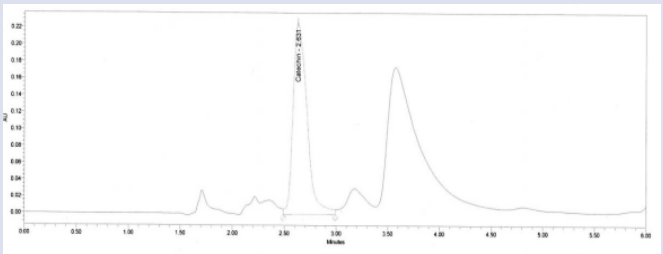

Figure 9: Chromatogram of ethyl acetate fraction.

Table 2: The level of catechin.

Sample	Concentration	RT	Area	Average of RT	Average of Area	SD	RSD	Content (µg/mL)
Ethyl acetate fraction	1 mg/mL	2.631	2131245	2.631	2154797.33	33870.68	1.57	309.77
		2.630	2139533					
		2.631	2193614					

compound.[11] According to Harborne[11] flavonoid compounds have different polarity depending on the number and position of the hydroxyl group. In general, flavonoids are divided into two major groups, namely aglycones flavonoid and glycosides flavonoid. Aglycones flavonoid are flavonoids which do not bind sugar to their structure while glycosides flavonoid are flavonoids that bind sugar to their structure. The presence of sugar in the flavonoid structure causes the glycosides flavonoid become polar compounds while the flavonoid aglycons are less polar. In the n-hexane filtrate, flavonoids content is undetectable because n-hexane is a non polar solvent. This is in line with several previous studies which state that aquadest is effective for extracting glycoside compounds, amino acids, and sugar.[20] Ethyl acetate is effective for extracting alkaloids, aglycons, and glycoside compounds,[20] sterols, terpenoids, and flavonoids.[21] Hexanes can solve non-polar compounds, such as lignin, wax, lipid, sterol, and terpenoids.[21]

Effectivity of ethyl acetate fraction was supported by total flavonoids and total phenolic content. Total flavonoid in ethyl acetate fraction also showed the biggest concentration compared with the others (Figure 4). Ethyl acetate filtrate has the biggest flavonoid content because the polarity of flavonoids is polar and less polar so that it is more easily extracted to semipolar solvents such as ethyl acetate. These results also correlate with the result of total phenolic contain. The ethyl acetate fraction contains the highest total phenolic. Ethanolic extract has higher total flavonoid content and total phenolic content than water fraction because the ethanolic extract has not been separated so that polar and semipolar compounds are still contained in ethanolic extract. Unlike the fraction of water that has undergone fractionation so the content contained in water fractions is polar compounds.

Antioxidant activity of ethanolic extract, ethyl acetate fraction and water fraction was carried out. The method used in antioxidant activity test is the DPPH radical uptake method because it is a simple, easy method, and uses a small amount of sample.[22] In addition, this method does not require a substrate because free radicals are available directly to replace the substrate. DPPH molecule is a free radical molecule in the presence of electrons delocalization around the molecule. The presence of antioxidant activity from the sample resulted in discoloration of the DPPH solution in methanol.[23] This method uses IC_{50} as a parameter to determine the concentration of antioxidant compounds that can inhibit 50% of free radicals. According to Jun *et al.*, as well as other text books,[24,25] antioxidant activity is categorized as a very powerful when IC50 <50 µg/mL, strong if IC50 values of 50-100 µg/mL, moderate at 101-250 µg/mL, and weak when IC50 250-500 µg/mL, and classified as inactive when the IC50 >500 µg/mL. The value of total flavonoid content and total phenolic content can be correlated with the total antioxidant activity which wasrepresented by the IC_{50} of DPPH. Ethyl acetate filtrate has the strongest antioxidant activity compared to ethanolic extract, water filtrate and ascorbic acid as positive control. But when viewed from the IC_{50} results, ethanolic extract, ethyl acetate filtrate and water filtrate are categorized as a very powerful antioxidant activity because the IC_{50} value is less than 50 µg/mL. The antioxidant capacity of flavonoid and phenolic compounds is essentially due to the presence of high activity as hydrogen or electron donor where a hydrogen atom from an aromatic hydroxyl (OH) group can be donated to a free radical.[26,27] The ethyl acetate filtrate which has the strongest antioxidant activity was measured for its catechin content and the results showed that the catechin content in ethyl acetate filtrate was 309.77 µg/mL.

CONCLUSION

The ethyl acetate fraction contains the largest flavonoid compounds compared to other extract so it can be used as a souce of antioxidant bioactive substances in green tea leaves.

CONFLICT OF INTEREST

The authors have no conflict of interest to declare.

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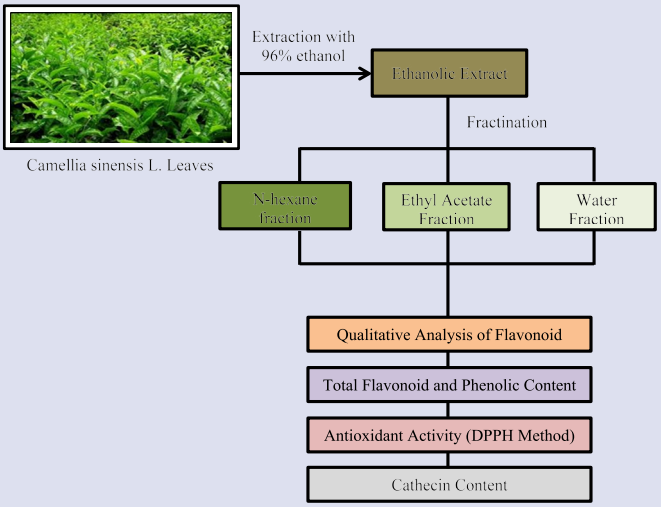
GRAPHICAL ABSTRACT



SUMMARY

Camellia sinensis L. leaves were extracted with 96% ethanol to obtain ethanolic extract. Ethanolic extract were freactinated with n-hexane, ethyl acetate and water to obtain n-hexane fraction, ethyl acetate fraction and water fraction. Ethanolic extract, ethyl acetate fraction and water fraction were contained flavonoid. The highest total flavonoid content and total phenolic content resulted from ethyl acetate fraction. Ethyl acetate fraction also has strongest antioxidant activity than the others with IC_{50} value of 3.926 µg/mL. From this study, it can be concluded that ethyl acetate fraction can be used as a souce of antioxidant bioactive substances in green tea leaves.

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Cite this article: Indarti K, Apriani EF, Wibowo AE, Simanjuntak P. Antioxidant Activity of Ethanolic Extract and Various Fractions from Green Tea (Camellia sinensis L.) Leaves. Pharmacog J. 2019;11(4):771-6.