

Original Article

Radical Scavenging Activity and Quercetin Content of *Muntingia calabura* L. Leaves Extracted by Various Ethanol Concentration

Ratih Dyah Pertiwi^{1,4}, Suwaldi², Ronny Martien², and Erna Prawita Setyowati^{3*}

¹Department of Pharmacy, Faculty of Health Sciences, Universitas Esa Unggul, Jln. Arjuna No. 9 A, Jakarta, 15110

²Department of Pharmaceutics, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta, 55281

³Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta, 55281

⁴Doctoral Program in Pharmaceutical Science, Faculty of Pharmacy, Universitas Gadjah Mada, Sekip Utara, Yogyakarta, 55281

*Corresponding author: Erna Prawita Setyowati | Email: erna_prawita@ugm.ac.id

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Abstract: *Muntingia calabura* is broadly cultivated and has become a common roadside tree in Indonesia that is known as "Kersen," or cherry. The current study aimed to determine the activities of the phytochemical constituent (i.e., phenolic and flavonoid total contents) with various concentrations of ethanolic extract of *M. calabura* leaves (EEMC) to determine the best one and the in vitro antioxidant activities and quercetin content using TLC Densitometry. The extraction was carried out by maceration with various concentrations of ethanol (96% v/v; 70% v/v and 50% v/v) to obtain the ethanolic extract of *Muntingia calabura*, L leaves. The determination of Quercetin was conducted by Thin Layer Chromatography (TLC) densitometry, and the total phenolic was analyzed with reagent Folin-Ciocalteu while the flavonoid content we identified by applying the colorimetric method. The antioxidant capacity was measured using the radical scavenging assay of 1,2-diphenyl-2-picrylhydrazyl (DPPH). The phytochemical study showed that phenolics, saponins, tannins, and flavonoids were present in all EEMC. The extract obtained by 96% ethanol showed the highest total antioxidant activity, reducing power and DPPH (2,2-diphenyl-1-picrylhydrazyl) radical scavenging activity. The same extract also exhibited the highest flavonoid content. However, the extract obtained by 70% ethanol showed the phenolic content, and the highest quercetin content was obtained by using 50% ethanol. The result from the EEMC analysis indicated that *Muntingia calabura* leaf extract contains essential bioactive compounds and shows potential as a source of antioxidants in *M. calabura* which uses 96% ethanol solvent and has the highest quercetin content in *M. calabura* using 50% ethanol solvent.

Keywords: DPPH, *muntingia calabura*, phenolic total, antiradical activity, TLC densitometry

1. INTRODUCTION

Medicinal plants are resources of a therapeutic device for lighting human ailments. About 80% of people in developing countries in the world rely on traditional therapy for their primary health care, and about 85% of traditional medicine involves the extracts of plants. Using the increase in the awakening of the health danger and toxicities associated with the abuse of synthetic drugs, the

interest in the use of plants and plant-based drugs has faced an increase around the world. However, many medicinal plants have not been researched to find out their pharmacological effects. A plant that has recently obtained therapeutic status is *Muntingia calabura* L [1].

M. calabura is widely conserved and therefore, has become a common roadside tree in Indonesia that is known as "Kersen,". *M. calabura* (Elaeocarpaceae), commonly known as cherry which contains phenol is a species in the genus *Muntingia* [2]. Meanwhile, in Indonesia, the use of *M. calabura* is still restricted, but it has been used to treat various diseases in the overseas. The roots of *M. calabura* have been consumed as an abortifacient in Malaysia and as an emmenagogue in Vietnam. In another country, Colombia, the flowers are infused and consumed as a tonic and tranquillizer. The methanol extract of *M. calabura* fruit has exposed potent DPPH quenching capacity [3].

Furthermore, phytochemical studies of various constituents of the plant have identified many bioactive flavonoids, sesquiterpenes, chalcones, and phenolic compounds [2]. The secondary metabolite classes present in the crude extracts of *M. calabura*, generally the flavonoid class, are known to be reliable for the plant's different bioactivities [4]. Past studies have proved flavones, flavanones, flavans, and biflavans to be the primary ingredients of this *M. calabura*, some of which have shown anti-platelet aggregation and cytotoxic activities [5]. However, the above research is mostly done in other countries than in Indonesia. Different sites for the growing of *M. calabura* plants will cause differences in the content of phenols and flavonoids. Besides, this research also aims to find the highest quercetin, phenols and flavonoids content from various variations of solvent concentration.

The current study is mainly focusing on the flavonoid and phenolic content of this plant. Flavonoids are the largest group of plant phenolics, for over half of the eight thousand naturally occurring phenolic compounds [6]. The main strength of dietary flavonoids is its antioxidant properties. Flavonoids are a group of natural antioxidants which are usually found in plants, fruits and vegetables. They are known to be the excellent scavengers of oxygen free radicals [7]. Phenolic and polyphenolic compounds comprise the main class of natural antioxidants present in plants, foods, and beverages [8]. Phytochemicals such as phenolics, carotenoids and flavonoids, have been exposed to possess functional properties such as antimicrobial and free radical scavenging activity. The phytoconstituents acts as a natural antioxidant and prevent free radical formation [9].

Flavonoids have continued beyond one hundred years and possess a broad spectrum of biological activities that might be able to influence processes which are not regulated in disease. Quercetin is a plant pigment that a potent antioxidant flavonoid and especially a flavanol. It is a flexible antioxidant known to possess protective abilities against damage tissue caused by several drug toxicities [10].

The determination of the total flavonoid and phenolic content of ethanol extracts from *Muntingia calabura* leaves (EEMC) with various concentrations and the examination on their potential antioxidant activities is essential to supply more scientific evidence for the research and development of the wild *M. calabura* tree. The main objective of this study is to find the best bioactive constituents of plant *Muntingia calabura*, the best quercetin content using TLC densitometry, the best antiradical activity using various concentrations of ethanol which could serve as a right candidate for the development of new antiradical agents.

2. MATERIAL AND METHODS

2.1. Plant Materials

M. calabura was collected in May 2017 from BALITRO (The Research Institute for Spices and Medicinal Plants) in Bogor and was determined by the Research Center for Biology–Indonesian Institute of Sciences (LIPI) in Cibinong, Bogor.

2.2. Chemical Materials

Ethanol 96%; 70%; and 50% (technical grade, C.V. Bratachem), Ascorbic acid (Sigma Aldrich), Gallic acid (Sigma Aldrich), HCl 2 N (technical grade, C.V. Bratachem), 2,2-Diphenyl-1-Pikrilhidrazil (Sigma Aldrich), Folin Ciocalteu reagent (Merck), Mess Buffer, Methanol 20% and 80% (technical grade, CV, Bratachem), and, H₂SO₄, Fe Cl₃ 1% (AlCl₃) 1% (w/v in ethanol) and Sulphuric Acid (H₂SO₄) 10% (v/v in water).

2.3. Extraction of *M. calabura* Leaves

Dried *M. calabura* leaves were grounded and extracted with various concentrations of ethanol (96%: 70%; 50%). The filtrate was evaporated to obtain the viscous extract [8].

2.4. Phytochemical Analysis

Chemical tests were carried out to the extract to screen for and identify bioactive chemical constituents in the medicinal plants, such as tannins, flavonoids, alkaloids, and saponins, using the standard procedures as described by Harborne [11] with slight modifications.

2.5. The Determination of Total Phenolic Contents

As suggested by Orak [12] that using gallic acid is the standard phenolic compound, the total phenolic content was determined by using the Folin–Ciocalteu reagent. Concentration was calculated using Gallic acid, as standard and the results were expressed as mg gallic acid equivalent/100 g wet weight. Gallic acid, in varying concentrations, were used to prepare a standard curve. This curve was used to relate the absorbance of the unknown samples to Gallic acid equivalents (GAE). Results were expressed as mg GAE in 1 g of dried sample (mg GAE/g). In short, 1 ml of extract solution was mixed with 45 ml of distilled water, then 1 ml of Folin–Ciocalteu reagent was added, and the contents of the flask were mixed thoroughly. After three minutes, 3 ml of Na₂CO₃ was added, and the mixture was allowed to stand for two hours. The absorbance was measured at 750–760 nm.

2.6. The Determination of Total Flavonoid Contents

The flavonoid total content was determined using a colorimetric method. The aluminum chloride colorimetric method was modified from the procedure suggested by Chang *et al* [13]. The total flavonoid content was determined using a standard curve with quercetin as the standard. Several milligrams of quercetin were dissolved in 80% ethanol and then diluted to 25, 50 and 100 µg/mL. The diluted standard solutions (0.5 mL) were separately mixed with 1.5 mL of 95% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate and 2.8 mL of distilled water. After incubated at room temperature for 30 min, the absorbance of the mixture was measured at 425 nm. The quantity of 10% aluminum chloride was reversed by the same amount of distilled water in blank. Similarly, 0.5 mL of ethanol extracts or 15 mL standard flavonoid solutions (100 µg/mL) were reacted with aluminum chloride for the determination of flavonoid content as described above.

2.7. Thin Layer Chromatography Study for Quantification of Quercetin

The standard and the sample were dissolved in ethanol and were filtered using Whatman Filter paper no. 41 before spotting on thin layer chromatography (TLC) plate. Each standard solution and samples were spotted on a 60 F254 silica gel plate and then eluted using a solvent system of chloroform, acetone, and formic acid (10:2:1) with track distance 0.50 cm and the migration distance was 90 mm. Detection was done under ultraviolet at 254 and 366 nm [14].

2.8. DPPH Antiradical Scavenging Assay

Assay of the antioxidant activity of extracts was carried out by spectrophotometric methods using 1,1-diphenyl-2-picrylhydrazyl (DPPH). The control used was ascorbic acid as a positive control, EEMC was prepared with the concentration of 1000 µg/ml, and 0.004% DPPH solution (0,4 mM in 10 ml methanol). Then, 300 µl of each sample solution with a concentration of 0; 5;10;20; and 25 µg/ml was mixed with 900 µl of DPPH solution. Positive control of ascorbic acid was prepared at the same concentration (0, 5, 10, 20, 25 and 25 µg/ml). Observations were made at minutes 1, 10, 20, and 30, using UV-Vis spectrophotometer at a wavelength of 500-550 nm [15].

The Radical Scavenging Activity (RSA) was calculated based on the decrease in DPPH absorption due to the addition of the test sample which is shown as follows:

$$\text{RSA (\%)} = \frac{(\text{Abs } 1 - \text{Abs } 2)}{\text{Abs } 1} \times 100\%$$

Where Abs 1 is absorbance value of DPPH blank and Abs 2 is absorbance value of sample.

3. RESULTS AND DISCUSSION

The material used in this research is *Muntingia calabura* leaves obtained from BALITRO (Research Agency of Spices and Medicinal Plants), Bogor. The results of determination that have been done in Center for Plant Conservation, Indonesian Institute of Sciences (LIPI), Cibinong, showed that the plants used in the research were a cherry plant (*Muntingia calabura* Linn.) *Muntingiaceae* family.

This study investigated a mixture of fresh and green *M.calabura* leaves. After harvested, they were washed and separated from their stems and twigs. The phytochemical constituents present in *M. calabura* plant extracts are shown in Table 1. The phytochemical study uncovered the presence of alkaloids, tannins, phenols, and flavonoids. Furthermore, the preliminary phytochemical studies showed the presence of phenolics, saponins, tannins and flavonoids in the extract of *Muntingia calabura*.

Table 1. Phytochemical constituents analysis of *Muntingia calabura*

Identification	Simplicia	EEMC 96%	EEMC 70%	EEMC 50%
Flavonoids	+	+	+	+
Saponins	+	+	+	+
Tannins	+	+	+	+
Phenols	+	+	+	+

*(+) indicates the presence of the phytochemical constituent.

*(-) indicates absence of the phytochemical constituent

The antioxidant compounds found in plants had different polarities, therefore, different solvents were used to isolate antioxidants. The antioxidant activity of the extract and the yield depended on the solvent [16]. Natural plant products are mainly divided into three major compounds including phenolic, terpenoids, and alkaloids. Accordingly, the study on preparation of plant extracts would be useful in the bioassay of the bioactive compounds. The extract's yield should depend on the polarity of solvents. Additionally, the solubility of the natural products and the solvent could also determine the yield. For example, polar solvents including methanol, acetone and ethanol are most commonly used to extract some flavonols, alkaloids, polyphenols and saponins [17].

The use of ethanol solvent with the different concentrations was intended to attract polar and non-polar chemical components in *M. calabura* leaves powder. The filtrate was concentrated using a rotary evaporator with a temperature of 80°C based on the boiling point of ethanol which is at 78°C [18]. The quantitative determination of phenolic compounds by using Folin–Ciocalteu (F–C) reactive is a broad method [19]. The quantitative determination of phenolic compounds result that was measured with a maximum wavelength of 762 nm for all variant concentrations solvent (96% v/v; 70% v/v and 50% v/v) were identified.

The standard curve for gallic acid was $y = 0.0110x + 0.0003$ ($R^2 = 0.9992$) where y is the peak area (mAU) and x is the concentration (mg mL⁻¹). The content of each compound was expressed as mg/100 g of fresh leaves (Table 2). The total phenolic contents of all various concentration solvents of EEMC (96% v/v; 70% v/v and 50% v/v) were respectively 120.36; 195.33 and 19.17 milligrams/grams gross crude extract (table 3). In contrast, the phenolic total content of EEMC 70% was higher than EEMC 96% and 50%. They should depend on the polarity of solvents. Besides, the level of phenolics in plant sources depends on factors such as cultivation techniques, cultivars, growing conditions, maturation processes, and processing and storage conditions among others [20].

The basic concept of the aluminum chloride colorimetric method is that aluminum chloride forms acid stable complexes with the C-4 keto group and the C-3 or C-5 hydroxyl group of both flavones and flavonols. Furthermore, aluminum chloride forms acid labile complexes with the ortho-dihydroxyl groups in the A- or B-ring of flavonoids (Chang et al., 2002). The flavonoid total contents of all variant concentration solvents of EEMC (96% v/v; 70% v/v and 50% v/v) were respectively 3.387; 0.06942 and 0.06004 % b/b (Table 3). It indicated that the highest flavonoid total content was the EEMC 96%.

Table 2. Linearity equations of reference ascorbic acid, quercetin and gallic acid

Number	Samples	Linier equations	R
1.	Ascorbic acid	$y = 7.6113x - 0.4239$	0.9989
2.	Quercetin	$y = 0,1046x - 0,0316$	0,9986
3.	Gallic Acid	$y = 0,011x + 0,0003$	0,9992

Table 3. Total Flavonoids content, Total Phenolic Content and IC 50 of EEMC 96% EEMC70% and 50%

Sample	Determination	Result	Unit
EEMC 96%	Total Flavonoids Content	3,387	%(w/w)
	Total Phenolic Content	120.36	mg/gram
	Antioxidant IC 50-DPPH	14.17	µg/ml
EEMC 70%	Total Flavonoids Content	0.0700	%(b/b)
	Total Phenolic Content	195.33	mg/gram
	Antioxidant IC 50-DPPH	26.58991	µg/ml
EEMC 50%	Total Flavonoids Content	0.0600	%(w/w)
	Total Phenolic Content	19.17	mg/gram
	Antioxidant IC 50-DPPH	15.36	µg/ml

The high content of phenolic and flavonoids in medicinal plants have been related to their antioxidant activities that play a role in the prevention of the development of the age-related disease, mainly caused by oxidative stress [21].

A technique of extraction from plants substantially affects the final quality of the herbal plants. Evaporator process at high temperature can destroy certain chemical compound in the extract and may adversely affect the efficacy of medicinal plants. Thereby a tool is needed to ensure the quality of the extract. Since the last decade, the chromatographic system has been known as the most important method for the determination of target and uncertain chemical compounds. A great number of TLC techniques have been developed and successfully adjusted into qualitative and quantitative antioxidants analysis.

In the thin layer chromatography experiment, the sample was adjusted to the thin layer as a band near the bottom edge of the layer. The separation in a closed chamber was fulfilled either by contacting the bottom edge of the layer with the mobile phase, which advanced through the layer by capillary forces, or by forcing the mobile phase to move through the layer at a controlled velocity by an external pressure source or centrifugal force. After that, the sample components were parted in space and their position and quantity were identified by scanning densitometry aided by the formation of easily detected derivatives by post-chromatographic [22].

Accordingly, the present study used TLC to examine the chromatographic pattern of the *M Calabura* leaves extract and the results are shown in Figure 1-6 and Table 4. The quercetin contents of all various ethanol concentrations (96% v/v; 70% v/v and 50% v/v) were respectively 0.79; 0.51; 2.29% (Table 4). The highest of quercetin content was EEMC 50%.

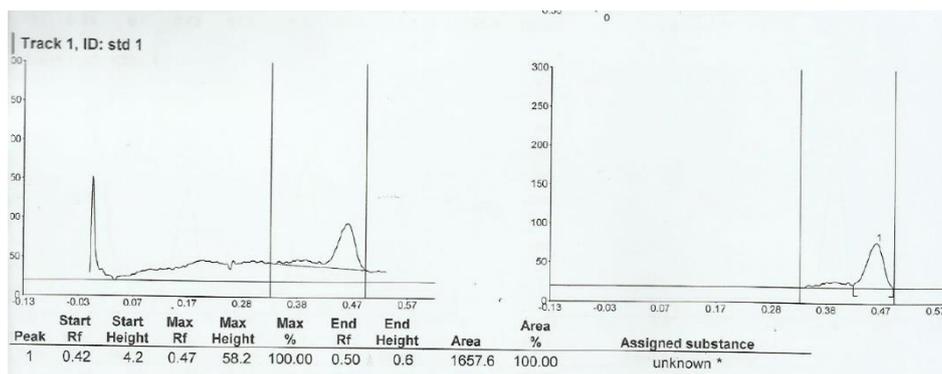


Figure 1. Thin layer chromatogram of Quercetin standard 1

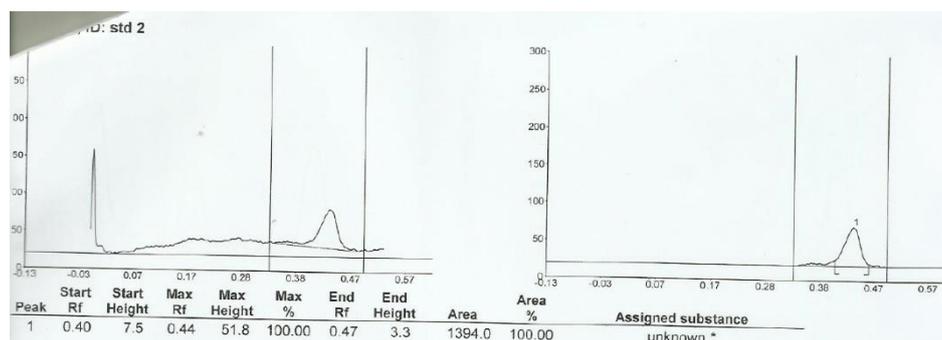


Figure 2. Thin layer chromatogram of Quercetin standard 2

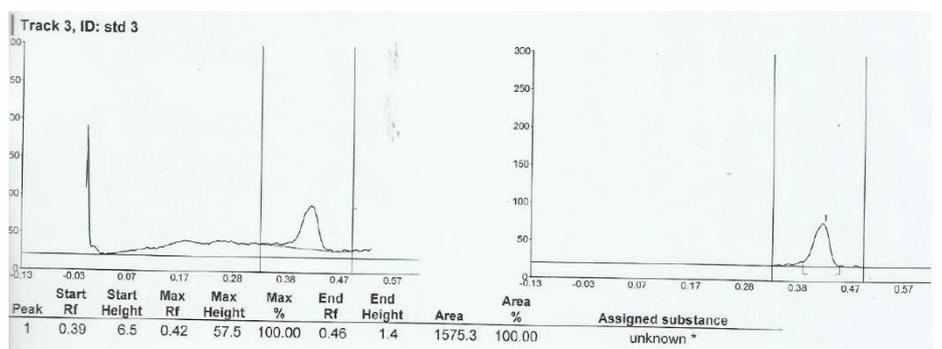


Figure 3. Thin layer chromatogram of Quercetin standard 3

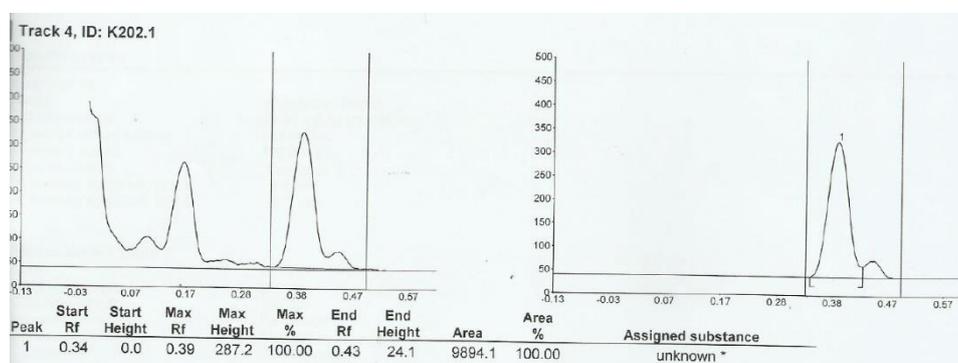


Figure 4. Thin layer chromatogram of Quercetin sample 1 (EEMC 50%)

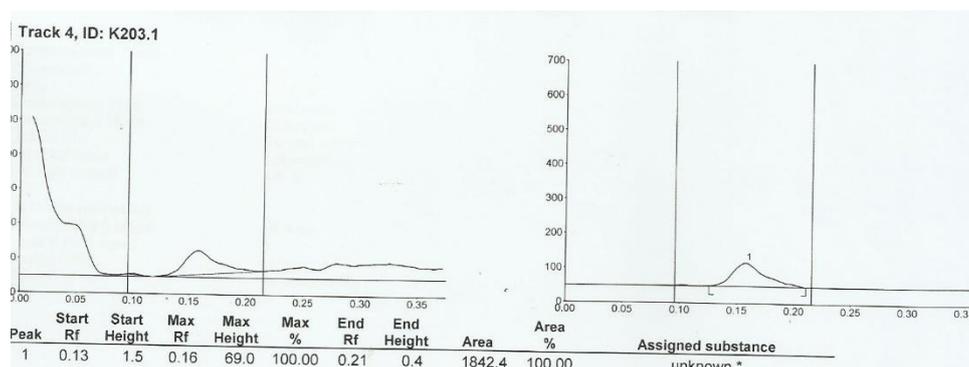


Figure 5. Thin layer chromatogram of Quercetin sample 2 (EEMC 70%)

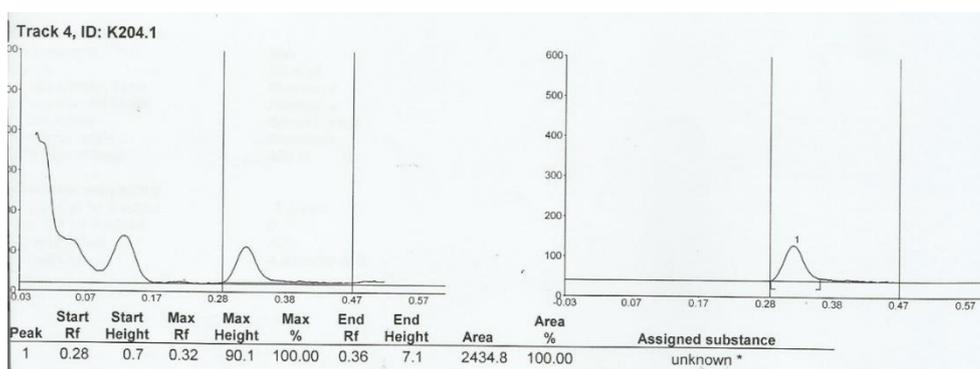


Figure 6. Thin layer chromatogram of Quercetin sample 3 (EEMC 96%)

Table 4. Quercetin content of EEMC 96%, 70% and 50% with TLC Densitometry

Number	Sample	Quercetin	unit
1.	EEMC 96%	0.79	%
2.	EEMC 70%	0.51	%
3.	EEMC 50%	2.29	%

In the present study, the percentage of inhibition was measured to determine the antioxidant activity of the extracts, which was able to inhibit free radicals. DPPH assay was considered to be a more valid, more comfortable and more economical method to evaluate the radical scavenging activity of antioxidants since the radical compound was stable and needed not to be generated. DPPH is nitrogen-centered free radical having a free electron which gives a strong absorption at 517 nm. Purple DPPH changed to yellow when its odd electron paired with the radical scavenger to reduce the DPPH-H. The decrease in the absorbance of DPPH free radical at 517 nm was because of the radical scavenger donated hydrogen [23]. Linearity equations and IC₅₀ of reference ascorbic acid and EEMC are shown in Table 5.

Table 5. Linearity equations and IC₅₀ of reference ascorbic acid and EEMC

Number	Samples	Linier equations	R	IC ₅₀ (µg/mL)
1.	Ascorbic acid	$y = 19,539x + 0,0682$	0,998	2,5554
2.	EEMC 96%	$y = 1,9125 + 3,9006$	0,9854	14,1700
3.	EEMC 70%	$y = 1.0422x + 22.288$	0,9941	26.58991
4.	EEMC 50%	$y = 2.5689x + 10.85$	0.9885	15.361

The result of the antioxidant activity test that was measured with a maximum wavelength of 524 nm for all variant concentration solvents (96% v/v; 70% v/v and 50% v/v) obtained average IC₅₀ value of 14.17000; 26.5899; 15.360 µg/mL respectively, while the result of an antioxidant activity test on ascorbic acid as a comparative standard showed an average IC₅₀ value of 2.5554 µg/mL. EEMC 96% showed a better IC₅₀ value compared to 50% ethanol and 70%. It indicated that various concentrations of solvent were influential in the antioxidant assay. The extract of *M. calabura* leaves proved that *M. calabura* contains important bioactive compounds and has the potential to be a source of antioxidant, especially the EEMC 96%.

4. CONCLUSION

Muntingia calabura leaf extract contains essential bioactive compounds and shows potential as a source of antioxidants in *M. calabura* which uses 96% ethanol solvent and has the highest quercetin content in *M. calabura* using 50% ethanol.

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