Journal of Advanced Research in Applied Sciences and Engineering Technology

Journal homepage:
https://semarakilmu.com.my/journals/index.php/applied_sciences_eng_tech/index
ISSN: 2462-1943

Measurement of Antioxidant Activity Combination of Robusta Coffee (*Coffea canephora*), Matoa Leaves (*Pometia pinnata*) and Stevia Leaves (*Stevia rebaudiana*) with Various Solvent Extractions

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ARTICLE INFO

Article history:
Received 24 January 2023
Received in revised form 18 June 2023
Accepted 23 June 2023
Available online 8 July 2023

Keywords:
Combination; Robusta coffee; Matoa leaf; Stevia leaf; Antioxidant

ABSTRACT

Antioxidants are chemical compounds that can donate one or more electrons to free radicals, so that these free radicals can be be suppressed. The human body does not have excess antioxidant reserves, so if there is excessive exposure to radicals, the body needs antioxidants. There is concern about the possibility of unknown side effects from synthetic antioxidants, causing natural antioxidants to become a much-needed alternative. The development of coffee drinks, growing rapidly and increasingly diverse. Some of them like the taste of pure coffee without any additives and many of them choose to add herbal plants, which have antioxidant properties to enhance their coffee drinks and also add natural sweeteners instead of sugar. This study aims to study the antioxidant activity of a combination of robusta coffee extract (*Coffea canephora*), matoa leaf (*Pometia pinnata*) and stevia leaf (*Stevia rebaudiana*), with various solvents, using the DPPH (1,1-Diphenyl-2-pikrihidrazil) method. The results showed that the ethanol extract of robusta coffee had antioxidant activity with an IC_{50} value of 18.96 ppm, the ethanol extract of matoa leaf had an IC_{50} value of 5.46 ppm, and the stevia leaf extract of ethyl acetate had an IC_{50} value of 17.54 ppm. The value of antioxidant activity in the combination of the three in comparison (4:4:2), has an IC_{50} value of 9.94 ppm. Robusta coffee, matoa leaves and stevia leaves are beverage that have very strong antioxidant activity. The results of the ANOVA test on the extraction various solvents and samples showed a value ($p < 0.01$) indicating that there was a significant difference.

1. Introduction

Indonesia is a country that is rich in natural compound resources with thousands of islands and vegetable wealth which still cannot be fully explored and utilized, especially for medicinal ingredients. Coffee is one of the plantation products that is quite popular, because coffee is currently one of the beverage that is in great demand by the public, especially young people in Indonesia.

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https://doi.org/10.37934/araset.31.2.8190

Thereare 3 types of coffee in Indonesia, including Robusta, Arabica and Liberika coffee. However, currently Robusta and Arabica coffee are one of the main commodities and are most in demand because the quantity and quality are always being developed [1].

In 2018, Indonesia's coffee production was 722.46 thousand tons, 73.06% or 527.80 thousand tons was robusta coffee while the remaining 26.94% or 194.66 thousand tons was Arabica coffee. Robusta coffee production centers in Indonesia, the average data for the last five years are the provinces of South Sumatra, Lampung, Bengkulu, East Java and Central Java. The Arabica coffee production centers in the same year were in the provinces of Aceh, North Sumatra, South Sulawesi, West Sumatra and West Java [2].

The coffee plant (Coffea spp.) belongs to a group of shrubs with the genus Coffea. Linnaeus was the first to describe the species of arabica coffee (Coffea arabica) in 1753. Now more than 120 coffee species have been identified but only one species, namely Coffea canephora or robusta coffee, is cultivated close to the quantity of arabica coffee worldwide [3].

The development of coffee drinks, growing rapidly and increasingly diverse. The many additional variations on coffee drinks make coffee connoisseurs have various tastes. Some of them like the taste of pure coffee without any additions and many of them choose to add herbal plants that have antioxidant properties to add their coffee drinks and also add natural sweeteners as a substitute for sugar.

Classification of coffee based on taxonomic levels, generally as follows: (https://www.itis.gov/servlet/SingleRpt/SingleRpt?search_topic=TSN&search_value=35189#null) [4,5]: Kingdom: Plantae, Sub Kingdom: Viridiplantae, Super Division: Embryophyta, Division: Tracheophyta, Sub Division: Spermatophytina, Class: Magnoliopsida, Order: Gentinales, Family: Rubiaceae, Genus: Coffee L. Species: Coffea arabica L, Coffea benghalensis B, Coffea canephora pierre, Coffea stenophylla G.Don, and Coffea liberica W. Bull.

In 2005-2008, Indonesia was the 4th largest coffee exporter in the world with a contribution value of 4.76%. The demand for Robusta coffee in Indonesia continues to increase from time to time. The coffee plant that is widely cultivated in Indonesia is Robusta coffee (90%) and the rest is Arabica coffee (10%). Robusta coffee is more widely cultivated because it is more resistant to leaf rust disease, has a high production rate, has easy growing and maintenance requirements [6,7].

Matoa leaf herbal plants (*Pometia pinnata*) which are plants from the Sapindaceae family have been widely used as traditional medicine by the society. In Lestyo research [8], matoa leaves (*Pometia pinnata*) contain flavonoid compounds and chemical compounds of flavonoids have been shown to have pharmacological effects as antibacterial, antioxidant and antifungal.

Matoa's classification are Kingdom: Plantae, Division : Magnoliophyta, Class : Magnoliopsida, Order: Sapindal, Family: Sapindaceae, Genus: Pometia, Species : P. pinnata [9].

Research results Lestyo *et al.,* [8], Rani Sauriasari *et al.,* [10] showed that the character of phenol compounds in matoa leaves is a compound belonging to the flavonoid group. Phenolic compounds are active compounds of secondary metabolites which are known to have several properties, namely as astringents, antidiarrheals, antibacterials , and antioxidants [10,11].

Excessive use of sugar can cause several side effects, such as damage to teeth and trigger diabetes. One way to overcome this is to use stevia leaves as an alternative natural sweetener [12].

In Indonesia, the stevia plant has not yet shown its real role as a sweetener source commodity. Many countries have used stevia sweetener to successfully appear as a trade commodity both locally and for export. When viewed from its potential, the stevia plant certainly has good prospects for development in Indonesia [12]. Addition of stevia leaves had a very significant effect on the moisture content, and had a significant effect on the color, scent, taste, and viscosity of instant herbal medicine [13].

The Stevia plant was discovered by a Paraguayan chemist named Dr. Moises Santiago Botany in 1903, so it was named Stevia rebaudiana Botany in honor of the discoverer of plants originating from tropical and sub-tropical regions in South America and Central America. The stevia plant is estimated to have entered Indonesia in 1977 on the cooperation of Japanese and Indonesian entrepreneurs. Stevia cultivation has been tried in several areas with an altitude of around 1000 meters above sea level, such as in the areas of Tawamangu, Sukabumi, Garut, Bengkulu, East Java and South Sulawesi [13].

Bawane's research [13], stevia leaves contain stevioside (5-10%) and rebaudioside A (2-4%) as a sweetener source in addition to other sweetener sources which are small in number such as rebaudiosides C and E (1-2%) and dulcosides A and C (0.4-0.7%), and minor glucosides, including flavonoid glycosides, coumarins, cinnamic acid, and some essential oils. The level of sweetness of stevia sugar is between 200-300 times that of sucrose. The sweetener source is about 14% of dried leaves and some of the sweeteners are similar in structure to the steviol aglycones connected at C-13 and C-19 to mono, di or trisaccharides consisting of glucose and rhamnose residues.

The use of stevia leaves gives paper main features (1), anti-hyperglycemic, anti-hypertensive, anti-caries, anti-inflammatory, and anti-cancer benefitsstevia, (2), value-added stevia joint products, eg. bread, dairy products, and beverages, (3), the effect of combiningstevia on the physicochemical, rheological, and nutritional properties of foods. [14]

According to Charoensin [15], free radicals start the initial stage or the initiation stage of a gene to be carcinogenic, because it requires antioxidant compounds that have free radical scavenging activity to prevent free radicals from initiating to produce substances that trigger degenerative diseases. Compounds that can function as antioxidants are flavonoids and polyphenols.

Free radicals are defined as atoms or molecules with one or more unpaired electrons and are unstable, short-lived and highly reactive to attract electrons from other molecules in the body to achieve stability causing potential damage to biomolecules by damaging the integrity of lipids, proteins and DNA which leads to increased oxidative stress such as neurodegenerative diseases, diabetes mellitus, cardiovascular disease, premature aging, and even cancer [16].

To prevent the accumulation of free radicals that can lead to the development of cancer, antioxidant compounds are needed to neutralize, reduce and inhibit the formation of new free radicals in the body by becoming electron donors for free radicals so as to make free electrons pair up and stop damage in the body. Antioxidants can be grouped into two groups, namely natural antioxidants and synthetic antioxidants. Several types of plants are a source of natural antioxidants, this can be found in several types of vegetables, fresh fruits, several types of herbs and spices. Antioxidants can be produced endogenously or exogenously to help neutralize free radicals present in the body. Endogenous antioxidants produced by the body include glutathione, ubiquinone, and uric acid. Meanwhile, exogenous antioxidants that are milder include vitamins C, E, and beta carotene [16].

The aim of the study was to determine the antioxidant activity and IC_{50} value of the combination of Robusta Coffee (Coffea canephora), Matoa Leaves (Pometia pinnata), and Stevia Leaves (Stevia rebaudiana), with ethanol, ethyl acetate, and water using the DPPH method.

Tests were carried out using the DPPH (1,1-Diphenyl-2-pikrihidrazyl) method, which is an effective and fast colorimetric method for calculating antiradical/antioxidantactivity. This chemical test is widely used in natural product research for the isolation of antioxidants and for testing the capacity of pure extracts and compounds to absorb free radicals. The DPPH method serves to measure single electrons such as hydrogen transfer activity as well as to measure free radical inhibitory activity [17,18].

2. Methodology

2.1 Research Procedures

Robusta coffee beans (Coffea canephora) which have been powdered, and preparation of Matoa leaves, and Stevia leaves obtained in a fresh condition, cleaned, then washed with clean running water, then the clean leaves are air-dried, then sorted dry. The dried simplicia was crushed using a grinder. Powdered simplicia is stored in a tightly closed and airtight container.

2.2 Creating Simplicia Extract

Simplisia was weighed as much as 100 gr. Then the simplicia is soaked in ethanol. Then the results of the soaking are filtered to separate the filtrate and residue. Soaking was carried out until the filtrate was close to clear. The filtrate was concentrated with a vacuum rotary evaporator at 50^{o}C to obtain a viscous ethanol extract, then the sample was weighed. The ethanol extract obtained was then fractionated using the liquid-liquid extraction method. A total of 10 g of ethanol extract was mixed with hot water at 70°C in 100 mL while stirring until all dissolved, put into a separatory funnel and added with 100 mL of ethyl acetate and shaken again until evenly mixed, then allowed to stand until 2 layers were formed, and separated. The water fraction was mixed again with 100 mL of ethyl acetate, stirred and then separated. The treatment was repeated 3 times. Evaporate with a vacuum rotary evaporator under vacuum at a temperature of 40°C until the condensed solvent does not drip and ethyl acetate (EA) fractionation is obtained. Fractionation with water to get the water fraction (A). The extracts and fractions were packaged in dark bottles and stored at 10 °C.

2.3 Calculation of % Yield Value

Prior to assessing the % yield, each filtrate from ethanol and ethyl acetate was evaporated using a rotary evaporator at a temperature of 40ºC-50ºC at a speed of 100 rpm to obtain an extract with a thick texture. Then it is poured into an evaporating cup to obtain ethanol extract and the ethyl acetate (EA) fraction and the water fraction (A). All extracts were weighed and the yield value was calculated. % yield value is calculated by a formula

$$\% \text{ yield value} = \frac{\text{Thick extract weight}}{\text{Extracted simplicia weight}} \text{ x } 100 \quad (1)$$

Fig. 1. Preparation of (a) Robusta coffee, (b) Matoa leaves, and (c) Stevia leaves

Fig. 1. Extracted of robusta coffee, matoa leaves, and stevia leaves

2.4 Preparing of DPPH Solution

4.0 mg of DPPH powder dissolved in 25 mL of methanol. After that, the solution was stored in a dark bottle. For each test a new solution is made.

2.5 Preparing of Test Solution

Further preparation of the sample was weighed as much as 7.1 mg, then dissolved with methanol up to 1000 ppm. Then 40, 80 and 200 μL of mother liquor were pipetted into test tubes that had been measured to obtain sample concentrations of 8, 40 and 200 ppm. Each tube was then added 1 mL of 1 mM DPPH solution and added with methanol to 5 mL. Once homogeneous, the tube containing the solution was incubated in a dark room for 30 minutes. Then the absorption was measured with a UV-visible spectrophotometer at a wavelength of 517 nm. The test was carried out three times for all concentrations of the test solution and the reference solution. This result is determined by the magnitude of the DPPH radical uptake inhibition by calculating the percentage of DPPH uptake inhibition.

2.6 Calculation of IC_{50}

After the absorbance is obtained, the inhibition of antioxidant activity by the extract can be known from the % inhibition value which is calculated using the equation

$$\%\ \text{inhibition} = \frac{(\text{Blank absorbance} - \text{Sample absorbance})}{\text{Blank absorbance}} \times 100\% \quad (2)$$

The IC_{50} value indicates the amount of inhibitor needed to achieve 50% inhibition of the enzyme. The IC_{50} value was determined through linear regression, where the x-axis shows the sample concentration and the y-axis shows the % inhibition. From the equation y = ax + b, the IC_{50} value can be calculated using the following formula

$$IC_{50} = \frac{50-a}{b} \quad (3)$$

Information:
y = % inhibition (50)
a = Intercept
b = Slope
x = Concentration of the test solution (K)

The data obtained from the UV-Vis spectrophotometer in the form of absorbance of control DPPH and DPPH after being reacted with sample and reference test solutions at various concentrations, is used to calculate the % inhibition. % Inhibition is used to obtain IC_{50} (17, 18, 19, 20).

2.6 Data Analysis

In this study, coffee extracts and simplicia from Matoa leaves and Stevia leaves were tested using the DPPH method to determine their antioxidant activity. Data analysis was carried out for the

selectivity test using the calculation of the IC_{50} value, namely the value indicating the concentration of the extract which can inhibit free radical activity by 50%.

To determine the effect of different solvents on the combination of robusta coffee beans, matoa leaves and stevia leaves on the value of antioxidant activity, a one-way ANOVA statistical test was carried out.

3. Results

The results of the antioxidant activity test obtained can be seen in Table 1.

Table 1
Inhibition Test Results (Mean, SD) Antioxidant Activity Robusta Coffee Matoa Leaf, Stevia Leaf and Ascorbic Acid Standard

No	Sample	Testing	Nilai IC_{50} (ppm)	Average	Standard deviation
1	Robusta ethanol extract	1	19,17	18,96	0.5
		2	19,37		
		3	18.39		
2	Robusta ethyl acetate extract	1	29,19	27,03	1,93
		2	26,44		
		3	25,46		
3	Robusta water fraction (Fahreza, Himmi)	1	21,78	20,13	1,43
		2	19,40		
		3	19,21		
4	Matoa leaf ethanol	1	5.71	5.46	0.23
		2	5.38		
		3	5.28		
5	Matoa leaf ethyl acetate	1	5.91	5.77	0.20
		2	5.86		
		3	5.54		
6	Matoa leaf water fraction (Carolin, Himmi)	1	5.82	5.62	0.27
		2	5.72		
		3	5.31		
7	Stevia leaf ethanol extract	1	34.14	33.36	0.68
		2	32.87		
		3	33.08		
8	Stevia leaf ethyl acetate	1	19.51	17.54	2.42
		2	18.26		
		3	14.84		
9	Stevia leaf water fraction	1	43.53	40.95	2.26
		2	40.04		
		3	39.29		
10	Combination of matoa leaf ethanol:coffee:stevia	1	10.33	9.94	1.08
		2	8.72		
		3	10.77		
11	Combination of matoa leaf water: coffee: stevia	1	12.94	12.58	0.33
		2	12.30		
		3	12.50		
12	Combination of ethyl acetate of matoa leaves:coffee:stevia	1	14.24	14.09	0.27
		2	13.77		
		3	14.25		
13	Ascorbic acid (standard)	1	4.59	4.58	0.02
		2	4.60		
		3	4.56		

Antioxidant activity was determined using the DPPH method. The DPPH method measures the ability of a sample to neutralize free radicals and calculates its IC_{50}. The concentration at which 50% of the DPPH free radicals can be reduced, is known as the IC_{50} value. Antioxidant activity will increase with a decrease in IC_{50} value. A chemical is considered very strong in antioxidant activity if the IC_{50} value is less than 50 ppm, the IC_{50} strong group is between 50-100 ppm, the moderate group is between 101-150 ppm, and the weak group is between 150-200 ppm [17-20].

The results of testing the antioxidant activity of the sample materials (Table 1) show that vitamin C as a standard has the best antioxidants. The very strong category of antioxidant activity is shown at IC_{50} values below 50 ppm. Table 1 above shows that the best antioxidant is the ethanol extract of arabica coffee with an IC_{50} value of 18.96 ppm. This IC_{50} value is almost the same as research by Nasirah N, Debi M, 2020 [21] with an IC_{50} value of 12.427 ppm including the strong category.

There is a difference with the research conducted by Ni Made SS 2020 [22] which obtained an IC_{50} value of 40.24 ppm. This difference could be due to the location where it grows, the samples in this study were obtained from the Kerinci area which has an average annual rainfall of 2,991 mm, an average temperature between 16°C to 28°C with an altitude of 3,805 m above sea level and is an active volcano. so that the soil conditions become fertile volcanic soil [23] whereas previous research was obtained from the Tambanan area, Bali which has an average rainfall of 263 mm, the average temperature ranges from an altitude of 2,276 m above sea level [24].

The results of the study in Table 1 above, the best IC_{50} in the ethyl ethanol fraction of matoa leaves 5.46 ppm, is included in the very strong category. This is almost the same as research [25], where the IC_{50} value is 1.403 µg/mL. There is a difference even though it is still in the same category, with research by Martiningsih W (2016) IC_{50} value of 45.78 ppm, this is the same as the coffee study above there are differences in where it grows and temperature, matoa leaves come from the Banyuasri, Singaraja. Research by Yusriyani, IC_{50} value of 43,895 with n-Hexane fraction due, to differences in solvents, matoa leaves come from Makassar [26], while in this study the samples were taken from Denpasar.

The results of the research in Table 1 above, the IC_{50} value of the very strong ethyl acetate fraction of stevia leaves is 17.54 ppm>, the ethanol extract of stevia leaves IC_{50} value is 33.36 ppm> and the water fraction of stevia leaves is 40.95 respectively. In the ethyl acetate fraction of leaves the best stevia IC_{50} value of 17.54 ppm, including strong antioxidant activity. In this study, an IC_{50} value of 17.54 ppm was obtained, the results were stronger in the value of antioxidant activity, this may be due to the growing location and temperature that is more suitable for stevia leaves.

The results of the study in Table 1 above, the combined IC_{50} value (4:4:2) of the ethanol extract of robusta coffee, matoa leaf, and stevia leaf showed the strongest IC_{50} value of 9.94 ppm>, the water fraction with an IC_{50} value of 12.58 ppm> fraction ethyl acetate 14.09 ppm. The IC_{50} was strongest in the combination of coffee, matoa and stevia in the ethanol fraction of 9.94 ppm. The value of antioxidant activity in the combination of the 3 is very strong. The IC_{50} value of matoa leaf extract can increase the antioxidant activity of arabica coffee and stevia leaf compared to the b value of arabica coffee and stevia extract alone. The results of the ANOVA test on the various solvents used and the samples showed a value ($p<0.01$) which indicated a significant difference between the solvents used and the resulting antioxidant activity values.

4. Conclusions

The best antioxidant activity of the combined ethanol extract (4,4,2) of robusta Coffee (*Coffea canephora*), Matoa Leaf (*Pometia pinnata*) and Stevia Leaf (*Stevia rebaudiana*) is at IC_{50} values respectively 9.94 ppm > 12.58 ppm > 14.09 ppm. The antioxidant activity of the combination of the

three drinks are included in the very strong category. The antioxidant activity of matoa leaf extract can increase the antioxidant activity of robusta coffee extract and stevia leaf compared to robusta coffee extract and stevia alone. The results of the ANOVA test on the various solvents used and the samples showed a value ($p < 0.01$) indicating that there was a significant difference.

The antioxidant activity of the combination of the three drinks is included in the very strong category. The antioxidant activity of matoa leaf extract can increase the antioxidant activity of robusta coffee extract and stevia leaf compared to the value of robusta coffee extract and stevia alone.

Acknowledgement
This research was funded by internal grant Universitas YARSI and YARSI Foundation.

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