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Total Flavonoid Content and Antioxidant Properties of Different Extraction Methods of Red Spinach Leaf (*Amaranthus tricolor* L.)

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ABSTRACT: Red spinach leaf (*Amaranthus tricolor* L.) has been well-proven to contain flavonoids that exhibit antioxidant activities. In this study, a comparison between maceration and ultrasonic methods has been carried out to evaluate the effect of these two methods on the level of red spinach leaf flavonoid. In this study, for maceration 1, 2, and 3 days while for the ultrasonic method 10-, 20-, and 30-minutes time duration have been used. The ratios of material and solvent were 1:5 and 1:10. The flavonoid content was determined with the help of a spectrophotometer at a maximum wavelength of 436.80 nm. The antioxidant activity was evaluated by the DPPH method to obtain the IC_{50}. The result of the study revealed that the highest flavonoid content of 4.27 mgQE/g and antioxidant activity (IC_{50}) of 110.47 ppm were obtained by an ultrasonic under conditions of 1:10 ratio and 30-minute extraction time. Results of the study suggested a significant difference ($p<0,05$) in the isolation of total flavonoid contents and antioxidant activities in the maceration and ultrasonic. In these two methods, ultrasonic was found to be more efficient than maceration because of minimum time, the highest flavonoid content and antioxidant activity could be obtained.

Keywords: ***Amaranthus tricolor* Linn.; maceration; ultrasonic; total flavonoid content; antioxidant activity; DPPH.**

Introduction

Red spinach leaf (*Amaranthus tricolor* Linn.) is a member of the *Amaranthaceae* family that has been widely consumed as food and supplements due to its high content of nutrition, minerals, and vitamins [1–3]. Red spinach leaf is reported to contain excellent amount of many compounds, such as dietary fiber, carbohydrates, moisture, and proteins, potassium, calcium, magnesium, iron, manganese, copper, zinc, chlorophyll *a*, chlorophyll *b*, ß-cyanins, total flavonoids, ß-xanthins, betalains, carotenoids, phenolics, ß-carotene, and vitamin C [4]. The remarkable content such as phenolics, ß-cyanins, flavonoids, ß-xanthins, and ß-carotene in red spinach leaf is responsible for its strong antioxidant activity [4]. Red spinach leaf also exhibits antibacterial activities [5,6]. The flavonoid of red spinach leaf, one of the factors for its antioxidant properties, has been well explored [7]. The total flavonoid in red spinach leaf has been found to be higher than in green spinach leaf (*Amaranthus viridis*) [6]. A flavonoid which exhibited antioxidant called anthocyanidins has been isolated from red spinach leaf extract [8].

Several studies have been published to explore the efficacy of red spinach leaf extract in recent times [9–11]. In such studies, the ethanol extract of red spinach leaf containing quercetin can be used for the therapy of hyperlipidemia in the induced Wistar rats [9]. The therapy dose of the ethanol red spinach leaf extract exhibited a gastroprotective effect in rats [10]. The ethanol red spinach leaf extract has hepatoprotective activity in rats [11]. The observed efficacy in the ethanol red spinach leaf extract is believed due to the antioxidant properties coming from its flavonoid content. Flavonoid is responsible for decreasing the free radicals that are involved in various pharmacological conditions. A study showed that the ethanol of *A. tricolor* L. extract has a high flavonoid amount [12]. Thus, the higher antioxidant properties in the ethanol of *A. tricolor* L. extract is due to the higher flavonoid

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content.

The extraction method is the most important step in processing plant raw materials in order to obtain the isolated target compound [13]. Flavonoids were commonly extracted using two types of extraction methods, which are conventional and modern [14]. The number of bioactive substances and extract quality can be affected by the extraction method [15]. In the previous study, the optimization of flavonoid content and antioxidant activities was carried out using the Soxhlet device by various solvents [6]. The methanol extract contained the highest antioxidant properties than in the other solvents [16]. The highest antioxidant properties of methanol extract is due to the flavonoids and phenols content [17].

Although several studies have been carried out to define the total flavonoid and antioxidant properties of *A. tricolor* L., the study on the comparison of different extraction methods in the total flavonoid content and antioxidant properties of *A. tricolor* L. is yet important to investigate. The main objective was to study the differences in the extraction technique under various of times and material-solvent to the total flavonoid contents and antioxidant properties of *A. tricolor* L. In this study, maceration and ultrasonic were used as conventional and modern extraction methods, respectively. The total flavonoid contents for each extraction technique were determined by the spectrophotometric method [18]. In addition, the antioxidant activities of leaf extract were also evaluated by the DPPH method.

Methods

Preparation of Amaranthus tricolor Linn.

Amaranthus tricolor L. leaves were collected on January 2023 from Bekasi, Indonesia (6°16'37.8"S 106°59'02.3"E). *A. tricolor* L. was authenticated in Badan Riset dan Inovasi Nasional (BRIN) in Cibinong, Indonesia. The *A. tricolor* L. fresh leaves were sorted, washed immediately, and then air-dried for 3 days at a room temperature (± 25 °C). The dried leaves were then ground to a fine powder and sieved to a size equal to 40 mesh.

Chemical and Reagents

Ethanol 70%, ethanol 96%, methanol, and aquadest in analytical grade were from PT. Smart-Lab Indonesia. $FeCl_3$, HCl, Magnesium powder, acetic anhydride, NaOH, chloroform, H_2SO_4, Mayer reagent, Dragendroff reagent, gelatin, $AlCl_3$, Potassium acetate, Quercetin, and DPPH free radical were from Sigma-Aldrich, USA.

Extraction Process

The two methods used in the extraction process were maceration and ultrasonic. Ethanol 70% was used as a solvent, with the time variation and the ratios of material and solvent for each procedure, as shown in Table 1.

Maceration Extraction

The dried powder (10 g) of *A. tricolor* L. leaves was weighed. The ethanol 70% was then added to 50 mL and 100 mL based on the ratio of material-solvent of 1:5 and 1:10 respectively. Then, each variation was left for various extraction times (1, 3, and 5 days) by stirring occasionally. The extracts were filtered and evaporated using an EYELA N-1200 BS of vacuum rotary-evaporator. The yield of each variation was calculated.

Ultrasonic Extraction

Sonication was performed with 40 kHz using an ultrasonic bath 1800 (Branson Ultrasonic Co., Brookfield, USA). The powder sample (10 g) was added ethanol 70% about 50 mL and 100 mL according to the ratio of material-solvent (1:5 and 1:10) respectively. Each variation was sonicated for 10, 20, and, 30 minutes. The extracts were filtered and concentrated by rotary-evaporator.

Table 1. Variation in the extraction time and ratio of material - solvents

Methods	Variation of extraction time	The ratio of material (g): solvent (mL)
Maceration (day)	1 3 5	1:5 1:10
Ultrasonic (minute)	10 20 30	1: 5 1:10

Phytochemical Evaluation

The phytochemical test was evaluated to detect the presence of secondary metabolites in the red spinach leaf extract. The metabolites identified include the detection of alkaloid, saponin, flavonoid, triterpenoid, steroid, phenolic, and tannin. The identifications were determined by the standard procedures from Indonesian Pharmacopeia and other publications [19–21].

Alkaloid Screening

Each 2 mL of extract sample was reacted by Dragrendroff and Mayer reagents. For Dragrendroff reagent, the sample is placed in aqueous HCl (5 mL), then filtered, and reacted with Dragendroff's solution. Alkaloid are indicated by the presence of red precipitate. For Mayer's reagent, a similar treatment is carried out on the sample and then a few drops of Mayer reagent are added. The formation of white or green color precipitate indicates the alkaloid [19,20].

Saponin Screening

Each 2 mL of extract sample is added with 4 mL of distilled water. Then, this solution will be mixed thoroughly and shaken vigorously. Saponin is detected if foam is produced and persisted for at least 10-15 minutes [19,20].

Phenolic Screening

Each 2 mL of extract sample was reacted with 2 mL of 5% $FeCl_3$.The phenolic is detected when the blue color is formed [19,21].

Flavonoid Screening

Each 2 mL of extract sample was added with certain drops of 20% NaOH to produce the yellow color. It was then added with certain drops of 70% of HCl to remove the formation of those yellow color. The flavonoid was indicated by the formation and the disappearance of the yellow color [19,21].

Triterpenoid Screening

Each 2 mL of extract sample was mixed with 0.5 mL for each acetic anhydride and chloroform. It was then added with few drops of concentrated sulfuric acid. The terpenoids was indicated by the presence of reddish-brown precipitate [19–21].

Steroid Screening

Each 2 mL of extract sample was added with 2 mL of chloroform. Then, it was added by 2 mL of concentrated H_2SO_4. The steroid will be indicated by the chloroform layer turned to red while the acid layer turned to green-yellow color [19,21].

Tannin Screening

Each 2 mL of filtered sample was added with 10% of alcoholic $FeCl_3$. The tannins were indicated by the formation of the black/ brownish blue [19–21].

Determination of Total Flavonoid Content

The total flavonoid content was examined by the aluminum chloride method, as described by Chang *et al.* [22]. Briefly, 10 mg of each maceration and ultrasonic extract were weighed, and added with ethanol 96% into a 10 mL flask to make 1000 ppm extract concentration. Then each extract was piped 0.5 mL added 0.1 mL $AlCl_3$ 10%, 0.1 mL Potassium acetate 1M, and ad 2.8 mL with distilled water, then shook until homogenous and incubated for the optimum time. Then, the samples have measured the absorbances at a maximum wavelength using a UV-1900 series spectrophotometer (Shimadzu, Kyoto, Japan). The linear regression curve was obtained by the Quercetin standard. The value was defined in terms of mg quercetin equivalent (QE) per gram of dry sample.

Table 2. The extraction yield of *A. tricolor* L. extracts (%, w/w)

Method	Time	The ratio of material (g) and solvent (mL)	
		1:5	1:10
Maceration	1	2.55	4.55
	3	6.22	9.51
	5	8.35	10.33
Ultrasonic	10	6.12	8.11
	20	9.22	12.25
	30	12.51	17.20

All determinations were conducted in triplicates and determined as mean ± SD.

Antioxidant Activities

The analysis of antioxidant activity was carried out using an in vitro DPPH assay adapted from Molyneux [23]. The stock solution of red spinach leaf extract was prepared at 1000 ppm. A quercetin standard stock was also served at 20 ppm. The extract solutions were 60, 80, 120, 140, and 160 ppm; while quercetin solutions were 2, 4, 6, 8, and 10 ppm. DPPH solution (1 mL, 0.2 mM) in ethanol was applied with the sample or quercetin in various concentrations (1 mL). The reaction was homogenized. The samples were then incubated under the dark condition for 30 minutes at room temperature. The absorbances of the reaction were then read at the maximum wavelength after undergoing decolorization by a UV-Vis spectrophotometer, which was 436.80 nm. The antioxidant activity of the quercetin standard was calculated for comparison. Ethanol was served as a blank, and the control solution containing ethanol and DPPH without any sample or standard was also served. Antioxidant activity was measured at the percent of inhibition relative to the control using the following equation:

$$\text{Antioxidant activity (\%)} = (A_{blank} - A_{sample} / A_{blank}) \times 100$$

A_{blank} = the absorbance of the control solution

A_{sample} is the absorbance of the extracts or quercetin standard.

All procedures were carried out in three replications, and the values were calculated in an average. The regression linear equation was generated by plotting the sample concentration (x) against the percentage of inhibition (y). The concentration which gives the inhibition of DPPH of 50% is defined as IC_{50}.

Statistical Analysis

The data obtained were analyzed using an ANOVA test. The statistical analyses were carried out to find out whether there were significant differences between the average counts of several data groups [24]. The dependent variables were total flavonoid content and antioxidant activity. The independent variables were maceration and ultrasonic methods. All experiments were performed in three replications. All values were evaluated using Statistical Package for Social Science (SPSS). The level of $p<0.05$ was defined as statistically significance.

Result and Discussion

Amaranthus tricolor L. leaves were extracted by maceration and ultrasonic which represent conventional and modern extraction methods respectively. The variation of time and ratios of material-solvent are important parameters during the extraction. The goal is to choose the method that can efficiently provide higher yield, total bioactive content, and activity. The yields obtained from red spinach leaf extract are shown in Table 2. In addition to the polarity factor of the targeted compound, ethanol was chosen as a solvent because flavonoid was proven to

Table 3. The phytochemical screening of *A. tricolor* L. extracts

Kelompok	Maceration (days)						Ultrasonic (minutes)					
	1:5			1:10			1:5			1:10		
	1	3	5	1	3	5	10	20	30	10	20	30
	M11	M31	M51	M12	M32	M52	U11	U21	U31	U12	U22	U32
Alkaloids	+	+	+	+	+	+	+	+	+	+	+	+
Phenolics	+	+	+	+	+	+	+	+	+	+	+	+
Flavonoids	+	+	+	+	+	+	+	+	+	+	+	+
Saponins	+	+	+	+	+	+	+	+	+	+	+	+
Tannins	+	+	+	+	+	+	+	+	+	+	+	+
Triterpenoids	-	-	-	-	-	-	-	-	-	-	-	-
Steroids	+	+	+	+	+	+	+	+	+	+	+	+

Note: (+) = detected; (-) = not detected; (M11, M31, M51) = Maceration 1 day, 3 days, and 5 days with the ratio of 1:5 as respectively; (M12, M32, M52) = Maceration 1 day, 3 days, and 5 days with the ratio of 1:10 as respectively; (U11, U21, U31) = Ultrasonic 10, 20, and 30 minutes with the ratio of 1:5 as respectively; and (U12, U22, U32) = Ultrasonic 10, 20, and 30 minutes with the ratio of 1:10 as respectively

be higher in the ethanol extract of red spinach leaf [25].

The highest yield by maceration method was found in 5 days under the ratio of 1:10 of material-solvent (10.33%). The yield of extraction by maceration in 1 and 3 days was about 2.55-9.51% w/w, which was lower than 5 days of maceration. It shows that the longer duration of maceration gives sufficient time for the solvent to solute the bioactive content in the samples [26]. The results of water content for the extract were in the range of 7.82-8.91%. These results meet the requirements for extract preparations, which is below 10% [27–29]. However, the yield of 5 days of maceration was still lower than the yield of 30 minutes of ultrasonic extraction (17.20% w/w). The results showed an increase in the ratio of material-solvent resulted in a higher yield of extracts. It shows that an increased amount of solvent can lead to more interactions of solute-solvent such that it produces an increased solubility of the substances.

Phytochemical identification was conducted to confirm the presence of secondary metabolites qualitatively. The results showed that alkaloids, phenolics, flavonoids, saponins, tannins, and steroids are present in all extracts as shown in Table 3. Meanwhile, none of the triterpenoids are detected in all extracts. The results showed that most of the phytochemical substances in *A. tricolor* L. leaves dissolved in ethanol 70% solvent. Ethanol is a universal solvent that is theoretically easier to enter the cellular membrane and solute the intracellular materials [30,31]. The differences in the extraction technique give similar types of phytochemical contents in the same extract [32].

Total Flavonoid Content

TFC was evaluated by the aluminum chloride method using quercetin as a standard. The color product from the reaction between aluminum chloride and flavonoid is evaluated at 436.80 nm of maximum wavelength. A linear regression curve of 2-6 ppm of Quercetin (y=0.0991x + 0.0014) with the coefficient of determination (R^2) value of 0.9952, was generated. TFC of *A. tricolor* L. extracts is shown in Figure 1. The highest TFC was found in U32 (Ultrasonic 30 minutes with the ratio of 1:10) at 4.27±0.02 mgQE/g extract which is approximately 1.2-fold more than M32 (Maceration 3-days with the ratio of 1:10). The highest TFC has the highest extraction yield of U32 at 17.20% as shown in Table 2. The results showed that the ultrasonic method under any variation produced higher TFCs than in maceration method in any variations. It revealed that ultrasonic is more efficient than maceration to solute bioactive compounds, including flavonoids. A similar result has shown that ultrasonic gave the highest percentage of phenolic than in maceration [33].

Analysis of Antioxidant Activity

The antioxidant capacity of the red spinach leaf extracts was determined by IC_{50}. IC_{50} is defined as the

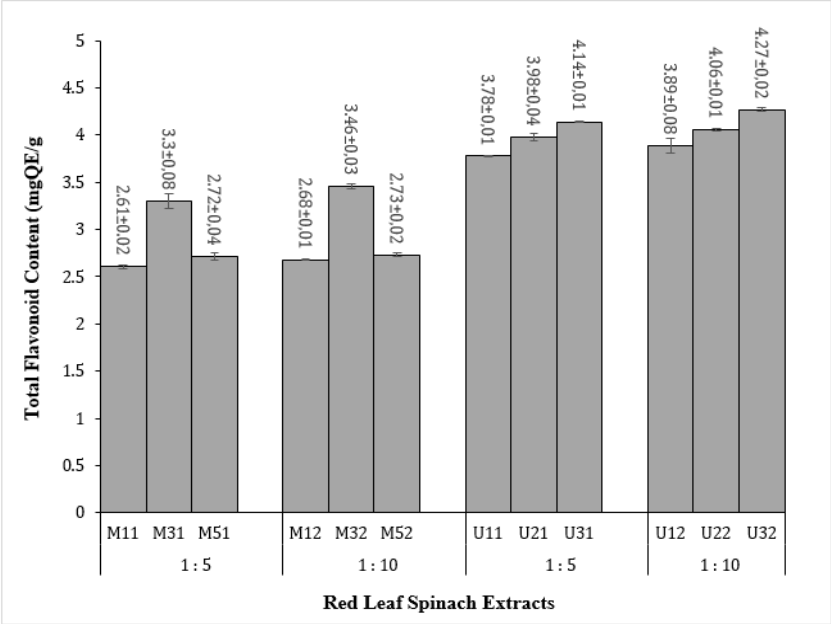

Figure 1. Total flavonoid content of *A. tricolor* Linn. leaves extracts

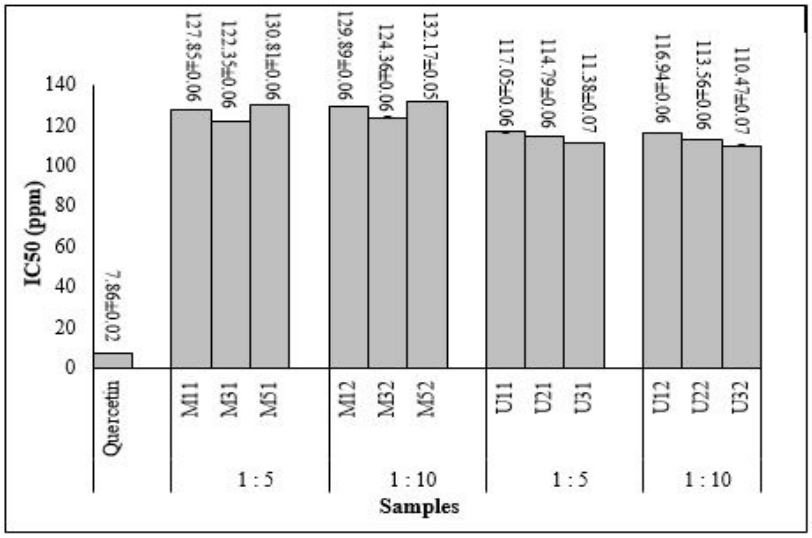

Figure 2. IC_{50} represents the antioxidant activity of *A. tricolor* Linn. leaf extracts as compared to Quercetin. Note: Data are expressed as mean ± SD (n=3)

concentration of the samples required to gain 50% of DPPH free radical inhibition. The high antioxidant capacity is indicated by the lower value of IC_{50}. The IC_{50} values are shown in Figure 2. For a comparison, a calibration curve of 2-10 ppm of Quercetin (y=4.6732x + 13.237) with the coefficient of determination (R^2) of 0.9945, was calculated. The IC_{50} value of the Quercetin standard is 7.86±0.06 ppm.

Antioxidant is a substance which inhibit oxidation producing free radicals [34]. The excess number of free radicals could generate oxidative stress [32]. Several varieties of chronic and degenerative diseases and some acute pathological conditions can potentially be caused by these oxidative stresses [35]. The DPPH assay is commonly used to calculate the antioxidant properties of compounds since it is considered as a valid accurate method [36]. Antioxidant properties of various *A. tricolor* L. extracts were investigated in this study. Among them, the highest antioxidant activity was U32 (IC_{50} = 110.47 ppm) as compared to the other extracts. The potency of antioxidant activity in the U32 is considered to be moderate since it has an IC_{50} value in the range of 100-150 ppm [18]. It is 14-fold lower as compared to the Quercetin. The U32 which has the highest radical scavenging activity is the extract which has the highest total flavonoid content (4.27±0.02 mgQE/g) and highest yield of extract (17.20%). In this study, the antioxidant activities obtained from the ultrasonic method are higher than maceration, as well as the TFC which is found higher in ultrasonic extracts than maceration extracts. It can be implied that the higher total flavonoid content (TFC) is responsible for the increase in antioxidant activity. In the ultrasonic method, the longer duration of ultrasonic produces higher antioxidant activity. It showed that a longer duration of the extraction process can increase the contact between solute and solvent, thus, increasing the solubility of compounds including flavonoids.

It is known that certain extraction techniques which involves high temperature and/or longer duration time, such as reflux heating, microwave, ultrasonic assisted extraction, and maceration, can possibly degrade the targeted compounds. The degraded compounds are unstable flavonoids, such as rhamnetin, myricetin, quercetin, and kaempferol [37]. In the maceration method, 5 days of maceration (132.17 ppm) has a lower antioxidant activity than 3 days of maceration (124.36 ppm). It is then followed by TFC data in which TFC in 5 days maceration (2.73±0.02 mgQE/g) was lower than 3 days of maceration (3.46±0.03 mgQE/g). It is possibly in some cases, the prolonged maceration may cause certain reactions that lead to the decrease in bioactive content [38,39]. A study showed that anthocyanins concentrations are decreased during extended maceration process [39]. The previous finding is in line with our study which showed that prolonged maceration (5-days) resulted in the decreased of flavonoid content and thus affect to the lower of antioxidant activity.

The statistical analysis has revealed that there was a significant effect of ultrasonic and maceration methods with variation of time and material-solvent ($p< 0.05$)

on the total flavonoid content and antioxidant activity. In this study, the significance value of 0.003 <0.05 was obtained suggesting that the results were significant. It can be concluded that the extraction methods with their variations can affect the total flavonoid contents and antioxidant activity of the red spinach leaf extract.

Conclusion

The best extraction method for red spinach leaf was the ultrasonic method using a 1:10 ratio of red spinach leaf to ethanol at 30 minutes of extraction time. The extract under this condition exhibited the highest yield of 17.20±0.055%, total flavonoid content of 4.27±0.02 mgQE/g, and antioxidant activity (IC_{50}) of 110.47±0.02 ppm. The higher total flavonoid content is responsible for the higher antioxidant activity of red spinach leaf extract. Hence, this study showed that ultrasonic was found to be more efficient than maceration because, within a minimum timeframe and less labor, the highest yield, TFC, and antioxidant activity could be obtained.

Conflict of Interest

The authors have no conflicts of interest regarding this investigation.

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