Effect of ultrasound-assisted extraction on total phenolics, flavonoids, and antioxidant activity of *Adenostemma lavenia* leaves

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Abstract. *Adenostemma lavenia* has been reported to possess significant value in traditional medicine and exhibit therapeutic potential. This study aimed to evaluate the extraction conditions of *A. lavenia* leaves using an ultrasound-assisted technique. A completely randomized factorial design with two factors (extraction time and ethanol concentration) was implemented. Total phenolic content (TPC) and total flavonoid content (TFC) were determined using the Folin-Ciocalteu and $AlCl_3$ complex assays, respectively. At the same time, antioxidant activity was assessed using 2,2-diphenyl-1-picrylhydrazyl (DPPH) and cupric ion-reducing antioxidant capacity (CUPRAC) assays. The Rank Spearman test evaluated the correlation between TPC, TFC, and antioxidant activity. The results indicated that the optimum conditions were 45 minutes of extraction time and 90% ethanol concentration, yielding a TPC of 11.94±0.11 mg tannic acid equivalents (TAE)/g extract, a TFC of 0.51±0.00 mg quercetin equivalents (QE)/g extract, antioxidant activity of 2,507.19±26.62 mg/L (IC_{50} of DPPH assay), and 133.50±0.71 mg/L (IC_{50} of CUPRAC assay). Antioxidant activity (CUPRAC assay) was significantly correlated with TFC in *A. lavenia* leaves. This study suggests that *A. lavenia* could be explored as a natural antioxidant.

1 Introduction

Medicinal plants have the ability to treat and prevent various diseases. Legetan warak (*Adenostemma lavenia*), a native Indonesian plant, has been used for generations as a medicinal herb. *A. lavenia* (Asteraceae family) is utilized to treat fever, cough, mouth ulcers, and sore throats [1]. Additionally, *A. lavenia* is frequently used in herbal remedies to treat

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pneumonia, hepatitis, and skin wounds [2]. According to reports, the leaf extract of *A. lavenia* has been found to exhibit antioxidant activity and other bioactivities [3, 4, 5]. Nurlela et al. [6] conducted a chemical profile analysis of *A. lavenia* using a liquid chromatography-tandem mass spectrometer (LC-MS/MS), which exhibited that *A. lavenia* contains a variety of secondary metabolites, including phenolics and flavonoids such as chlorogenic acid, dicaffeoylquinic acid, kaempferol, rhamnetin, and eriodictyol 7-O-sophoroside. These phenolics and flavonoids are abundant in *A. lavenia* leaves, exhibit various bioactivities, notably antioxidant activity, which helps enhance the body's defence against diseases caused by free radicals.

Bioactive compounds extraction, such as phenolics and flavonoids of *A. lavenia* leaves is a crucial step in harnessing their potential. Various methods have been employed, including maceration and ultrasound-assisted extraction (UAE). The UAE method has proven effective in improving extraction efficiency by reducing solvent volume and extraction time. Moreover, UAE can be performed at lower temperatures to prevent heat-induced damage. The mechanical effects of acoustic cavitation enhance UAE efficiency by disrupting plant cells, thereby increasing mass transfer and solvent penetration into plant material. To achieve high extraction efficiency, optimizing ultrasonic operational parameters, including solvent type and concentration, extraction time, and temperature [7].

Yazici [8] conducted the Asteraceae plant *Taraxacum assemanii* extraction optimization. The study indicated that the UAE method, when applied for 30 minutes, enhanced the total phenolic content (TPC) by 36.66% and the total flavonoid content (TFC) by 21.55% compared to the TPC and TFC yielded by maceration extraction conducted for 1 hour. Another study investigating the extraction of *Artemisia herba-Alba* (Asteraceae family) demonstrated that utilizing the UAE method for 50 minutes resulted in a 72% improvement in the DPPH-IC_{50} value compared to maceration extraction, which required 4 hours of agitation [9]. These findings suggest that the UAE method can increase TPC, TFC, and its antioxidant activity.

Several factors, including solvent concentration and extraction duration, can significantly influence the results of UAE extraction. For instance, a study on *Stevia rebaudiana* leaves showed that using the UAE method with 50% ethanol for 5 minutes resulted in the highest TPC and antioxidant activity compared to other solvents (25% ethanol and water) and extraction durations (10 and 15 minutes) [10]. The UAE method can be further optimized by adjusting the solvent concentration and extraction time. However, the specific effects of extraction duration and solvent concentration on the extraction of *A. lavenia* leaves using the UAE method have not been previously reported. Therefore, it is necessary to conduct a study that evaluates the extraction of *A. lavenia* leaves using the UAE method with varying extraction time and solvent concentration to produce high TPC, TFC, and antioxidant activity of *A. lavenia* leaves extract.

2 Materials and methods

2.1 Materials

Adenostemma lavenia leaves were procured from the Biopharmaca Conservation and Cultivation Station, Tropical Biopharmaca Research Center at IPB University, Bogor, West Java, Indonesia. The geographical coordinates of the collection site are approximately 6°32'25.47" N latitude and 106°42'53.22" E longitude, at an altitude of approximately 142.60 meters. The reagents utilized were of analytical-grade quality.

2.2 Extraction

The air-dried leaves of *A. lavenia* underwent pulverization into powder using a mechanical grinder. Subsequently, the powdered leaves were quantified and placed into an Erlenmeyer flask, combined with ethanol, and subjected to extraction employing the ultrasound-assisted extraction (UAE) technique utilizing a UAE apparatus (Sonics VCX 759, USA). Following filtration with Whatman filter paper, the extract was vacuum-dried using a rotary evaporator operating at 50°C until the solvent was entirely evaporated. The study was conducted using a randomized complete factorial design that included two variables: the duration of extraction (30 and 45 minutes) and the ethanol concentration (50, 70, and 90%), with ratio of sample and solvent 1:10. Thus, there were six sample variations: T1C1 (30 minutes, 50%), T1C2 (30 minutes, 70%), T1C3 (30 minutes, 90%), T2C1 (45 minutes, 50%), T2C2 (45 minutes, 75%), and T2C3 (45 minutes, 90%).

2.3 Total phenolic content determination

The method for quantification the overall amount of phenolic compounds in A. lavenia leaves, as described by Batubara et al. [11], was carried out with some adjustments. In brief, a mix of 10 µL of leaf extract and 10 µL of 10% Folin–Ciocalteu reagent, along with 20 µL of 10% Na2CO3 and 150 µL of distilled water, was prepared for each microplate well. Following extensive mixing, the mixture was allowed to incubate for 30 minutes at room temperature. Absorbance readings were then taken using a microplate reader at 750 nanometers (Epoch Biotek, Winooski, VT, USA). The amount of phenolic content was determined using the gallic acid calibration curve, with results expressed as milligrams of gallic acid equivalent per gram of dried extract (mg GAE/g extract). Each sample was measured three times to provide accurate results.

2.4 Total flavonoid content determination

The determination of Total Flavonoid Content (TFC) followed the procedure outlined by Batubara et al. [11] with slight adjustments. Specifically, a solution of leaf extract of 60 µL was dispensed into individual wells of a microplate, along with 10% $AlCl_3$ (10 µL), CH_3COOK (10 µL), and distilled water (120 µL). Subsequently, the solution was meticulously mixed and left to incubate at room temperature for 30 minutes. Absorbance readings were then recorded at 415 nanometers using a microplate reader (Epoch Biotek, Winooski, VT, USA). To ensure the accuracy and reliability of the total flavonoid content determination, a calibration curve was developed using quercetin as the standard. This curve was utilized to calculate the amount of flavonoids present in the dried extract, expressed in milligrams of quercetin equivalent per gram of extract (mg QE/g extract). Each sample was subjected to triplicate analyses.

2.5 DPPH assay

DPPH (2,2-diphenyl-1-picrylhydrazyl) assay evaluated the antioxidant activity through a free radical scavenging mechanism. DPPH procedure, as outlined by Prastya et al. [12] with modifications, was followed. In brief, various concentrations of each extract (50, 100, 150, 200, 250 mg/l) were added into a 0.1 mg/ml DPPH solution (in a 1:2 volume ratio) to initiate the reaction necessary for constructing a calibration curve. The absorbance was measured after a 30-minute incubation period at 515 nm with UV-visible spectrophotometer (Shimadzu UV-1780). An ethanol solution of DPPH served as the control, and quercetin acted as the reference standard. Triplets of analyses were carried out for each extract and the standard,

including those that evaluated the antioxidant activity of the extracts. The percentage inhibition of DPPH absorbance was calculated using the following equation to determine the antioxidant activity of each extract:

$$\%\ \text{inhibition}\ (\frac{\text{mg}}{\text{l}}) = \frac{(\text{A control} - \text{A sample})}{\text{A control}} \text{x} 100\% \quad (1)$$

The IC_{50} value, which indicates the concentration of the sample required to scavenge the DPPH radicals of 50%, was determined using the calibration curve when the percentage of inhibition reached 50%.

2.6 CUPRAC assay

An assay known as cupric ion-reducing antioxidant capacity (CUPRAC) was employed to determine the antioxidant activity through the measurement of reducing power. CUPRAC was conducted as Irawan et al. [13] described with minor modifications. Briefly, various concentrations of each extract were added into a CUPRAC solution (volume 1:1). Subsequently, each solution was homogenized using ethanol pa. Following the homogenization process, the solutions were allowed to incubate at 37°C for 30 minutes. The absorbance was then measured using a UV-visible spectrophotometer (Shimadzu UV-1780) at 459 nanometers, which was also applied to a standard butylated hydroxytoluene (BHT) solution. The equation for calculating the reducing activity is as follows:

$$\%\ \text{reducing power}\ (\frac{\text{mg}}{\text{l}}) = \frac{(\text{A sample} - \text{A control})}{\text{A sample}} \text{x} 100\% \quad (2)$$

The curve produced a linear equation (Y=bX+a; Y for the % reducing power, b for slope, X for the solution concentration, and a for intercept). The IC_{50} value was obtained from the sample concentration (X) when the % reducing power (Y) was 50%.

2.7 Statistical Analysis

The outcomes were depicted as the average ± standard deviation, derived from three repetitions of each trial. The use of a two-way analysis of variance (ANOVA) was employed to assess variations in mean values. The relationship between TPC, TFC, and antioxidant activities was assessed by Rank Spearman correlation coefficient. The p-value threshold for significance was set at <0.05 for all tests conducted.

3 Results

3.1 Total phenolic and total flavonoid content

The *A. lavenia* leaf extract exhibited the highest total phenolic content in the T1C2 treatment, which involved an extraction time of 30 minutes and an ethanol concentration of 70%. This yielded a phenolic content of 15.39±0.15 mg TAE/g, as shown in Table 1. When the duration of extraction was increased further to 45 minutes, the total phenolic content decreases. In contrast, the maximum overall flavonoid content in the *A. lavenia* leaf extract was identified in the T2C3 treatment, which involved 45 minutes of extraction time and 90% of ethanol concentration, resulting in a flavonoid content of 0.51±0.00 mg QE/g (Table 1).

Table 1. Total phenolic and total flavonoid content in ethanol extracts of *Adenostemma lavenia* leaves

	Total phenolic content (mg TAE/g extract)	**Total flavonoid content (mg QE/g extract)**
T1C1	8.37±0.37[a]	0.26±0.02[a]
T1C2	15.39±0.15[e]	0.43±0.00[c]
T1C3	12.19±0.28[c]	0.27±0.01[a]
T2C1	9.26±0.03[b]	0.34±0.01[b]
T2C2	14.49±0.35[d]	0.49±0.01[d]
T2C3	11.94±0.11[c]	0.51±0.00[e]

Note: Superscripts with different values in the specified column indicate a statistically significant difference ($p \leq 0.05$)

3.2 Antioxidant activities

Table 2 illustrates the outcomes of our antioxidant assay that utilized the DPPH method. These results show IC_{50} values greater than 500 mg/l for all sample treatments, indicating a very weak antioxidant activity [14] A lower IC_{50} value indicates higher antioxidant activity. Furthermore, according to Table 2, the greatest antioxidant activity of the *A. lavenia* extract, as determined by the cupric ion reducing antioxidant capacity (CUPRAC) method, was found in the T1C1 treatment. This treatment involved an extraction duration of 30 minutes and a solvent concentration of 50%. A smaller IC_{50} value indicates more potent antioxidant activity. Therefore, the antioxidant activity of the T1C1 treatment falls into the moderate category. With an extract concentration of 110.50±4.95 mg/L, T1C1 was able to reduce copper (II) ions (Cu^{2+}) in the CUPRAC solution by 50%.

Table 2. Antioxidant activities of *Adenostemma lavenia* extracts.

	IC_{50} DPPH (mg/l)	**IC_{50} CUPRAC (mg/l)**
T1C1	3,259.96±16.21^{d}	110.50±4.95^{a}
T1C2	2,634.95±38.63^{b}	126.00±4.24^{bc}
T1C3	2,401.41±52.71^{a}	112.50±7.78^{a}
T2C1	3,270.00±64.06^{d}	118.00±4.24^{ab}
T2C2	2,854.96±56.34^{c}	136.00±5.66^{c}
T2C3	2,507.19±26.62^{a}	133.50±0.71^{c}

Note: Superscripts with different values in the specified column indicate a statistically significant difference ($p \leq 0.05$)

3.3 Correlation of Total phenolic, total flavonoid, and antioxidant activities

The Spearman test results, summarized in Table 3, indicated a correlation between the antioxidant activity and the total flavonoid content using the CUPRAC method. The correlation coefficient is -0.89*, suggesting that the total flavonoid content significantly contributes to the antioxidant activity determined by the CUPRAC method, with an inverse relationship. This condition means that as the flavonoid content increases, the IC_{50} value decreases, indicating enhanced antioxidant activity. The impact of active components, like flavonoids, on the antioxidant activity of the extract is influenced by their presence. No other variables displayed significant correlations, as their ρ values were below 0.05.

Table 3. Antioxidant activities of *Adenostemma lavenia* extracts.

	p-value			
	TPC	**TFC**	**IC_{50} DPPH**	**IC_{50} CUPRAC**
TPC	-	0.31	0.45	-0.60
TFC	0.31	-	0.03	-0.89*
IC_{50} DPPH	0.45	0.03	-	-0.14
IC_{50} CUPRAC	-0.60	-0.89*	-0.14	-

Note: * = The correlation between the variables is statistically significant at 0.05 level

4 Discussion

Ultrasonic Assisted Extraction (UAE) method primarily relies on acoustic cavitation as its driving mechanism. This procedure creates a sequence of rarefactions and compressions in the solvent molecules, which ultimately result in bubble formation as a consequence of temperature and pressure variations [15]. If the extraction time is extended, it causes an increase in temperature, leading to higher degradation rates of phenolics and a subsequent decline in phenolic content [16].

The distribution of phenolic compounds within the plant cells is determined by their solubility, which is influenced by their polarity [17]. Solvents like ethanol possess substantially lower polarity than water, enhancing the solubility and diffusion of phenolics in plants. Water-alcohol mixtures have proven more effective in extracting phenolic compounds [18]. Water functions as a swelling agent for the matrix of plant, enhancing the contact surface area, whereas ethanol facilitates to break the bonds between the solutes and the matrix. Our results, which align with a the previous study, demonstrate that a 70% ethanol concentration in solvents leads to improved extraction yields, a finding that can be attributed to the polarity of the phenolics present in the samples [19].

However, when the duration of extraction was increased further to 45 minutes, the total flavonoid content increases (Table 1). This observation can be attributed to the longer and more intensive contact time between the solvent and the material, which facilitates the diffusion of chemical components aided by ultrasonic waves. The impact of cavitation and mechanical vibration caused by ultrasonic waves can disrupt the cell wall layer, enhancing solvent penetration into the cells and making it easier to extract chemical compounds, thereby increasing the total flavonoid yield [20]. Hydrophilic phenolic compounds are primarily located in cell vacuoles. In contrast, other compounds, including water-insoluble polyphenols, flavonoids, and lignins are deposited in the cell wall through hydrophobic interactions with polysaccharides and proteins [21]. Therefore, flavonoids are more effectively extracted in a water-ethanol mixture with a higher ethanol concentration, as demonstrated in this study at 90% ethanol concentration.

The average phenolic content of the ethanol extract of *A. lavenia* leaves in this study was 11.94 mg TAE/g, while the total flavonoid content was 0.38 mg QE/g. The total phenolic content was higher than the total flavonoid content. These findings align with our earlier research, which reported a TPC of 14.40 mg GAE/g and a TFC of 4.73 mg QE/g in the methanol extract of *A. lavenia* leaves [22]. Similarly, Baharuddin et al. [23] observed that TPC of *Clinacanthus nutans* leaves (herb) was higher than the total flavonoid content. Since flavonoids are a subgroup of phenolic compounds, TPC is generally higher than TFC. Phenolic compounds such as lignans, phenolic acids, tannins, flavonoids , and stilbenes are the primary metabolites in plants [24].

A variety of chemical tests have been utilized alongside cutting-edge, automated detection techniques to evaluate the antioxidant capacity of substances. These techniques include evaluating scavenging activity against various free radicals or reactive oxygen species (ROS), measuring reducing power, assessing metal chelation abilities, and other specialized techniques. Radical scavenging methods, such as DPPH (2,2-diphenyl-1-picrylhydrazyl) assay, are commonly used and well-established spectrophotometric techniques for assessing the antioxidant properties of foods, compounds, and extracts derived from plants. The popularity of this assay can be attributed to their sensitivity, speed, simplicity, and reproducibility [25].

Based on DPPH method (Table 2), *A. lavenia* leaves extracts have a very weak antioxidant activity. This observation suspected that flavonoid glycosides in the *A. lavenia* leaf extract are responsible for the low antioxidant activity. Some flavonoid glycosides are known to have a reduced capacity to scavenge free radicals [26]. Shafek et al. [27] reported

that two newly identified kaempferol 3-O-glycosides from *Solenostemma argel* exhibited lower DPPH free radical scavenging ability than kaempferol. Additionally, some terpenoid and steroid compounds present in the extract cannot donate hydrogen atoms to scavenge DPPH free radicals, as demonstrated by Torres-Martínez et al. [28], the compounds limonene, linalool, menthone, and pulegone from *Satureja macrostema* exhibited low or nearly negligible antioxidant activity when evaluated using DPPH and ABTS methods.

Another contributing factor could be the limitations of the DPPH assay. In the process of DPPH radical scavenging, antioxidants interact with DPPH radicals through a rapid transfer of electrons (ET) and a slower transfer of hydrogen atoms (HAT) [29]. Although transfer of electron occurs quickly, it is slower than the reactions between antioxidants and 2,2-azinobis 3-ethylbenzthiazoline-6-sulfonic acid radicals (ABTS) due to limited access of phenolics to the radical site on the DPPH molecule. The obstacle of accessibility impedes all reactions, especially the transfer of hydrogen, which is necessary for the formation of a complex involving a hydrogen bond between the the N lone pair and α-C—H radical. Additionally, instability of the DPPH reagent, particularly under exposure to light, can decrease antioxidant activity [30].

Based on the results (Table 2), the compounds in the *A. lavenia* leaf extract exhibit more optimal antioxidant activity through a reduction mechanism compared to a free radical scavenging mechanism. The reason for this outcome is that the CUPRAC reagent is more stable and readily available than other chromogenic reagents, such as DPPH and ABTS. Additionally, this method has the capability to measure both hydrophilic and lipophilic antioxidant compounds, such as α-tocopherol and β-carotene [31]. Moreover, the CUPRAC reagent efficiently oxidizes thiol-based antioxidants rapidly, whereas ET-based assays, including the ferric reducing antioxidant power (FRAP) method, cannot measure specific thiol antioxidants, such as glutathione. This difference could be due to the electronic configuration of Cu (II), which allows faster kinetics.

Additionally, the CUPRAC assay is performed under favorable conditions of pH 7.0, unlike the acidic pH of 3.6 in the FRAP method or the basic pH of 10.0 in the Folin–Ciocalteu assay. At pH values below the physiological pH of 7.4, antioxidants' reducing ability may be impaired due to the protonation of antioxidants. On the other hand, when the pH level is higher, the acid dissociation of phenols can increase the reduction capacity, which may result in inaccurate measurements of the total antioxidant capacity. The CUPRAC reagent also benefits from a favorable redox potential, being selective due to its lower redox potential compared to the ferric–ferrous couple in the presence of phenanthroline or similar ligands. The Cu(II, I)–Nc redox pair's standard potential is approximately 0.6V, which is close in value to that of ABTS+/ABTS, which is also 0.68V. Consequently, compounds not actual antioxidants, such as citric acid and simple sugars, cannot undergo oxidation when reacting with the CUPRAC reagent [32].

According to the Spearman correlation shown in Table 3, there is a significant relationship between the total flavonoid content and the antioxidant activity measured by the CUPRAC method, with an inverse correlation observed. Flavonoids react with copper ions (Cu^{2+}) in the CUPRAC reagent solution. During this reaction, flavonoids undergo oxidation while copper ions are reduced to copper (I) oxide (Cu^{+}) [33]. Consequently, the flavonoid content in the extract positively correlates with its antioxidant activity. The previous study reported that flavonoids and alkaloids were the most prevalent compounds identified in the extract of *A. lavenia* [6]. Those flavonoids including 3-O-acetylpinobanksin (flavonoids), pectolinarigenin (flavones), eriodictyol 7-O-sophoroside (flavanones), and formononetin (isoflavones). 3-O-acetylpinobanksin and eriodictyol 7-O-sophoroside have potent antioxidant activities [34, 35]. The highest antioxidant capacities were found for certain phenolic and flavonoid compounds, e.g., epicatechin gallate, rosmarinic acid, quercetin, epigallocatechin, catechin, epicatechin, caffeic acid, gallic acid, chlorogenic acid, and rutin,

when measured using the CUPRAC assay. This effectiveness is attributed to the optimal electron transfer, which is dependent on the quantity and positioning of hydroxyl groups, as well as the level of conjugation across the entire molecule [36]. Other variables did not show a significant correlation, as the ρ value was less than 0.05. Therefore, the recommended UAE extraction condition in this study is the one that produces the highest total flavonoid content (T2C3), with a 45 minutes extraction time and a 90% ethanol solvent concentration.

5 Conclusion

In conclusion, the variations in solvent concentration and the duration of extraction using the ultrasound-assisted method have a significant impact on the levels of total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity found in *Adenostemma lavenia* leaves. The optimal conditions for extraction were 45 minutes of extraction duration with 90% ethanol concentration. Under these conditions, the TPC was 11.94 ± 0.11 mg TAE/g extract, the TFC was 0.51 ± 0.00 mg QE/g extract, the antioxidant activity measured 2,507.19 ± 26.62 mg/L (IC_{50} of the DPPH assay), and 133.50 ± 0.71 mg/L (IC_{50} of the CUPRAC assay). The Spearman rank test demonstrated a significant correlation between antioxidant activity (as measured by the CUPRAC assay) and TFC in *A. lavenia* leaves. The outcomes suggest that *A. lavenia* may serve as a viable natural source of antioxidants.

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