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Research article

Total Phenolic Content and Antioxidant Capacity from Stems and Leaves of *Andrographis paniculate* in Different Solvent Combinations

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Abstract

Andrographis paniculata, a member of the Acanthaceae family, is renowned for its secondary metabolites and bioactivity. Optimization of the extraction of these compounds can be achieved by selecting the appropriate solvent. In this research, extraction was carried out using water, acetone, and ethanol as solvents, both individually and in combination. The study aimed to evaluate the total phenolic content (TPC) and antioxidant capacity of the stems and leaves of *Andrographis paniculata* using solvent extraction. The research employed a completely randomized design with three replications, using dry powder of *A. paniculate* as the sample. TPC was analyzed using the Folin-Ciocalteu method, while antioxidant capacity was evaluated via the FRAP and ABTS assays. The results showed that the water-acetone solvent (50%:50%) produced the highest TPC (7.4±0.50 mg GAE/g DW) and FRAP antioxidant capacity (41.96±1.11 µmol TE/g DW). The highest antioxidant capacity using the ABTS method was obtained with the ethanol-acetone combination (50%:50%), with a value of 4.26±0.02 mol TE/g DW. A positive correlation between TPC and FRAP antioxidant capacity was observed (r = 0.6821), indicating that the phenolic content of *Andrographis paniculata* is strongly linked to its FRAP-based antioxidant activity. Overall, the extraction solvent combination significantly influenced the TPC content and antioxidant capacity of *Andrographis paniculata* stems and leaves.

Keywords: *Andrographis paniculata*; antioxidant; ethanol; acetone; total phenolic; water

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1. Introduction

Andrographis paniculata is a plant that belongs to the Acanthaceae family, with a height of approximately 30 to 110 cm, dark green stems, and leaves that measure up to 8 cm in length and 2.5 cm in width. The plant prefers moist and shady habitats and is widely distributed across Asia countries including Indonesia, China, Myanmar, Malaysia, Thailand, Hong Kong, the Philippines, and Bangladesh (Akbar, 2011; Benoy et al., 2012; Kumar et al., 2012; Hossain et al., 2014; Hossain & Urbi, 2016). Research on *A. paniculata* has been extensive, highlighting its various bioactive properties, such as antibacterial, antihyperlipidemic, antidiabetic, antioxidant, and anticancer properties (Sule et al., 2011; Sivananthan, 2013). The aerial parts and roots of the plant contain various metabolites including terpenoids, flavonoids, xanthones, proteins, and noriridoids. Among these, andrographolide, the plant's most well-known diterpenoid lactone, has been identified as a key bioactive compound (Okhuarobo et al., 2014).

The extraction of phytochemical compounds from crude plant material is a crucial step in separating these compounds for further analysis and application (Salomon et al., 2014). Various extraction methods, such as ultrasonic-assisted extraction (UAE), are commonly employed due to their efficiency and ability to obtain metabolites from different plant materials (Assami et al., 2012; Liu et al., 2015; Ma et al., 2016; Rocha et al., 2017). This method has several advantages, including high extraction yields, low extraction temperature, and shorter extraction time (Dey & Rathod, 2013). Choosing the appropriate solvent is equally critical, as previous research has demonstrated that solvent selection influences the extraction of phenolic compounds and antioxidant capacity in *A. Paniculata*. For example, ethanol has been shown to be more effective than water for extracting bioactive compounds from this plant (Adedapo et al., 2015). Similarly, Majhi et al. (2023) reported that while water has polar properties, it is limited in its ability to break hydrogen bonds effectively, necessitating the use of organic solvents to improve extraction efficiency.

Previous studies have focused on individual solvents for extracting compounds from *A. paniculata*, but the combined use of solvents has not been explored extensively. This study seeks to fill that gap by evaluating the effect of solvent combinations on the extraction of phenolic compounds and antioxidant capacity. The objective of this research is to determine which solvent or solvent combination optimizes the total phenolic content (TPC) and antioxidant capacity of *A. paniculata*. In this study, three solvents with varying polarity levels—water, ethanol, and acetone—were used in different combinations to identify the optimal solvent system that provides the strongest correlation between TPC and antioxidant capacity.

2. Materials and Methods

2.1 Sample preparation

The study was carried out at the Research Laboratory of IPB University in Bogor, Indonesia from February to May 2023. The plant used in this research was *Andrographis paniculata*, which was sourced from the Tropical Biopharmaceutical Research Center (TropBRC) garden of LPPM IPB University. The stems and leaves of the plant used as the research samples were dried in an oven at 40°C for leaves and 50°C for stems for 24 h. The two parts of the plant were then ground into 80 mesh powder (Nurcholis et al., 2022).

2.2 Sample extraction

To obtain *A. paniculata* plant extract, a UAE (ultrasound-assisted extraction) method based on Nurcholis et al. (2022) with modifications was used. The sample used was dry powder of the *A. paniculate* plant resulting from a composite of leaves and stems (1:1) made in 60 mesh size. The extract was prepared by adding 4 g of *A. paniculata* plant powder to 20 mL of solvent in an Erlenmeyer flask. The experiment design was completely randomized design with three replications. The types of solvents used consist of water, ethanol and acetone. The compositions of the solvents used in this study were water 100% (w); water-ethanol 50%:50% (we); water-acetone 50%:50% (wa); ethanol 100% (e); ethanol-acetone 50%:50% (ea); acetone 100% (a); and water-acetone-ethanol 33.33%:33.33%:33.33% (wae). The mixture was sonicated for 30 min at room temperature, centrifuged at 10,000 × *g* at 4°C for 15 min, and the supernatant was separated from the pellet. The volume was adjusted to obtain an extract concentration of 0.2 g/mL. All sample extracts obtained were stored at 4°C for further analysis.

2.3 Total phenolic content analysis

The Folin-Ciocalteu method, as described by Nurcholis et al. (2022), was utilized to determine the total phenolic content. To perform the assay, 20 µL of sample extract was combined with 120 µL of 10% Folin-Ciocalteu in a microplate and incubated in the dark for 5 min. Then, 80 µL of 10% Na_2CO_3 was added to the solution, and the mixture was incubated in the dark for an additional 30 min. The absorbance of the solution was measured at a wavelength of 759 nm using a nano spectrophotometer (SpectrostarNano BMG LABTECH). To establish a calibration curve, a gallic acid standard with concentrations ranging from 0 to 300 ppm was used.

2.4 Antioxidant activity quantification

2.4.1 FRAP (Ferric Reducing Antioxidant Power) assay

The present study utilized the FRAP method, as originally described by Nurcholis et al. (2022), with some modifications. To prepare the FRAP reagent, 10 µM tripyridyl-s-triazine (in 40 mM HCl) and 20 mM $FeCl_3$ were dissolved in acetate buffer (pH 3.6) in a ratio of 1:1:10 (v/v/v). The reagent was stored in the dark for 30 min before use. A total of 10 µL of sample extract was then mixed with 300 µL of FRAP reagent in a microplate and incubated in the dark for 30 min. Absorbance was measured at a wavelength of 593 nm using a nano spectrophotometer (Spectrostar Nano, BMG LABTECH). To establish a calibration curve, Trolox was used with concentrations ranging from 0 to 400 µM.

2.4.2 ABTS (2,2-Azinobis-3-ethylbenzothiazoline-6-Sulfonic acid) assay

The method for measuring the antioxidant capacity of ABTS was based on a modification of the procedure described by Nurcholis et al. (2022). To prepare the ABTS reagent, 7.7 mM ABTS (w/v in distilled water) and 2.4 mM $K_2S_2O_8$ (w/v in distilled water) were mixed in a ratio of 2:1 (v/v) and incubated in the dark at room temperature for 6 min. The absorbance of the reagent should be in the range of 0.7±0.02 with the addition of distilled water. The sample extract (20 µL) was then reacted with 280 µL of ABTS reagent in a microplate and incubated in the dark at room temperature for 6 min. The absorbance was measured using

a nano spectrophotometer (SPECTROstar Nano BMG LABTECH) at a wavelength of 734 nm. The antioxidant capacity of ABTS was calculated based on standard Trolox concentrations of 0-500 µM.

2.5 Statistical analysis

The data were analyzed using the ANOVA method in IBM SPSS Statistics 25, resulting in a mean±standard deviation (SD) of three replications. The Tukey test was conducted with a confidence level of $\alpha = 0.05$, and the Pearson correlation test was used to determine the relationship between TPC and antioxidant capacity, which was visualized as a graph using GraphPad Prism 8.

3. Results and Discussion

Phenolic compounds are a type of secondary metabolite produced by plants to defend themselves (Nakatani, 2000; Zaynab et al., 2018). In this study, the Folin-Ciocalteu method was used to measure the total phenolic content. The method involves mixing the Folin-Ciocalteu reagent with phenolic compounds in an alkaline medium, resulting in the formation of a blue complex with a maximum absorbance of 765 nm. The absorbance is directly proportional to the total phenolic content, which is determined as gallic acid equivalent (Molole et al., 2022). The total phenolic content (TPC) obtained from *A. paniculata* extract based on solvent combinations showed different results (Figure 1a). The water-acetone solvent combination produced the highest total phenolic content (7.40±0.50 mg GAE/g DW), while the lowest was produced by 100% ethanol solvent, with a value of 0.95±0.02 mg GAE/g DW.

In this study, the combination of solvents used came from variations in the dependent variable so it was not possible to determine the right formula to optimize the total phenolic and antioxidant content. However, this research can be used as an initial reference to determine what solvent combinations need to be optimized for further research such as response surface methodology design. The combination of solvents used in extracting plant compounds has a prominent role in extracting phenolic compounds. Generally, acetone is more effective in extracting phenolic compounds with high molecular weights such as tannins (Mokrani & Madani, 2016). In this case, the water-acetone solvent combination produced the highest yield among other solvents. Besides that, the water-acetone solvent combination showed significantly different results from the 100% acetone solvent. These results indicate that adding water can increase the yield of phenolic compounds during extraction. Water is classified as a polar solvent (Rajhard et al., 2021), while acetone is classified as a semi-polar solvent (Adaramola & Onigbinde, 2016). The combination of the two solvents typically produces a semi-polar solvent, which suggests that most of the phenolic compounds extracted from the leaves and stems of *A. paniculata* were semi-polar. This is supported by the principle of "like dissolves like," which states that polar solvents dissolve polar compounds (Zhuang et al., 2021). It is worth noting that using 100% ethanol and 100% water solvents tends to result in a low yield of phenolic compounds. This further supported that the phenolic compounds extracted from *A. paniculata* were semi-polar.

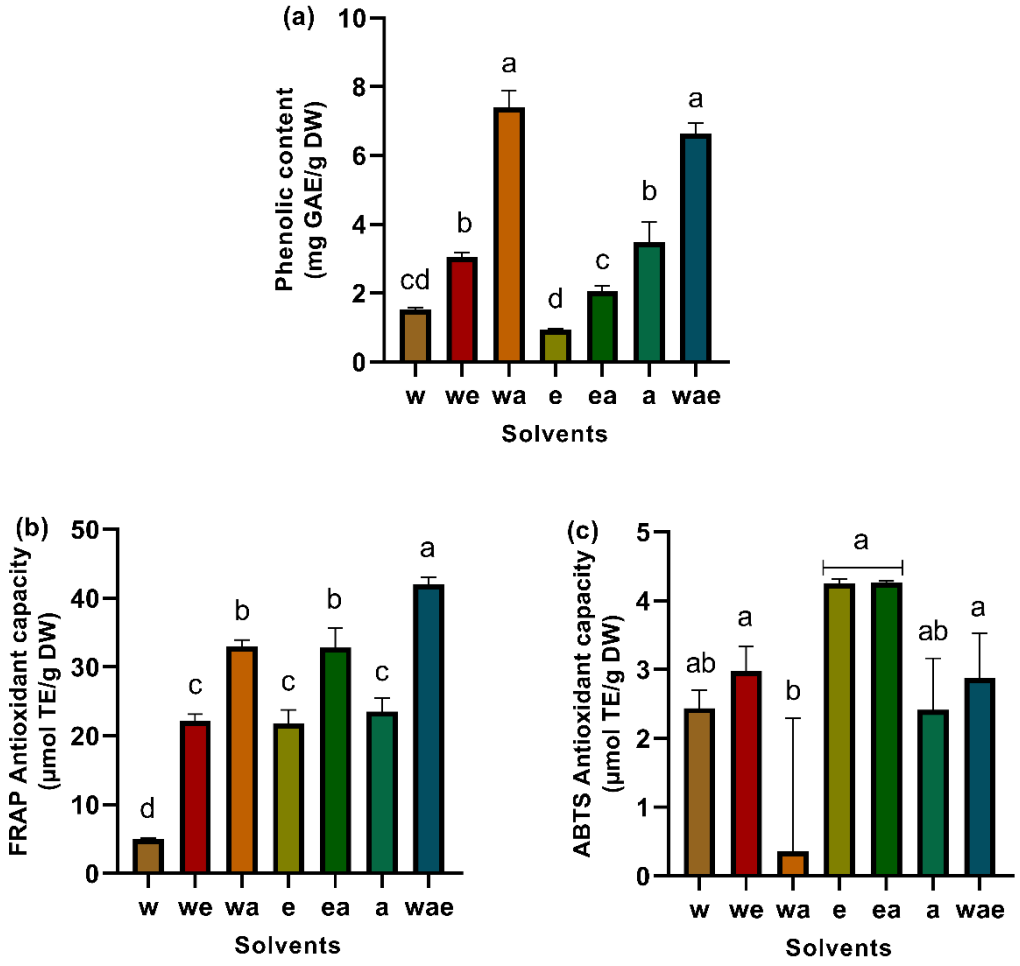

Figure 1. Evaluation results of total phenolic content (a), FRAP antioxidant capacity (b), and ABTS antioxidant capacity (c) from *A. paniculata* extract. Each value is presented as the mean of three replicates±standard deviation (SD). Mean values marked with different letters illustrate significant differences ($p < 0.05$) in Tukey's test. w = water, e = ethanol, and a = acetone.

The antioxidant capacity of the extracts was evaluated using the FRAP (Ferric Reduction Antioxidant Power) and ABTS (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) methods. The FRAP method measures the ability of antioxidant compounds to reduce Fe^{3+} ions to Fe^{2+}, indicating their antioxidant power. Compounds with reducing power tend to act as antioxidants by donating electrons or hydrogen atoms to stabilize free radicals, making the radical compounds more stable (Raharjo & Haryoto, 2019). The ABTS method assesses the ability of antioxidant compounds to neutralize free radicals by donating protons, indicated by the fading of the greenish-blue color to colorless as the ABTS radical cation is reduced (Al-Hmoud et al., 2014; Sukweenadhi et al., 2020).

The highest antioxidant capacity, as measured by FRAP, was found in the water-ethanol-acetone solvent mixture (41.96±1.11 µmol TE/g DW). The lowest capacity was observed in 100% water solvent (5.01±0.17 µmol TE/g DW) (Figure 1b). The highest antioxidant capacity for ABTS was found in the ethanol-acetone solvent combination (4.26±0.02 µmol TE/g DW) (Figure 1c). The antioxidant capacity obtained from the ethanol-acetone solvent combination was not significantly different from that of the other solvents tested, including 100% ethanol, water-ethanol-acetone, and water-ethanol. The water-acetone solvent combination produced the lowest ABTS antioxidant capacity (average of 0.36±1.93 µmol TE/g DW) among all the solvent combinations tested. The two methods used in this research have distinct mechanisms, leading to significantly different antioxidant capacities. In the FRAP method, the ability of a natural extract to reduce iron (FRAP) indicates that the extract contains electron-donating compounds, which can react with free radicals to form more stable products that can halt radical chain reactions (Raharjo & Haryoto, 2019). The antioxidant capacity of an extract is strongly related to the solvent used. Differences in the polarity of solvents result in variations in the antioxidant potential (Boeing et al., 2014). Additionally, the unique bioactive groups that each solvent can extract contribute to the differences in the antioxidant capacity obtained through both the FRAP and ABTS methods (Ngo et al., 2017).

The antioxidant capacity of *A. paniculata*, as determined by FRAP method, was found to be positively correlated with its total phenolic content. This correlation was evaluated through a Pearson correlation coefficient analysis, which revealed a significant positive relationship between TPC and FRAP (r = 0.6821) in this study (Figure 2a). This finding is consistent with previous research by Zhang et al. (2018), who reported a high positive Pearson correlation value (r = 0.90) between TPC and FRAP in vegetable juice. These results suggest that the majority of TPC compounds extracted successfully were also FRAP antioxidants. While a significant negative correlation (r = -0.676) was obtained from the relationship between TPC and ABTS antioxidant activity (Figure 2b). The Pearson correlation value between TPC and ABTS obtained in this study showed a value of opposite sign to the study of Wootton-Beard et al. (2011). Wootton-Beard et al. (2011) found a positive correlation (r = 0.89) between TPC and ABTS from vegetable juice. The negative correlation indicates that the phenolic compounds extracted from the leaves and stems of *A. paniculate* had a power reducing antioxidant mechanism, such as FRAP (Munteanu & Apetrei, 2021), and did not possess a free radical scavenging mechanism, such as ABTS (Atere et al., 2018). The FRAP method involves the reduction of metal ions (such as Fe3+ to Fe2+), and this is achieved using antioxidant compounds (Gliszczyńska-Świgło, 2006; Müller et al., 2011). Meanwhile, in the ABTS method, ABTS· radicals interact with antioxidants to reduce them and become more stable (Ilyasov et al., 2020), resulting in the formation of a green-blue color that can be measured at a maximum wavelength of 734 nm (Prior et al., 2003; Calvindi et al., 2020). The Pearson correlation results suggest that the higher the total phenolic content obtained, the greater the antioxidant capacity as measured by FRAP. Conversely, the higher the TPC, the lower the antioxidant capacity (measured by ABTS) produced by *A. paniculata*.

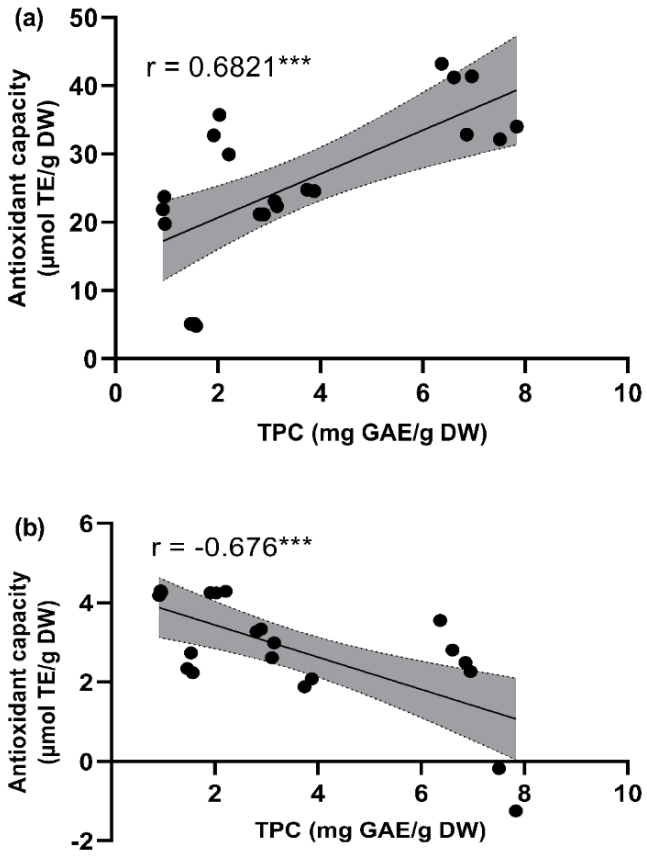

Figure 2. Simple linear correlation of total phenolic content and antioxidant capacity of FRAP (a) and ABTS (b) methods obtained from *A. paniculata* extract. r, coefficient value of Pearson correlation, *** represents highly significant at $P < 0.05$.

4. Conclusions

The evaluation of the total phenolic content and antioxidant capacity of *A. paniculata* using a combination of water, ethanol, and acetone solvents resulted in significant effects. Among the solvent combinations, the water-acetone solvent combination showed the highest efficiency in extracting TPC, with an average of 7.40±0.50 mg GAE/g DW. The water-ethanol-acetone solvent combination produced the highest FRAP antioxidant capacity, at 41.96±1.11 µmol TE/g DW. Meanwhile, the ethanol-acetone solvent combination resulted in the highest ABTS antioxidant capacity, at 4.26±0.02 µmol TE/g DW. Additionally, the TPC extracted from the leaves and stems of *A. paniculata* showed a stronger correlation with FRAP antioxidant capacity compared to ABTS antioxidant capacity, indicating that the phenolic content is more closely related to the antioxidant activity measured by the FRAP assay.

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6. Conflicts of Interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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