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Antioxidant Activity, Total Phenolic Content, and Total Flavonoid Content of Methanol Extract of *Vernonia elaeagnifolia* Leaves Using ABTS and DPPH Assays

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ABSTRACT

Vernonia elaeagnifolia, also known as *Vernonia elliptica*, is a member of the Asteraceae family and is known to contain phenol and flavonoid compounds. The study examining the activity of these active compounds in this plant is limited. Therefore, this study aimed to evaluate the phytochemical profile, total phenolic content (TPC), total flavonoid content (TFC), and antioxidant activity of the methanol extract of *V. elaeagnifolia* leaves. Phytochemical screening was conducted using tube tests, while TPC and TFC were measured using the Folin-Ciocalteu and aluminium chloride colorimetric methods, respectively. Antioxidant activity was assessed using ABTS and DPPH assays. Phytochemical screening revealed the presence of alkaloids, saponins, flavonoids, and polyphenols in the methanolic extract. Quantitative analysis showed that TPC and TFC values were 81.26 ± 0.62 mg GAE/g and 54.02 ± 0.70 mg QE/g, respectively. The extract showed moderate antioxidant activity, with IC_{50} values of 139.81 ± 0.62 µg/mL (ABTS) and 166.62 ± 3.72 µg/mL (DPPH), which were significantly different. These findings suggest that the ABTS assay is more effective in evaluating the antioxidant activity of *V. elaeagnifolia* leaf extract compared to the DPPH assay, and *V. elaeagnifolia* leaves have potential as a source of natural antioxidants. Further isolation and characterization of active compounds are recommended.

INTRODUCTION

The human body naturally produces free radicals, like reactive oxygen species (ROS) and reactive nitrogen, through its own systems, exposure to certain chemicals, or during certain health conditions (Jamshidi-kia et al., 2020). The reactive species have the potential to interact with other compounds in the human body, resulting in tissue injury that can induce diseases such as cancer and Alzheimer's disease (Selvaraj et al., 2025). If the level of these reactive compounds is elevated, then oxidative stress will occur, which will increase the risk of cancer and neurodegenerative diseases.

Increased levels of endogenous or exogenous antioxidants are one of the ways to reduce oxidative stress (Panova & Tatikolov, 2023). Antioxidants are molecules that can donate electrons to radicals and neutralize them. Hence, they can slow down or prevent cell damage. Exogenous antioxidants can be synthetic and natural. Synthetic antioxidants can cause side effects with long-term use (Amarachukwu Uzombah, 2022). Hence, natural antioxidants could be a solution.

Most of the natural antioxidants were obtained from plants. *Vernonia elaeagnifolia* is a climbing plant from tropical and subtropical regions, including Indonesia. This species grows hanging and spreading along the side of the road or building, to protect from sun exposure (Lau and Frohlich, 2012). This plant belongs to the Asteraceae family, which has been used in folk medicine and exhibited antioxidant, antimicrobial, and anti-inflammatory properties

(Rolnik & Olas, 2021). Plants of the same genus, specifically *Vernonia amygdalina*, also known as African leaves, have been reported to exhibit antioxidant activity. Study on *V. amygdalina* extract using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) and ABTS methods showed an IC_{50} value of 13.54 ± 0.10 µg/mL (Karlina et al., 2023) and 94.83 µg/mL (Hussen & Endalew, 2023), respectively.

Previous research by Wulandari et al. (2024) investigated the phytochemical profile and biological activities of ethanolic leaf extracts of *V. elaeagnifolia*. Their findings confirmed the presence of alkaloids, flavonoids, phenolics, tannins, and saponins in this species (Wulandari et al., 2024). The ethanol extract of the leaves showed moderate antioxidant properties with an IC_{50} value of 147.28 µg/mL. However, the total phenolic content (TPC) and total flavonoid content (TFC) of *V. elaeagnifolia* leaf extracts have not been reported yet, leaving a knowledge gap regarding the quantitative relationship between phytochemical content and antioxidant potential.

The present study was conducted to complement and expand on the findings of Wulandari et al. (2024). In this study, methanol was employed as the extraction solvent, which is reported to be more efficient in extracting polar phenolic compounds than ethanol (Iglesias-Carres et al., 2019). This study also enhances the understanding of the antioxidant potential of *V. elaeagnifolia* leaves by measuring TPC and TFC, and comparing ABTS and DPPH results.

METHODS

Sample Extraction

Vernonia elaeagnifolia leaves were collected from Kartasura, Sukoharjo, and determined at the Biology Laboratory, Universitas Muhammadiyah Surakarta, with document number 036/A-E-I/LAB.BIO/II/2024. The collected leaves were dried in a drying cabinet at 60°C, then ground with a blender. The powdered leaves were maserated in methanol (1:10) for 3x24 hours at room temperature. After 72 hours, the macerate was separated from the residue by filtration, the residue was re-macerated for 24 hours with methanol at room temperature. The combined methanol extract was concentrated using an evaporator and water bath until a thick extract was obtained, and the percentage yield was calculated.

Phytochemical Screening

Phytochemical screening of the methanolic leaf extract of *V. elaeagnifolia* was performed using the tube test according to a previous reported method (Ngibad, 2019). Alkaloid identification was conducted using Mayer's, Dragendorff's, and Wagner's reagents; the formation of characteristic precipitates indicated positive results. Saponins were detected by shaking the extract with hot water and observing the formation of persistent froth. At the same time, flavonoids were identified after heating with methanol, acidification with hydrogen chloride and Mg powder, and observing color development. Tanins and polyphenols were confirmed by the formation of dark green or blue-green coloration upon addition of ferric chloride solution.

Determination of Total Phenolic Content

Total phenolic content (TPC) was determined using the Folin-Ciocalteu method which was modified from Hatami et al. (2014). A total of 500 ⌈L of the methanolic leaf extract (1000 µg/mL) was mixed with 2.5 mL of a Folin-Ciocalteu reagent and incubated for 5 minutes. Two milliliters of 1.6% sodium carbonate were added to neutralize the mixture, and then incubated in the dark for 120 minutes at room temperature. The absorbance was measured at 795 nm using a UV-Vis spectrophotometer Shimadzu 1280 (Hatami et al., 2014). A standard calibration curve of gallic acid standard at five levels (20-100 µg/mL) was used to calculate the TPC. The result was expressed as mg equivalents of gallic acid per gram extract (mg GAE/g).

Determination of Total Phenolic Content

Total flavonoid content (TFC) of the methanolic extract of *V. elaeagnifolia* leaves was determined using the aluminum chloride colometric method. A total of 500 ⌈L of the methanolic leaf extract (1000 µg/mL) was mixed with 1.5 mL of methanol, 0.1 mL of 10% $AlCl_3$, 0.1 mL of potassium acetate, and 2.8 mL of distilled water. The mixture was incubated at room temperature for 30 minutes, and then the absorbance was measured at ⌊ maximum (463 nm) using a UV-Vis spectrophotometer

Shimadzu 1280 (Kumar et al., 2017). A standard calibration curve of quercetin standard at five levels (20-100 µg/mL) was used to quantify the TFC. The result was expressed as mg of quercetin equivalents per gram of extract (mg QE/g).

Antioxidant Activity Assays

DPPH radical scavenging assay

DPPH solution was prepared by dissolving 24 mg of DPPH in 100 mL of methanol. The solution was left in the dark for 30 minutes. The DPPH solution stock was diluted with methanol until the absorbance was obtained around 0.973 at 517 nm. The DPPH radical scavenging activity was evaluated by taking 1 mL of each extract solution (400, 200, 100, 50, and 25 µg/mL) and adding 3 mL of DPPH. Three milliliters of DPPH solution in 1 mL of methanol was given as a standard. The solutions were left for 30 minutes at room temperature in the dark. The absorbance was measured at 517 nm. The radical-scavenging activity (RSA) was calculated using the following formula (Baliyan et al., 2022):

$$RSA = [(Ac - As) \mid Ac] \cdot 100\%$$

where: Ac is the absorbance of the standard (DPPH or ABTS solutions mixed with solvent) and As is the absorbance of testing solutions (DPPH or ABTS solutions mixed with extracts). The IC_{50} value was determined from the dose-response curve. Vitamin C was used as a positive control.

ABTS radical scavenging assay

ABTS radical cation ($ABTS^{\cdot+}$) solution was prepared by dissolving 384 mg of ABTS in 10 mL of distilled water (7 mM), then mixing 5 mL of this solution with 88 µL of 140 mM potassium persulfate to produce a final mixture of approximately 6.9 mM ABTS and 2.4 mM potassium persulfate. The mixture was left in the dark for 12-16 hours. The solution was diluted to an absorbance of 0.70 ± 0.02 at 734 nm before use. Extract solutions were added to the $ABTS^{\cdot+}$ solution, incubated for 6 minutes, and the absorbances were measured at 734 nm (Kusumorini et al., 2022). Radical-scavenging activity was calculated as described for DPPH, and the IC_{50} value was determined. Vitamin C was used as a positive control.

RESULT AND DISCUSSION

Vernonia elaeagnifolia leaf extraction was carried out using maceration to obtained the active compounds. This method was quite simple, where the dried and powdered leaves was soaked in methanol for several days to obtain the desired compounds. The solvent will penetrate the walls of the extracted materials, then enter the cell cavity that contains the active compound, so that the compound will be attracted (Mathews et al., 2024). Maceration of *V. elaeagnifolia* leaves produced a concentrated extract that was dark-green in color with a yield of 10.47%w/w.

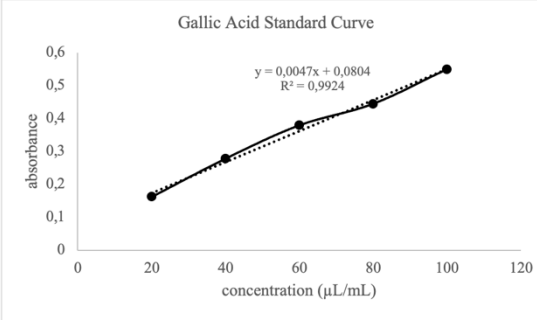
Table 1. Phytochemical screening of the methanol extract of *V. elaeagnifolia* leaves

Phytochemical constituent	Reagent	Result
Flavonoids	Mg + HCl	+
Polyphenols	$FeCl_3$	+
Alkaloids	Dragendorff, HCl + Mayer, Wagner	+
Tannins	Gelatin 1%, NaCl 10%	-
Saponins	HCl 2N	+

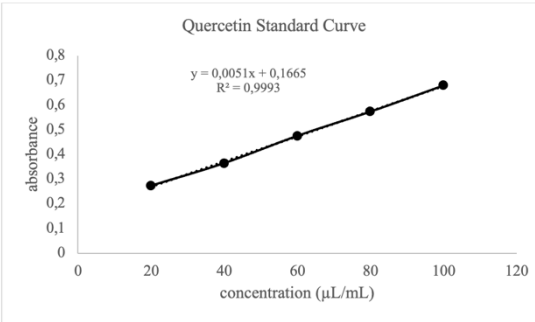
+ : present, - : absent

Phytochemical screening, using tube test, confirmed the presence of alkaloids, saponins, flavonoids, and polyphenols, but tannins were not detected (**Table 1**). These findings are consistent with Wulandari et. al. (2024), suppporting the reproducibility of the plant's secondary metabolite profile. The presence of these multiple phytochemical groups may produce synergistic antioxidant effects on *V. elaeagnifolia* extract (Hassanpour & Doroudi, 2023). The quantity of electron-donating substituents (-OH and $-OCH_3$) and their arrangement on phytochemical substances that contribute to antioxidant activity significantly influence antioxidant capacity (Moazzen et al., 2022). Therefore, the analysis of TPC, TFC, and antioxidant activity is crucial to confirm the contribution of these phytochemical constituents to the overall antioxidant capacity of the leaf extract.

The measurement of TPC in the methanolic leaf extract was carried out using gallic acid as a reference standard because this compound is able to form complex bonds with Follin-Ciocalteu reagent. Hydroxyl groups and conjugated double bonds in each benzene ring of gallic acid are responsible for this bond (Hilma et al., 2021). Meanwhile, in TFC measurements, quercetin was used as a reference standard because it is a flavonol found in various types of plants and has very strong antioxidant capabilities (Vollmannová et al., 2024). Based on the absorbance values of 5 series of gallic acid and quercetin concentrations, the standard curve equations were obtained as presented in **Figure 1**. Both curves provided a correlation coefficient (R2) value approaching 1, indicating linear calibration curves and a positive relationship between the concentration of gallic acid or quercetin and the absorbance value (Schober et al., 2018).



(A)



(B)

Figure 1. Standard calibration curves of gallic acid (A) and quercetin (B)

Quantitative analysis revealed the TPC and TFC values of 81.26±0.62 mg GAE/g and 54.02±0.70 mg QE/g, respectively (**Table 2**). These results indicate that the methanol extract of *V. elaeagnifolia* leaves had higher phenolic content than flavonoids. The TPC is related to antioxidant activity, which a higher TPC will exhibit higher antioxidant activity (Hikmawanti et al., 2020). Compared to plant extracts from the same genus, namely *Vernonia amygdalina*, the TPC value of *V. elaeagnifolia* was slightly higher. The TPC and TFC values of *V. amygdalina* leaves ethanolic extract were 70.33±2.11 mg GAE/g and 63.82±0.99 mg QE/g, respectively (Alara et al., 2019).

Table 2. TPC and TFC calculation of methanol extract of *V. elaeagnifolia* leaves

Parameter	Results (mean ± SD, n=3)	Reference Standard
TPC	81.26 ± 0.62 mg GAE/g VME	Gallic acid
TFC	54.02 ± 0.70 mg QE/g VME	Quercetin

Antioxidant activity of *V. elaeagnifolia* leaf extract was assessed using ABTS and DPPH assays. The ABTS method relies on the interaction between antioxidants and the pre-formed ABTS radical cation, where antioxidants reduce the activity of ABTS, leading to the inhibition of ABTS radicals. This process is marked by a color change from blue to green at low concentrations and fading at higher concentrations (Ilyasov et al., 2020). The IC_{50} of the methanol extract of *V. elaeagnifolia* leaves determined by the ABTS assay was 139.81±0.62 µg/mL (n=3), whereas vitamin C, used as a positive control, had an IC_{50} of 12.56±0.10 µg/mL (n=3) (**Figure 2**). An independent t-test in Excel was used to assess whether there was a significant difference between these values. The results indicated a significant difference ($p<0.001$, two-tailed), with vitamin C showing notably higher antioxidant activity than *V. elaeagnifolia* extract.

The DPPH method is based on electron transfer. When a purple DPPH solution reacts with an antioxidant (an electron donor), the DPPH radicals experience a decrease in absorbance, indicated by the solution's purple color fading and a yellowish color formed by the picryl groups (Platzer et al., 2021). Based on the linear equation of the relationship between extract concentrations and %DPPH inhibitions

in **Figure 2**, the IC_{50} value of the methanolic leaf extract of *V. elaeagnifolia* was 166.62±3.72 µg/mL (n=3). In comparison, vitamin C, serving as a positive control, exhibited an IC_{50} value of 11.77±0.02 µg/mL (n=3). According to the independent t-test, these two values were significantly different (p<0.001, two-tailed). This finding was consistent with the results of the ABTS assay, which showed that vitamin C exhibited significantly higher antioxidant activity compared to the methanolic leaf extrac.

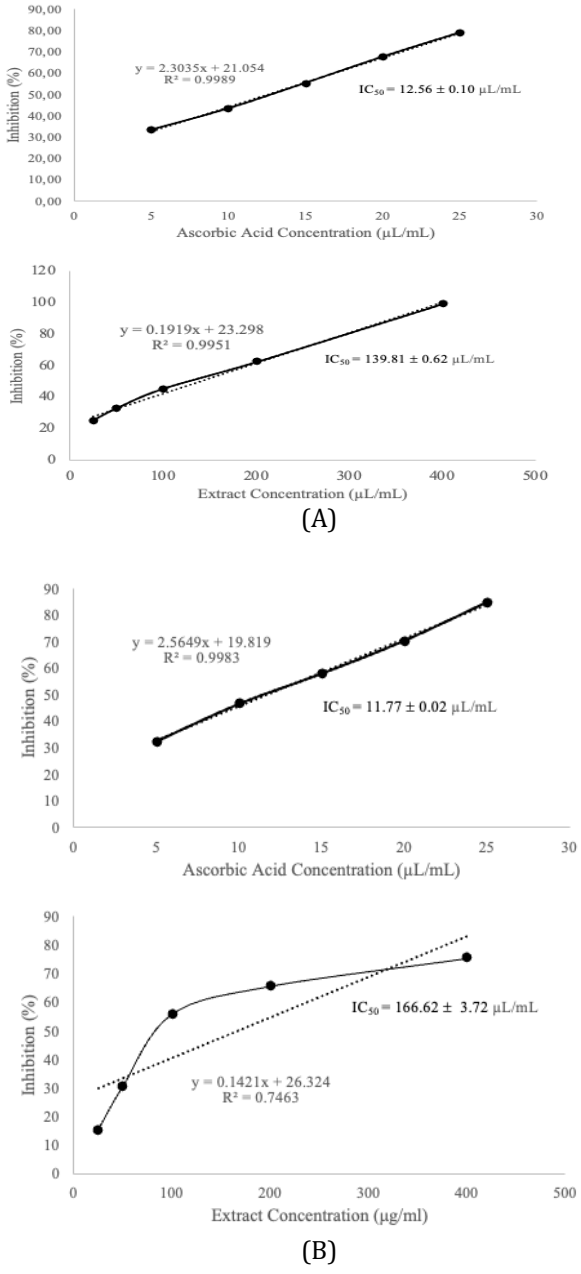


Figure 2. Antioxidant activities of ascorbic acid and methanol extract of V. elaeanigfolia leaves using ABTS (A) and DPPH (B) assays

Furthermore, an independent t-test was also conducted to compare the antioxidant activities obtained from ABTS and DPPH assays. The statistical analysis revealed a significant difference between these two assays (p<0.01, two-tailed). This result suggests that ABTS assay tends to show higher antioxidant potential of methanol extract of *V. elaeagnifolia* leaves compared to the DPPH assay. A previous study of *V. amygdalina* leaves ethanolic extract also showed a higher IC_{50} on the ABTS assay (198.87 ± 1.76 µg/mL) than the DPPH assay (321.37±3.19 µg/mL) (Alara et al., 2019). The differences observed between ABTS and DPPH assays reflect not only assay-specific sensitivities but also the diverse and possibly synergistic composition of phytochemicals within the extract. It can be assumed that some active compounds in the *V. elaeagnifolia* leaves extract do not react with the DPPH reagent but react more easily with the ABTS reagent, such as dihydrochalcones and flavanones (Platzer et al., 2021). However, the IC_{50} values from these two assays indicate that the extract has moderate antioxidant activity (Kusumawati et al., 2021). These findings suggest that phenolics serve as the major contributors to the antioxidant activity of *V. elaeagnifolia* leaves, while flavonoids and other non-phenolic compounds may act synergistically.

CONCLUSIONS

The methanol extract of *V. elaeagnifolia* leaves contained various bioactive compounds, such as polyphenols, flavonoids, alkaloids, and saponins. Quantitative analysis showed relatively high levels of TPC (81.26±0.62 mg GAE/g) and TFC (54.02±0.70 mg QE/g). The antioxidant evaluation showed that the extract had moderate activity, with IC_{50} values of 139.81±0.62 µg/mL in the ABTS method and 166.62±3.72 µg/mL in the DPPH method. The use of the ABTS assay is more suitable for evaluating the antioxidant activity of *V. elaeagnifolia* leaf extract than the DPPH assay, as it provides a lower IC_{50} value. These findings support the potential use of *V. elaeagnifolia* leaves as a natural source of antioxidants, which could be further explored for development in nutraceutical or pharmacetical formulations.

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AUTHORS' CONTRIBUTIONS

Asti Arum Sari: Writing – original draft, Validation, Methodology, and Investigation. Febri Wulandari: Writing – review and editing, and Conceptualization. Minda Ustavia Adiningsih: Writing and Investigation.

CONFLICT OF INTERESTS

The authors confirm that there are no known conflicts of interest related to this publication.

ETHICAL CONSIDERATION

Ethical issues (including plagiarism, data fabrication, double publication, etc) have been completely observed by the author

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