Evaluation of Antioxidant Potential of Andrographis paniculata Leaf and Stem Extracts: Effects of Growing Site Altitude

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**Abstract.** *Andrographis paniculata* (sambiloto) is widely used in traditional medicine and contains flavonoids with reported antioxidant properties. Environmental conditions, such as altitude, may influence the concentration and activity of these compounds. This study examined the effect of growing altitude on the antioxidant activity of *A. paniculata* extracts. Samples from Tuban (lowland) and Malang (highland) were extracted by maceration with 70% ethanol, yielding 16.18% and 16.20%, respectively. Antioxidant activity was assessed using the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay. The Tuban extract (ApT) showed 47.04% inhibition at 319.46 µg/mL, insufficient for IC₅₀ determination, while the Malang extract (ApM) yielded an IC₅₀ of 264.42 ± 54.29 µg/mL, classified as weak activity. Although highland plants demonstrated relatively greater activity than lowland plants, the overall weak antioxidant effect indicates that *A. paniculata* may not serve as a strong primary source of antioxidants. These findings highlight the need for further studies to identify specific active compounds, explore synergistic effects with other phytochemicals, and evaluate alternative conditions or formulations that may enhance its antioxidant potential.

# INTRODUCTION

Indonesia is an archipelago with a tropical climate and is located between the Australian and Asian continents, so it has very varied geographical conditions. This can affect the variety of biodiversity it has, which is a source of natural materials for its people in their daily needs, including food ingredients or medicinal ingredients for various diseases. The growth of plants of the same type can be influenced by the varied growing environment, including the content of chemical compounds produced, both in terms of quantity and composition [1, 2]. Environmental factors such as temperature, soil type and conditions, altitude, and humidity are believed to affect plant morphology. When environmental influences outweigh genetic factors, plants growing in different locations may exhibit distinct morphological characteristics. Previous studies have indicated that altitude is among the key factors affecting plant growth [3].

*Andrographis paniculata,* commonly known as Sambiloto in Indonesia, is an herbaceous plant belonging to the family Acanthaceae. Often referred to as the Bitter King because it has a distinctive bitter smell and taste. For generations, A. paniculata has been traditionally employed to treat a wide range of ailments, including cancer, diabetes, leprosy, bronchitis, skin disorders, flatulence, colic, influenza, and malaria. In China, it has also been used for detoxification purposes [4, 5]. Phytochemical analyses have shown that A. paniculata contains numerous bioactive compounds with potential pharmacological effects, such as flavonoids, quinoids, xanthones, tannins, alkaloids, and other chemical constituents [6].

Herbal medicines represent a preferred alternative therapy for local populations in developing countries. Indonesia, with its rich natural resources, offers numerous sources of bioactive compounds with antioxidant properties. This is supported by scientific studies reporting the bioactivity of various Indonesian traditional herbal medicines [7]. Plants are a source of diverse biological activities, including antidiabetic, antioxidant, and antimicrobial potentials [8]. Substances with potential bioactivity are generally collected as secondary metabolites in all plant tissues, but their concentration ranges depend on the plant part, the event, the atmospheric conditions, and the corresponding growth phase. The existence of these bioactive components is in demand for potential benefits for human health [9]. Many plants react to fluctuating environmental atmospheric conditions by producing antioxidants. Crude extracts from plants have important antioxidants and bioactive compounds suitable for inhibiting oxidation processes caused by oxidizable substrates. Antioxidants function by scavenging and neutralizing reactive oxygen species, including hydrogen peroxide, superoxide, and nitric oxide [10, 11].

A. paniculata exhibits strong antioxidant properties and effective free radical scavenging activity. Free radicals are molecules or atoms containing at least one unpaired electron, which typically enhances their chemical reactivity. They are generated endogenously during energy production and can also arise from environmental exposures such as ultraviolet radiation, smoke, and pesticides. Accumulating evidence indicates that free radicals play a role in the development of various diseases, including cancer, diabetes, cardiovascular disorders, and aging [12].

Antioxidants can act directly in vivo, while the body also depends on its internal defense systems. Enzymes in the body, such as reduced superoxide reductase (SOD) and glutathione (GSH), which play a role in assimilating toxins into two parts and require micronutrient cofactors, for example, selenium, iron, copper, zinc, and manganese, for ideal catalytic activity. Intake and assimilation of essential minerals decline with age, which can affect the internal antioxidant immune system. Supplementation with external antioxidants or enhancing the body's internal antioxidant immunity has been shown to be a beneficial strategy in responding to the stress properties caused by oxidation. Plants have long been a source of external antioxidants [13]. Reactive oxygen species (ROS) are naturally generated oxygen-based free radicals, which can become harmful when produced in excess and not effectively removed; they can trigger biomolecular oxidation [14]. The extraction of active constituents from the leaves and stems of A. paniculata was carried out using samples obtained from locations with different altitudes, Tuban (representing lowlands) and Malang (representing highlands), and was reported. The extracts were subsequently evaluated for their antioxidant activity using the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and their IC₅₀ values were determined. This allowed assessment of the influence of growing altitude on the antioxidant potential of A. paniculata extracts.

# MATERIALS AND METHOD

**Materials**

The tools used in this study were a maceration apparatus, beaker glass, filter paper, spatula, dropper pipette, glass funnel, 1000 µL micropipette, 100 µL micropipette, yellow tip (Onemed 500 µL), blue tip (Onemed 1000 µL), Eppendorf, analytical balance (Fujito FSAR 210), rotary evaporator (Buchi R-210, Switzerland), and UV-Vis (Genesys UV-Vis Spectrophotometer, Thermo Fisher Scientific, Madison, I, USA). The leaves and stems of *A. paniculata* were collected from Tuban Regency and Malang Regency, East Java, Indonesia. Ethanol 70%, methanol p.a., methanol, gallic acid, and DPPH reagent (Rey's Laboratory) were used.

# Extract Preparation

Sambiloto (*A. paniculata)* samples were taken from 2 locations with different altitudes, namely lowlands (<250 meters above sea level) obtained from Mrutuk village, Tuban, with an altitude of 0 - 25 meters above sea level. Then highlands (>250 meters above sea level) were obtained from Tegalweru village, Malang. The leaves and stems of *A. paniculata* were cleaned, dried at room temperature, and ground into a fine powder. A total of 50 grams of *A. paniculata* samples from Tuban (ApT) and *A. paniculata* samples from Malang (ApM) that had been extracted using the maceration technique in 70% ethanol (500 mL) over a period of 2×24 hours. The obtained extract was filtered and concentrated using a rotary evaporator until a thick consistency was achieved, after which it was weighed to determine the yield of each extract [15].

# DPPH Radical Scavenging Assay

The DPPH antioxidant activity of each extract was determined by dissolving 10 mg of sample in 1 mL methanol. A stock solution of 1.19 mg DPPH in 50 mL methanol was prepared, and 33.33 µL of crude extract solution was mixed with 1 mL of DPPH radical solution (6 × 10⁻⁵ M). After 20 min incubation at 37 °C, the absorbance was measured at 517 nm using a UV–Vis spectrophotometer, with methanol as the blank and gallic acid as the positive control. Antioxidant activity was calculated using Equation (1) [15], [16]. The assay was performed in triplicate. For ApM, further testing with multiple concentrations allowed IC₅₀ determination, whereas ApT was excluded because its inhibition at 319.46 µg/mL did not exceed 50%. It should be noted that only one concentration was initially tested for both extracts, limiting comparability. Moreover, environmental conditions at the collection sites (temperature and humidity) and botanical validation (voucher specimen) were not recorded, which may influence reproducibility.

Inhibition (%) = [(Ab – As)/Ab] x 100% (1)

Furthermore, the extract, which has a good DPPH inhibition (%) was measured for its 50% inhibitory concentration (IC50) value (μg/mL). The value of IC50 was presented as the quantity of the extracts to react with half of DPPH radicals. It means the lower the value of IC50, the better its biological activity [17]. IC50 is a sample concentration required to capture 50% of DPPH free radicals; this is measured by solving a gradient variable pair response model [13].

# RESULT AND DISCUSSION

**Extraction Yield**

This study was conducted to see the effect of altitude on the antioxidant activity of Sambiloto (*A. paniculata*) extract. In this study, the samples used were *A. paniculata* leaves and stems obtained from 2 areas with different altitudes. The first sample was lowland (<250 masl) obtained from Mrutuk village, Tuban (ApT) with an altitude of 20 masl. Then the second sample was medium land (>250 masl) obtained from Tegalweru village, Malang (ApM). Prepare samples of ApT and ApM leaves and stems that have been cleaned, dried at room temperature, and then ground. ApT and ApM samples weighed 50 g. Extraction was carried out by the maceration method using 70% ethanol solvent, as much as 500 mL per sample. The results of organoleptic tests on ethanol extracts showed organoleptic results in the form of a blackish green color influenced by tannins in *A. paniculata*. This shows that the organoleptic in the form of color and odor of ethanol extract preparations of *A. paniculata* leaves is influenced by the addition of ethanol extract to *A. paniculata* leave [18]. Tannin is a phenolic compound. Maceration was carried out for 2 x 24 hours. The maceration results obtained a thick, blackish-green extract with a distinctive bitter odor (Figure 1). The thick extract is then calculated as a percentage yield to determine the amount of extract produced. The mass of ApT extract obtained was as much as 8.0886 g with a yield of 16.18%, and ApM extract as much as 8.0980 g with a yield of 16.20%. ApT and ApM thick extracts are not much different. From the results obtained, there was no difference in the yield, indicating that the height of the growing place had little effect on the amount of extract produced.

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| --- | --- |
| Two small bottles with black liquid  AI-generated content may be incorrect.**(a)** | Two small bottles with black liquid  AI-generated content may be incorrect.**(b)** |

**Figure 1**. Thick extract of *A. paniculata* from maceration with 70% ethanol solvent (a) ApT (b) ApM

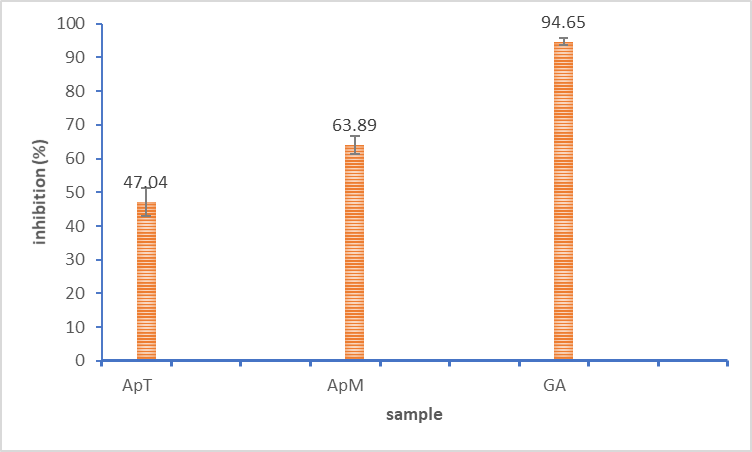
**DPPH Radical Scavenging Assay**

Antioxidant activity tests of ApM and ApT extracts were carried out using the DPPH method. This technique is one of the commonly used methods because it is practical, simple, and relatively low cost to measure the antioxidant potential of a compound. The principle of this method is to test the ability of compounds to neutralize free radicals or act as hydrogen atom donors. The stable radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) is commonly employed to evaluate the antioxidant capacity of natural substances, including those found in foods, pharmaceuticals, and complex biological systems [10, 16].

DPPH is a type of stable free radical centered on a nitrogen atom, with the ability to accept electrons or hydrogen atoms from other compounds to form stable diamagnetic molecules. The reaction between DPPH and a reducing agent produces electron pairs that form hydrazine compounds. The reduction in DPPH radical color intensity occurs in direct proportion to the number of electrons or hydrogen atoms donated by antioxidant compounds. This reaction converts the solution’s color from purple, representing the radical form, to yellow, representing the non-radical form. The DPPH assay is recognized as a simple and rapid method for evaluating antioxidant capacity, with absorbance measured using a UV–Vis spectrophotometer [10, 17].

The DPPH (2,2-Diphenyl-1-Picrylhydrazyl) radical scavenging assay is widely applied to assess the antioxidant potential of plant extracts, as it evaluates their capacity to donate electrons or hydrogen atoms in neutralizing DPPH radicals [19]. In the present study, this method was employed to compare the antioxidant activities of A. paniculata extracts collected from two altitudes, namely Tuban (lowland, ApT, 20 masl) and Malang (highland, ApM, >250 masl). The results demonstrated significant differences in their radical scavenging potential, which can be attributed to variations in phytochemical composition influenced by environmental factors such as altitude, UV exposure, and temperature [2, 18].

Gallic acid was employed as the reference standard in this study. In the preliminary DPPH assay at a concentration of 319.46 µg/mL, the ApM extract (highland) demonstrated an inhibition of 63.80%, whereas the ApT extract (lowland) achieved only 47.04% inhibition (Figure 2). As the positive control, gallic acid exhibited an antioxidant activity reaching 94.65%, confirming the assay’s validity [12]. While the ApT sample did not inhibit 50% at that concentration, it was continued to the IC50 calculation for only the ApM sample. This shows that the % inhibition of the ApM extract (highlands) is better at inhibiting (> 50%) compared to the ApT extract (lowlands). The fact that ApT did not reach 50% inhibition suggests that lowland conditions may lead to lower accumulation of antioxidant compounds compared to highland-grown plants [3]. The ApM extract was continued to the IC50 value calculation stage.



**FIGURE 2**. DPPH radical scavenging ability of ApT, ApM, and gallic acid (positive control) extracts at a concentration of 319.46 μg/mL

Based on the results of concentration and inhibition interpolation (Table 1), the IC50 value for ApM was 264.42 ± 54.29 µg/mL, classifying it as a weak antioxidant (IC50 > 250 µg/mL) [17]. Which suggests limited pharmacological potential as a primary antioxidant source. In contrast, gallic acid (IC50 = 0.35 ± 0.04 µg/mL) exhibited very strong activity, serving as a benchmark [16]. Linear regression equation was used to determine concentrations of antioxidant [22]. Gallic acid contains an aromatic ring substituted with three hydroxyl groups and one carboxyl group, which are crucial for its effectiveness in scavenging free radicals. Results from the DPPH assay indicated that the ApM extract (highland) exhibited the strongest potential to neutralize DPPH radicals. These results indicate that extracts from the highlands have better antioxidant activity than those from the lowlands. The high IC50 of ApM suggests that while the extract has detectable antioxidant activity, it may require further purification or optimization to enhance its potency [15].

**TABLE 1.** IC50 of ApM extract and gallic acid (positive control)

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| --- | --- |
| Sample | IC50 (μg/mL) |
| ApM | 264.42 ± 54.29 |
| ApT | 319,46 |
| GA (positive control) | 0.35 ± 0.04 |

Antioxidant activity in plants is greatly influenced by the content of bioactive compounds contained in the plant. Generally, the higher the altitude of a plant, the better its antioxidant activity. This is closely related to environmental factors, such as air temperature. In the lowlands, air temperatures tend to be higher so that the capacity of the air to hold water vapor increases. As a result, the relative humidity of the air decreases, especially during the day. In addition, the intensity of sunlight is also an important factor. Low light intensity can be caused by shade, either from clouds, trees, or other forms of shade [21].

Environmental stress and secondary metabolite production: plants grown at higher altitudes (e.g., Malang) experience greater UV radiation, lower temperatures, and oxidative stress, which stimulate the synthesis of protective secondary metabolites such as flavonoids, phenolics, and terpenoids [1, 18]. Flavonoids (e.g., andrographolide in *A. paniculata*) are known for their radical scavenging ability due to hydroxyl groups that donate hydrogen atoms to the DPPH [5, 6]. Phenolic compounds contribute to antioxidant activity by stabilizing free radicals through redox reactions [11]. Similar trends were observed in other medicinal plants, where high-altitude samples showed higher antioxidant activity due to increased polyphenol content [2, 3]. For example, Lallo et al. (2022) reported that *Alpinia galanga* extracts from higher elevations had significantly stronger DPPH scavenging activity than lowland samples [2].

The high IC50 value (264.42 µg/mL) suggests that crude ApM extract may not be potent enough for direct therapeutic use. Fractionation or compound isolation (e.g., using column chromatography) could help identify more active antioxidant constituents [6, 12]. Maceration with 70% ethanol was used here, but other solvents (e.g., methanol, acetone) or techniques (e.g., ultrasound-assisted extraction) might improve yield and bioactivity [10]. Comprehensive phytochemical profiling using HPLC or LC-MS analysis could identify specific bioactive compounds (e.g., andrographolide and flavonoids) responsible for the observed antioxidant effects [5, 6]. Natural compounds obtained from plant extracts or in pure form offer endless possibilities for drug development, thanks to their exceptional chemical diversity [23].

# CONCLUSION

Extraction using the maceration method with 70% ethanol produced thick, blackish-green extracts with yields of 8.0886 g (16.18%) for the Tuban sample (ApT) and 8.0980 g (16.20%) for the Malang sample (ApM). Antioxidant testing showed that ApM exhibited weak activity with an IC₅₀ of 264.42 ± 54.29 µg/mL, while ApT was not analyzed further because its inhibition at 319.46 µg/mL was only 47.04%, below the threshold for IC₅₀ determination. These results suggest that altitude affects the antioxidant potential of Andrographis paniculata; however, the study is limited using a single assay (DPPH), reliance on crude extracts, and the overall weak activity observed. Future research should include fractionation, variation of extraction solvents, and compound profiling to provide a clearer understanding of altitude-related differences in phytochemical composition and antioxidant properties.

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