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Antibacterial Activity of *Vernonia amygdalina* Leaf Extract and Fraction against the Growth of *Escherichia coli* Bacteria

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Abstract: *Vernonia amygdalina* leaves are known to possess antibacterial activity, which is commonly evaluated using ethanol extracts. However, solvent polarity plays a crucial role in extracting secondary metabolites that contribute to antibacterial effects. This study aimed to evaluate the antibacterial activity of *Vernonia amygdalina* leaf extracts obtained using different solvents. The extraction was performed by maceration using methanol and ethyl acetate. Three maceration processes were conducted using the same amount of leaf powder: methanol (M1), ethyl acetate (M2), and methanol followed by fractionation (M3). The M3 extract was further fractionated using n-hexane and ethyl acetate. Antibacterial activity was tested using the disc diffusion method. The results showed that M1 and the ethyl acetate fraction of M3 exhibited higher antibacterial activity than M2, as indicated by inhibition zone diameters of 8.56 mm (M1), 5.50 mm (M2), and 7.71 mm (M3). These differences are likely due to variations in the types and amounts of secondary metabolites extracted by each solvent. Further studies are recommended to investigate the correlation between secondary metabolite content and antibacterial activity of *Vernonia amygdalina* and other herbal plants.

Keywords: Antibacterial activity; Herbal extract; Maceration; Secondary metabolites

1. INTRODUCTION

Escherichia coli are beneficial bacteria in the digestive system, but they can also be pathogenic to humans and animals. These bacteria can cause diarrhea, particularly if food is unhygienic (contaminated with *E. coli*). According to the 2023 Indonesian Health Survey (SKI), the prevalence of diarrhea based on diagnosis/symptoms across all age groups in Indonesia is 4.3%, making this disease a serious concern [1].

Many studies have examined the treatment of diarrhea, particularly in inhibiting the growth of *E. coli*, by testing herbal plants. One herbal plant with potential antibacterial activity, particularly against *Escherichia coli*, is the African leaf (*Vernonia amygdalina*). A study has tested the inhibitory ability of *Vernonia amygdalina* leaves to inhibit the growth of *E. coli*. The study reported that ethanol extract of African leaves can inhibit the growth of *Escherichia coli*, with an inhibition rate of 16.60±0.17 mm at 80% concentration [2]. However, this study did not document the results of the *E. coli* inhibition test using ethanol extract of African leaves. Another study reported that methanol extract was also able to inhibit the growth of *E. coli*. The study stated that methanol extract of African leaves produced an inhibition of 23 mm [3], but also did not provide documentation of the inhibition results.

Many studies have shown that the presence of flavonoid compounds in plants can contribute to their antibacterial properties [4]. One study found that using ethyl acetate as a solvent during extraction yielded the highest flavonoid content compared to other solvents [5]. This study attempts to link the results of previous studies to optimize the use of African leaves as an antibacterial agent

(specifically against *E. coli*). This study aims to demonstrate the inhibitory effects of methanol extract, ethyl acetate extract, and ethyl acetate fraction on the growth of *E. coli*.

2. MATERIALS AND METHODS

The *Vernonia amygdalina* leaves to be studied were harvested from a cultivation area in Ponorogo City, East Java. The harvested leaves underwent post-harvest processing, including wet sorting, washing, chopping, drying, and dry sorting. Before being studied, the leaves were ground and sieved with a 40-mesh sieve. The authenticity of the cultivated plants used for research has passed taxonomic tests in the Plant Systematics Laboratory of the Faculty of Biology, Gadjah Mada University. To serve as a guideline for research using these plants, microscopic examinations were conducted on the leaves, which were then processed into simple preparations. Therefore, if other researchers purchase leaf powder claiming to be *Vernonia amygdalina* leaves, they can use the microscopic results from this study to verify the leaves authenticity.

2.1. Materials and Tools

2.1.1. Materials

The extraction process involved *Vernonia amygdalina* leaves, several organic solvents, including methanol (p.a), ethyl acetate (p.a), and filter paper (Whatman). The antibacterial activity test required materials including pure cultures of the test bacteria, Mueller–Hinton agar (Granulated GM173-500G Himedia), Mueller–Hinton broth (Granulated GM391-500G Himedia), physiological saline solution (85% NaCl) (p.a), cotton swabs (Onemed), blank disks (MN Germany), and tetracycline (Super tetra).

2.1.2. Tools

The tools used in the sample preparation process are knife/scissors, basin, and sample drying rack. The extraction process requires tools such as blender (Philips), no. 40 mesh sieve (Stainless), beaker glass (Herma), porcelain cup, measuring cup, separating funnel (Herma), maceration vessel (Herma), glass stirrer, analytical balance (Ohaus), rotary evaporator (RE Series), and water bath (Memmert). The antibacterial activity test requires tools such as several glassware, an incubator (Memmert), an Ose (Biologix), a paper disc (Advantec), a microscope (Binokuler XSZ 107BN), a disposable petri dish (Onelab), and a vernier caliper (Mitutoyo).

2.2. Extraction

Three maceration vessels were prepared. Each vessel was filled with 150 g of African leaf powder. Vessels 1 and 3 were extracted with Methanol solvent, while vessel 2 was extracted with Ethyl acetate solvent. All extraction processes required 550 mL of solvent. The extraction method used was maceration. The extraction process was carried out for 3 x 24 hours or 3 days. After 3 days of maceration, filtration and evaporation were carried out with a rotary evaporator and continued with a water bath. The extraction results from the third vessel were continued with a fractionation process. The sequence of the fractionation process was that the methanol extract was fractionated with hexane solvent and then with ethyl acetate. The ethyl acetate fraction was then concentrated for further antibacterial testing. All extraction results were calculated for yield.

$$\text{Yield (\%)} = \frac{\text{Extract weight}}{\text{Weight of leaf simplicia}} \text{ X } 100\% \text{ [6]}$$

2.3. Antibacterial test

Antibacterial testing of African leaf extracts and fractions was performed using the disc diffusion method [7], [8]. Test bacterial isolates were collected using a loop and suspended in physiological saline solution to a turbidity equivalent to 0.5 McFarland (~1–2 × 108 CFU/mL). A 100 µL suspension of each test bacterium was spread evenly on the surface of Mueller–Hinton agar. Sterile paper discs (6 mm) were impregnated with the sample extract or fraction at a concentration of 100 mg/mL, with 20 µL per disc. The discs containing the extract or fraction were placed on the surface of Mueller–Hinton agar containing the test isolates. The test was performed three times, with a gentamicin disc as a positive control and a solvent-containing disc as a negative control. Gentamicin is an antibiotic used for infections caused by Gram-negative bacteria and *E. Coli* are Gram-negative bacteria [9], [10].

Incubation was carried out at 30°C for 24 hours. The diameter of the inhibition zone was measured in millimeters using a vernier caliper. In this bacterial testing phase, data on *Escherichia coli* inhibition results will be obtained from the methanol extract, ethyl acetate extract, and ethyl acetate fraction. These three *Escherichia coli* inhibition data will be compared to determine which has the best inhibitory ability. According to Davis and Stout (1971), antimicrobial inhibition is categorized based on the diameter of the inhibition zone: Weak (W) ≤ 5 mm, Moderate (M) 5–10 mm, Strong (S) 10–20 mm, and Very Strong (VS) ≥ 20 mm [4].

3. RESULTS AND DISCUSSION

According to the Indonesian Herbal Pharmacopoeia, *Vernonia amygdalina* leaf powder contains several marker fragments, including the lower epidermis with stomata, covering hairs, vascular bundles with reticulated-type thickening, mesophyll with scale hairs and also sclerenchyma [11].

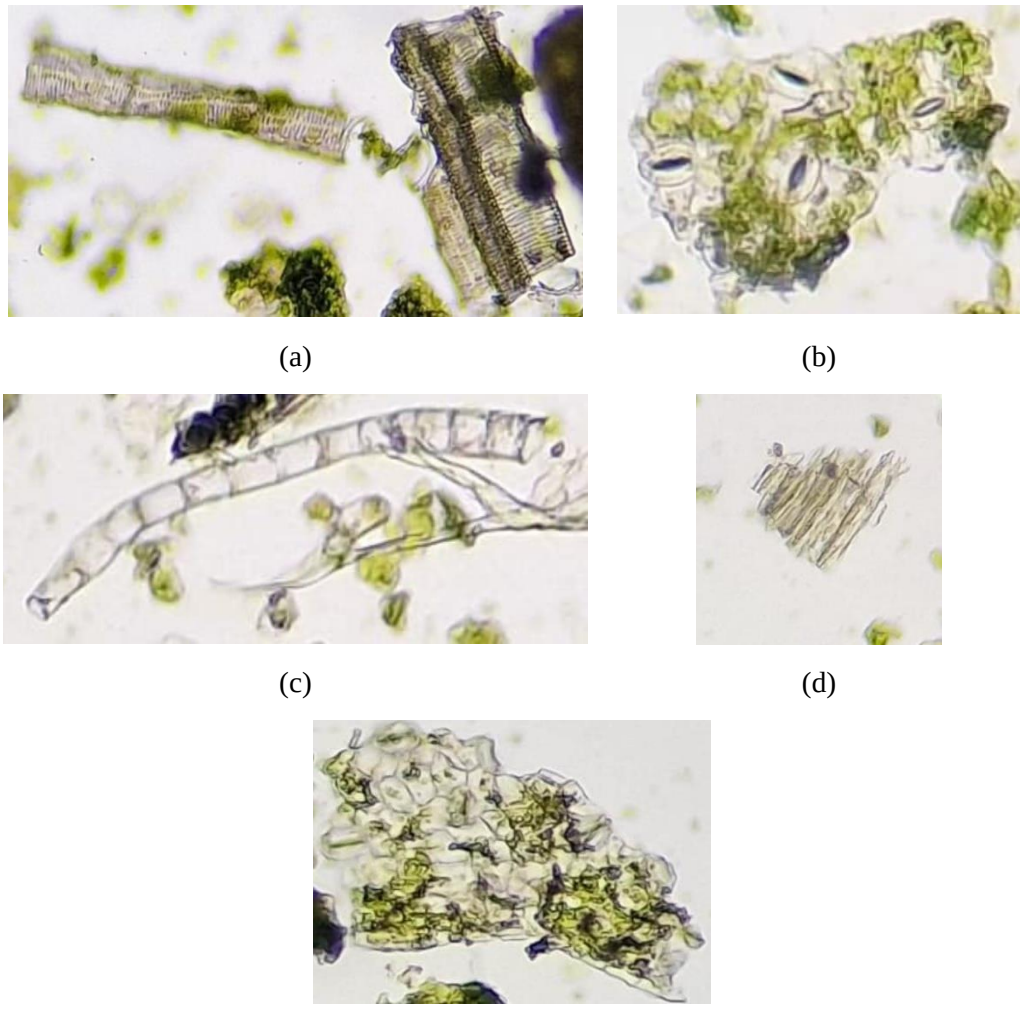
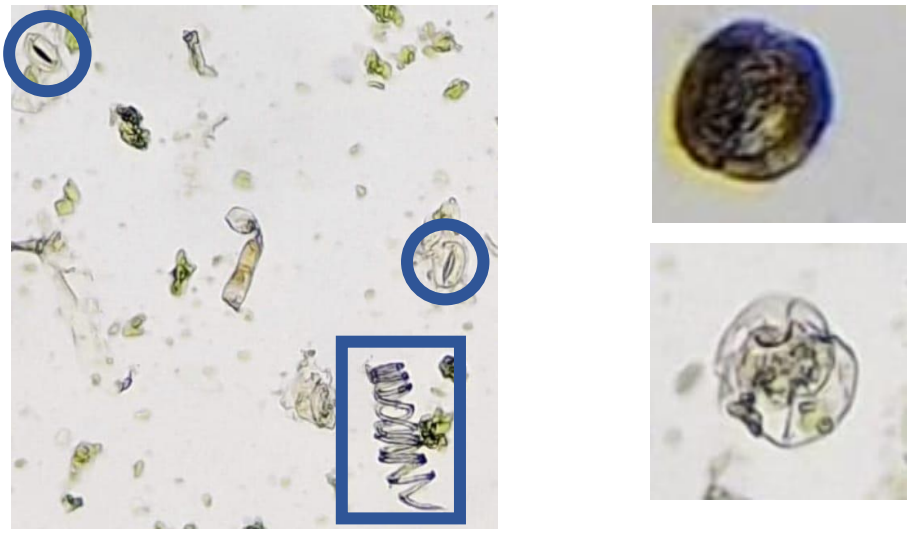


Figure 1. *Vernonia amygdalina* leaf powder marker fragment; (a) vascular bundles with reticulated-type thickening, (b) the lower epidermis with stomata, (c) covering hairs, (d) sclerenchyma, and also (e) mesophyll with scale hairs.

However, several fragments not mentioned in the Indonesian Herbal Pharmacopoeia but found in this study may provide additional data in assessing the authenticity of *Vernonia amygdalina* leaves. These fragments include rosette-shaped calcium oxalate crystals, freely detached spiral-shaped vascular bundles, and stomata detached from the lower epidermis (Figure 2). Therefore, this leaf powder contains not just one type of vascular bundle, but two types: the reticulate type and the spiral type. The differences between these various types of vascular bundles can be seen in Figure 4.

Stomata are modified leaf epidermal cells with two guard cells that allow the exchange of water vapor and gases. They are located primarily on leaves (can be above or below the leaf), stems, and rhizomes [12]. In general, there are five types of stomata: anomocytic, anisocytic, parasitic, diacytic, and actinocytic. Anomocytic stomata are those in which the guard cell is surrounded by a specific number of cells that do not differ from the rest of the epidermis in shape or size. Anisocytic stomata are those in which each guard cell is surrounded by three neighboring cells of varying size. Parasitic stomata are those in which each guard cell is joined to one or more neighboring cells, with their longitudinal axis parallel to the axis of the neighboring cells and the aperture. Diacytic stomata are those in which each guard cell is surrounded by two neighboring cells, with cell walls that form a right angle to the longitudinal axis of the stoma. Actinocytic stomata are those in which each guard cell is surrounded by neighboring cells that spread out in a radius [13], [14]. The stomata type of Vernonia amygdalina leaves is estimated to be anomosic. The stomata structure of *Vernonia amygdalina* leaves consists of guard cells surrounded by epidermal cells and can be seen in Figure 3.



(a) (b)

Figure 2. Additional explanation of the marker fragments from *Vernonia amygdalina* leaf powder, (a) stomata detached from the lower epidermis and freely detached spiral-shaped vascular bundles, (b) rosette-shaped calcium oxalate crystals.

Trichomes, often referred to as covering hairs, are commonly found on leaves and stems. They come in several types: star-shaped, multicellular hairs, single-celled needle-shaped, and multicellular needle-shaped [15]. The covering hairs on *Vernonia amygdalina* leaves are multicellular needles.

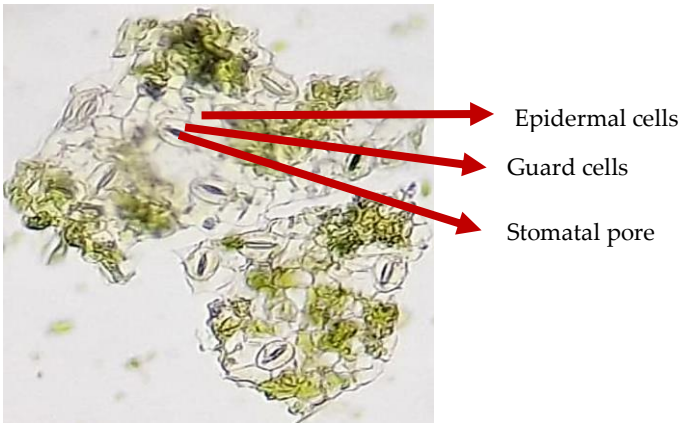


Figure 3. Stomata of *Vernonia amygdalina* leaves.

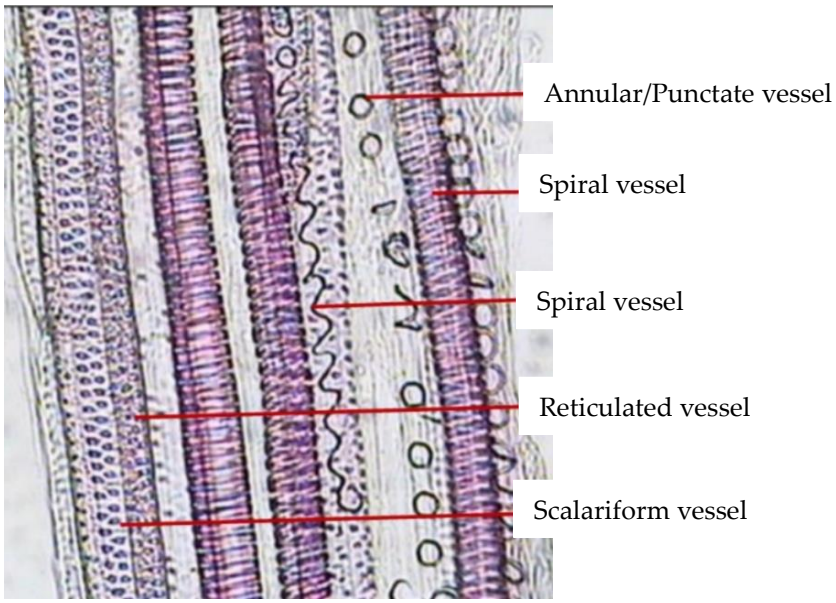


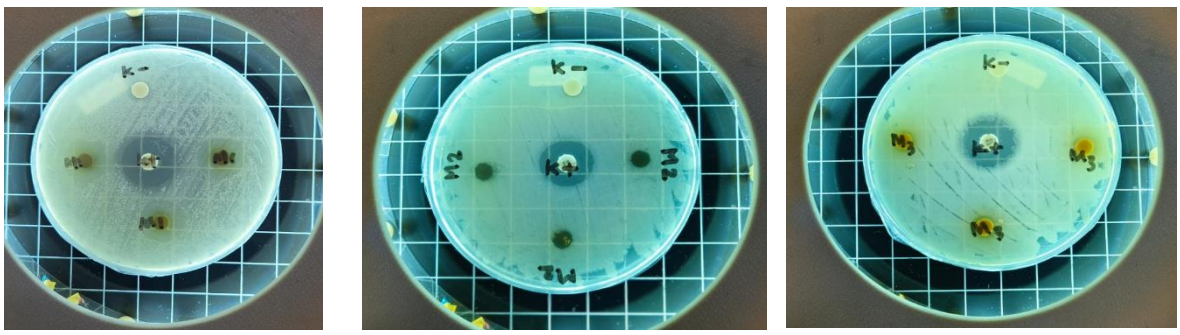
Figure 4. Various types of vascular bundles fragments (https://www.numerade.com/).

Vernonia amygdalina leaf powder after maceration and other parts that have been further processed through fractionation, yield results are obtained (Table 1).

Table 1. Results of yield calculations from extraction and fractionation

Sample	Weight of powder (g)	Extract/fraction (g)	Yield (%)
Methanol extract	150	75.45	50.3
Ethyl acetate extract	150	19.47	12.98
Ethyl acetate fraction	150	3.93	2.62

From this study, it can be concluded that the extraction process using methanol solvent produces a higher yield than the extraction process using ethyl acetate solvent. This is because methanol solvent is more often known as a universal solvent. This solvent is universal because it has two sides, namely one side is polar (-OH group) and the other side is non-polar (-CH_3) [16]. So, it is possible that the extracted compounds are non-polar, semi-polar, and polar compounds. For ethyl acetate solvent is semi-polar, so the compounds that can be extracted by this solvent are limited to semi-polar compounds. This is in accordance with the concept of "like dissolves like."



(a) (b) (c)

Figure 5. Antibacterial test results of *Vernonia amygdalina* leaf extract and fractions; (a) methanol extract, (b) ethyl acetate extract, and (c) ethyl acetate fraction.

Table 2. Inhibitory value of *Vernonia amygdalina* leaf extract and fraction

Sample	Inhibition zone diameter (mm)			Final score (mm)	Inhibition category
	R1	R2	R3		
Methanol extract	8.44	8.24	9.00	8.56±0.39	Moderate
Ethyl acetate extract	5.50	5.50	5.50	5.50±0	Moderate
Ethyl acetate fraction	8.80	7.00	7.52	7.71±0.93	Moderate
Positive control	19.00 (M1)	16.40 (M2)	17.82 (M3)		

Description
R1: First test point
R2: Second test point
R3: Third test point

From this study, it can be concluded that the methanol extract and ethyl acetate fraction can inhibit the growth of *E. coli* quite well compared to the ethyl acetate extract. However, all three test samples are included in the moderate inhibition category. Between the methanol extract and ethyl acetate extract have different inhibitory abilities, but when viewed from the various secondary metabolite contents are actually the same. According to previous research, methanol extract and ethyl acetate extract contain secondary metabolites, including flavonoids, phenols, tannins, alkaloids, and saponins [18]. The negative control (paper discs with added solvent) showed no inhibition, with no clear zone. However, the positive control had a larger clear zone than the *Vernonia amygdalina* leaf extract and fraction. This suggests that the inhibition of the *Vernonia amygdalina* leaf extract and fraction is still inferior to commercially available antibacterial drugs.

Some secondary metabolites that have the potential as antibacterials include saponins, tannins, and flavonoids. Saponins can increase membrane permeability, thereby causing cell hemolysis. Tannins can inhibit the growth of bacteria (low concentrations) and are also able to coagulate bacterial protoplasm (high concentrations). Flavonoids can bind proteins, thereby disrupting cell metabolic processes [19]. Actually, methanol extract and ethyl acetate extract both have these three compounds (flavonoids, tannins, and saponins), but their inhibitory abilities are different. This is thought to be due to the presence of polar secondary metabolites, particularly tannins, that are less extractable with ethyl acetate. This is because tannins are more polar due to their high hydroxyl content [20]. Flavonoids and saponins can be extracted with both polar and semipolar solvents. This is supported by the yield values, where the methanol extract yielded a higher yield than the ethyl acetate extract [21], [22].

4. CONCLUSION

The inhibitory power of the methanol extract and ethyl acetate fractions is approximately the same, but the ethyl acetate extract is less effective in inhibiting *E. coli*. From this study, it can be concluded that extracting herbal plants for antibacterial activity is best done using methanol as a solvent. This aims to maximize the extraction of all compounds with antibacterial potential (flavonoids, saponins, and tannins). For further research, it is recommended to examine the relationship between the levels of antibacterial compounds and the antibacterial properties of herbal plants.

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