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The Potential of Bitter Leaf (*Vernonia amygdalina*) in Herbal Medicine as Anti-Inflammatory Agent

Potensi Daun Pahit (Vernonia amygdalina) dalam Pengobatan Herbal sebagai Agen Anti-inflamasi

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Abstract

The inflammatory process involves increased vascular permeability, protein denaturation, and membrane alteration. Flavonoids are a class of secondary metabolites with biological and pharmacological activities, including anti-inflammatory. The bitter leaf, *Vernonia amygdalina* (VA), are used in various alternative medicine in several countries, including Indonesia. Chemical components in VA leaf are polyphenolic compounds, flavonoids, terpenes, and coumarins. The previous study exhibited that the anti-inflammatory activities of plants are closely related to the levels of polyphenols and flavonoids. Therefore, this study aimed to evaluate the anti-inflammatory activity of VA leaf and determine the total flavonoid-phenolic content of VA leaf ethanolic extract. The extraction method used maceration by ethanol as a solvent. The anti-inflammatory activity was measured by protein denaturation inhibition properties. The total flavonoid and phenolic content were determined by colorimetric and Folin-Ciocalteu methods, in which both procedures were measured by UV-Visible spectrophotometry at maximum wavelength. The results showed that VA leaf ethanolic extract has an anti-inflammatory effect with a half inhibition concentration (IC50) of 346.23 µg/mL. The total flavonoid and phenolic content of the ethanolic extract of VA leaves were 25.62 mgQE/g extracts and 21.47 mgGAE/g extract, respectively. Thus, the ethanolic extract of VA leaves can potentially be developed as an anti-inflammatory agent in herbal medicine.
Keywords: anti-inflammatory, flavonoid, gallic acid, phenolic, quercetin

Abstrak

Proses inflamasi melibatkan peningkatan permeabilitas vaskular, denaturasi protein, dan perubahan membran. Flavonoid merupakan senyawa metabolit sekunder dengan aktivitas biologis dan farmakologis potensial, termasuk sebagai antiinflamasi. Daun pahit, Vernonia amygdalina (VA), digunakan dalam berbagai pengobatan alternatif di beberapa negara, termasuk Indonesia. Komponen kimia dalam daun VA adalah senyawa polifenol, flavonoid, terpen, dan kumarin. Studi sebelumnya menunjukkan bahwa aktivitas anti-inflamasi tanaman terkait erat dengan kadar polifenol dan flavonoidnya. Oleh karena itu, penelitian ini bertujuan untuk mengevaluasi aktivitas antiinflamasi daun VA dan mengetahui kandungan flavonoid-fenolik total pada ekstrak daun VA. Metode ekstraksi menggunakan maserasi dengan etanol sebagai pelarut. Aktivitas antiinflamasi diukur dengan menggunakan metode penghambatan denaturasi protein. Kandungan total flavonoid dan fenolik ditentukan dengan metode kolorimetri dan Folin-Ciocalteu. Kedua prosedur tersebut diukur pada spektrofotometri UV-Visible pada panjang gelombang maksimum. Hasil penelitian menunjukkan bahwa ekstrak etanol daun VA memiliki efek anti-inflamasi dengan konsentrasi daya hambat 50% (IC_{50}) sebesar 346,23 µg/mL. Kandungan total flavonoid dan fenolik ekstrak etanolik daun VA berturut-turut adalah 25,62 mgQE/g ekstrak dan 21,47 mgGAE/g ekstrak. Dengan demikian, ekstrak etanol daun VA berpotensi untuk dikembangkan sebagai agen anti-inflamasi dalam pengobatan herbal.
Kata kunci: *anti-inflamasi, asam galat, fenolik,* flavonoid, quercetin

INTRODUCTION

Inflammation is the body's defense response to eliminate the cause of damage to tissues or cells, cleanse tissue from the remnants of damage, and build new tissue. Causes of inflammation include infection, foreign bodies, physical trauma, extreme temperatures, and chemical causes that cause tissue

damage (Chen et al., 2018). The new tissue construction is intended to replace damaged tissue. However, damaged cells are not always replaced by the same functional cells, so the former damaged tissue may be replaced by fibrous tissue, which causes scarring (Adler et al., 2020; Wynn & Ramalingam, 2012).

The inflammatory process involves increased vascular permeability, protein denaturation, and membrane alteration (Gunathilake et al., 2018). Protein denaturation is a process in which proteins lose their tertiary and secondary structure through external compounds, such as strong acids and bases, concentrated inorganic salts, organic solvents, and heating (Kumar & Jain, 2014). In general, proteins lose their biological function when there is protein denaturation. Inflammation due to protein denaturation causes cells to release several phospholipids, including arachidonic acid. After the arachidonic acid is free, it will be activated by several enzymes, including cyclooxygenase and lipoxygenase. These enzymes convert arachidonic acid into unstable forms (hydroperoxides and endoperoxides) and are further metabolized into leukotrienes, prostaglandins, prostacyclin, and thromboxane (Ullah et al., 2014). Prostaglandins and leukotrienes are responsible for the symptoms of inflammation (Jo-Watanabe et al., 2019).

Currently, commercially available anti-inflammatory products are dominated by synthetic chemical compounds, which often cause side effects for patients. Meanwhile, natural compounds have long been used in medicine because their pharmacological effects are comparable to synthetic compounds with minimal side effects. Thus, the search for natural compounds that have an anti-inflammatory effect is expected to be an alternative to overcome inflammation. One of the plants known to have many benefits in alternative medicine is the bitter leaf. *Vernonia amygdalina* (VA), a bitter leaf, belongs to the Asteraceae family and grows widely in Africa. So, in Indonesia, this plant is known as the African leaf. VA leaves are used in various alternative medicine in several countries, especially in Africa. Chemical components in VA leaf are polyphenolic compounds, flavonoids, terpenes, and coumarins. The results showed that VA has several potentials, including antioxidant, antibiotic, and anti-cancer. Plant antioxidant and anti-inflammatory activities are closely related to the levels of carotenoids, polyphenols, and flavonoids (Cho et al., 2020; Fawwaz et al., 2020). A kind of flavonoid in VA is luteolin which has been reported to have strong antioxidant activity. Furthermore, the results showed that luteolin could prevent the production of pro-inflammatory cytokines (Wang et al., 2020).

Extract from VA leaves can be developed in herbal medicine because the chemical components in VA leaf are essential in treating various diseases, including flavonoid and phenolic components. Flavonoid and phenolic compounds are secondary metabolites of plants with polyphenolic structures, which are synthesized through the polypropionate pathway and the molecular component of phenylalanine (Tungmunnithum et al., 2018). Flavonoids (Figure 1) are a class of secondary metabolites with biological and pharmacological activities, including anti-inflammatory, antibacterial, allergic, and cytotoxic activities. The exact mechanism of flavonoids' action in vitro is unknown, but various studies have shown that the anti-inflammatory activity of flavonoids works in overcoming protein denaturation (Panche et al., 2016).

The total flavonoid determination was carried out using the $AlCl_3$ method. The addition of $AlCl_3$ in the sample forms a complex between $AlCl_3$ and quercetin (Figure 2), which can be analyzed using UV-Visible spectrophotometry at a wavelength of 435 nm. This method can detect the presence of flavonoid compounds of flavone and flavanol groups (Tristantini & Amalia, 2019).

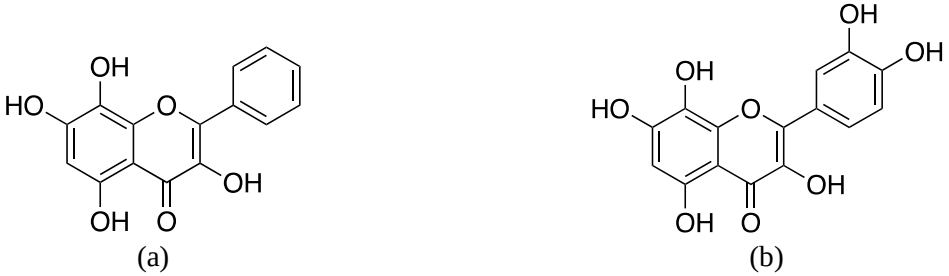

Figure 1. The Structure of Flavonoid (a) and Quercetin (b)

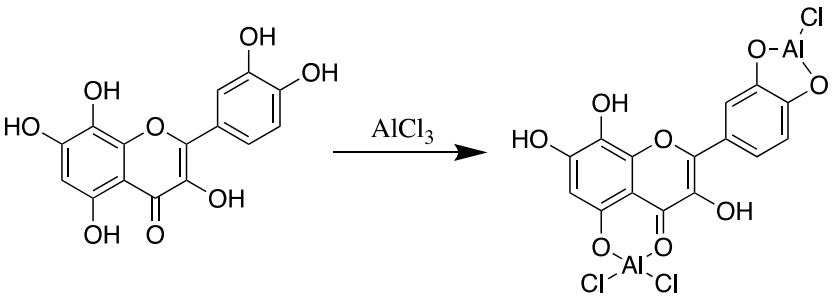

Figure 2. The Complex Reaction Between Quercetin and $AlCl_3$

Efforts in developing herbal medicine products are performed through the initial evaluation of natural compounds. These evaluations can be in the form of determining the active chemical components and activity tests in vitro and in vivo. The development of herbal medicinal products is possible in Indonesia, considering that the technology needed to develop herbal medicine products is adequate in several national pharmaceutical industries. Therefore, this study investigated the potential of ethanolic extract of VA leaves growth in Makassar-Indonesia on anti-inflammatory effect. This study examined the levels of flavonoids and phenolics in VA leaves extract. This study also provided an overview of the potential of VA leaves as an anti-inflammatory so that they can be a source of information for further researchers in developing herbal medicine products for VA plants.

METHODS

Chemicals and Sample

The chemicals, including solvents, were analytical grade and purchased commercially. Gallic acid, quercetin, ascorbic acid, sodium diclofenac, Folin-Ciocalteu reagent, and other chemicals were purchased from Merck Co. (Darmstadt, Germany). Deionized water was obtained through a Millipore-Q50 Ultrapure water system (Sartorius). Bovine serum albumin (BSA) was obtained from Biowest (Nuaillé, France). The stock solution of gallic acid and quercetin (1000 µg/mL) was prepared, respectively by dissolving 10 mg into 10 ml ethanol. Stock standard solutions were used for calibration standards preparation. The bitter leaves, VA, were obtained in Makassar City. The Division of Botany, Laboratory of Pharmacognosy and Phytochemistry, Faculty of Pharmacy, Universitas Muslim Indonesia, Makassar-Indonesia, confirmed the sample.

Extraction

The sample was washed with water and then dried by air-dried. The dried samples were sorted and then cut into small pieces and powdered. A total of 50 g of sample was macerated with 500 mL of 96% ethanol at room temperature for three days. The liquid extract was filtered and concentrated under reduced pressure to obtain a dry extract. The extract obtained was then calculated as the yield:

$$\% \text{ yield} = \frac{\text{total weight of extract}}{\text{total weight of sample}} \text{ x } 100\ \% \quad (1)$$

Determination of Anti-Inflammatory Activity

The anti-inflammatory activity was evaluated by the protein denaturation method, as described previously, with slight modifications (Dharmadeva et al., 2018). The sodium diclofenac 4000 µg/mL was diluted to prepare concentration series (100; 200; 400; 800; 1600 µg/mL). Each 50 µL of sodium diclofenac was filled up to reach a total volume of 5 mL with 0.2% BSA. The mixture was incubated at room temperature for 30 minutes, heated at 72 °C for 3 minutes, and allowed to stand at room temperature for 25 minutes. Furthermore, the solution was vortexed and measured the absorbance by UV-Visible spectrophotometry at the maximum wavelength of 660 nm. The same procedure was done for the VA leaves extract (1000; 2000; 4000; 8000; and 16000 µg/mL). The percentage of protein denaturation

inhibition was calculated by the following formula, AC: absorbance of the negative control; AS: absorbance of the samples.

$$\% \text{ inhibition} = \frac{A_C - A_S}{A_C} \times 100\% \quad (2)$$

Determination of Total Flavonoid Content (TFC)

The TFC of the VA leaves extract was examined by the aluminum chloride ($AlCl_3$) colorimetric method (Chang et al., 2020). Quercetin (10; 15; 20; 25; 30; 35 µg/mL) was used as a reference standard to obtain a calibration curve. For each concentration of quercetin (1 mL) was added 1 mL of 2% aluminum chloride ($AlCl_3$) and 120 mM potassium acetate (CH_3COOK), respectively. Following the 30 minutes incubation at room temperature, the absorbances were determined using the UV-Visible spectrophotometric method at the maximum wavelength at 435 nm. The best fit of quercetin data was determined by linear regression using the following equation: y = bx + a, where y = absorbance, b = slope, x = concentration, and a = intercept.

The same procedure was performed on the VA leaves extract 5 mg/5 mL triplicate. The absorbance of the extract was plotted in the linear regression equation of quercetin. The TFC was expressed as mg of quercetin equivalents (QE) per g extract (mgQE/g). The calculation of total flavonoid levels is based on the following formula:

$$\text{Flavonoid total (mgQE/g)} = \frac{\text{Volume sample (L) x Initial concentration (x)}}{\text{Weight of extract}} \quad (3)$$

Determination of Total Phenolic Content (TPC)

The TPC of the VA leaves extract was examined using the Folin-Ciocalteu assay as previously described (Nayaka et al., 2020). Gallic acid (9; 11; 13; 15; 17 µg/mL) was used as a reference standard to obtain a calibration curve. Each concentration of gallic acid (0.5 mL) was added Folin-Ciocalteu reagent (2 mL), homogenized, and incubated for 5 minutes at room temperature. Into the mixture, 7% sodium carbonate (Na_2CO_3) (2 mL) was added and then incubated for 30 minutes at room temperature. The absorbances were determined at 765 nm by UV-Visible Spectrophotometry, in which distilled water was used as a blank. Gallic acid was used to construct a standard curve. The best fit of gallic acid data was determined by linear regression using the following equation: y = bx + a, where y = absorbance, b = slope, x = concentration, and a = intercept.

The same procedure was performed on the VA leaves extract 5 mg/10 mL triplicate. The absorbance of the extract was plotted in the linear regression equation of gallic acid. The TPC was expressed as mg of gallic acid equivalents (GAE) per g extract (mgGAE/g). The calculation of total phenolic levels is based on the following formula:

$$\text{Phenolic total (mgGAE/g)} = \frac{\text{Volume sample (L) x Initial concentration (x)}}{\text{Weight of extract}} \quad (4)$$

RESULTS AND DISCUSSION

The VA leaves were extracted by the maceration method, which was very compatible with the AV leaves extract. The solvent used in the extraction, ethanol, allowed the flavonoid and phenolic compounds in AV leaves to be extracted perfectly. The yield on triplicated VA leaves extraction showed a value of 37.37%.

Determination of Anti-Inflammatory Activity

The anti-inflammatory activity of VA leaves ethanolic extract was evaluated using the protein denaturation inhibition method by heating. Temperature is one of the factors that can cause protein denaturation. BSA was used in the protein denaturation assay due to its sensitivity and stability in thermal conditions compared to other albumin indicators (Raoufinia et al., 2016). The BSA was prepared by dissolving it in the tris buffer saline (TBS) at pathological pH (6.2-6.5) to maintain the pH. The TBS is the common buffer used in biological, biochemical, and molecular biology applications. The ability of

VA extract to inhibit denaturation during heating was determined by the turbidity of the mixture as measured on a UV-Visible spectrophotometer at a wavelength of 660 nm. As a non-steroidal anti-inflammatory drug, Diclofenac sodium was chosen as a positive control due to its ability to inhibit protein denaturation (Ullah et al., 2014).

The anti-inflammatory study showed that the VA extract could inhibit protein denaturation, as shown in Table 1. The linearity exhibited a high correlation between concentration and inhibition. The half inhibitory concentration (IC_{50}) of VA extract and sodium diclofenac were 346.23 µg/mL and 33.86 µg/mL, respectively. These results exhibited that the VA extract could be an anti-inflammatory agent. A study showed that compounds inhibiting protein denaturation greater than 20% are considered anti-inflammatory and can be developed for further study (Novika et al., 2021). The previous study showed that the acetone extract of VA leaves at 100 and 200 mg/kg doses has anti-inflammatory activity by significantly reducing edema in rats. Its anti-inflammatory activity was comparable to indomethacin (Adedapo et al., 2014). The anti-inflammatory efficacy of a VA methanol extract was examined, and the extract was found to lower infiltrating leucocytes, protein concentration, and malondialdehyde levels while remaining unaffected by glutathione and superoxide dismutase (Onasanwo et al., 2017).

Table 1. Anti-inflammatory activity of sodium diclofenac and VA leaves extract

Initial Concentration (µg/mL)	Final Concentration* (µg/mL)	Absorbance (660 nm)	Inhibition (%)	Linearity	IC_{50} (µg/mL)
			Sodium diclofenac		
0	0	1.79	0	y = 1.0663x + 13.901	33.86
100	1	1.52	15.26		
200	2	1.51	15.70		
400	4	1.48	17.82	R^2 = 0.9961	
800	8	1.38	22.99		
1600	16	1.24	30.79		
			VA leaves extract		
0	0	1.79	0	y = 0.9309x + 17.803	346.23
1000	10	1.45	18.99		
2000	20	1.45	19.27		
4000	40	1.40	21.55	R^2 = 0.9979	
8000	80	1.33	25.45		
16000	160	1.21	32.63		

*Concentration after mixed with BSA

Determination of Total Flavonoid Content (TFC)

The standard solution absorbances of quercetin and the ethanolic extract of VA leaves were started by running a suitable wavelength for the standard quercetin solution. The running results obtained are 435 nm. The absorbance of the standard quercetin solution was used to construct a linear regression curve, as shown in Figure 3.

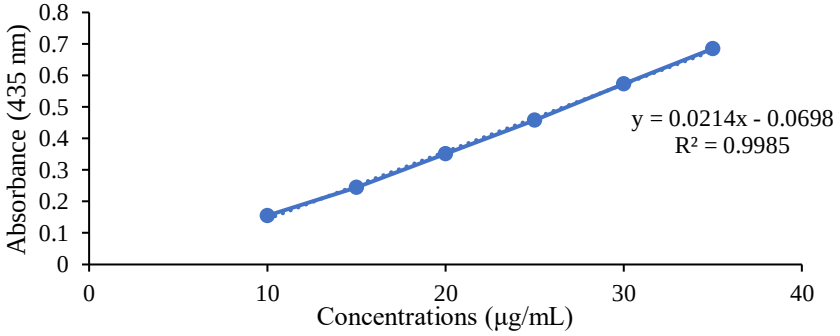

Figure 3. The Linier Regression Curve of Quercetin

The TFC determination of the VA leaves extract was based on the absorbance data of the sample obtained triplicate to ensure data accuracy. The absorbance of the extract was then plotted into a linear regression equation, as stated in Figure 3. The TFC of the VA leaves extract was 25.62 mgQE/g extract, meaning that each g contains 25.62 mg of flavonoid, which is equivalent to quercetin, as shown in Table 2. This result exhibited a similar level to the previous study, which contains 22.53 ± 0.92 mgQE/g (Harahap et al., 2021).

Table 2. Total flavonoid content of VA leaves extract

Replication	Absorbance (y)	Initial Concentration (mg/L)	Total Flavonoid (mgQE/g)	Average (mgQE/g)
1	0.46	25.09	24.85	
2	0.49	26.57	26.36	25.62
3	0.47	25.86	25.65	

Determination of Total Phenolic Content (TPC)

Determination of TPC in the VA leaves extract used gallic acid as a standard solution because it is one of the natural phenols with good stability. Gallic acid is a phenolic compound derived from hydroxybenzoic acid, a simple phenolic acid (Kumar & Goel, 2019). A reaction occurred between the Folin-Ciocalteu reagent and phenolic compounds in the sample, forming a molybdenum-tungsten complex in determining the total phenolic content of VA leaves extract. This reagent oxidizes phenolics (alkali salts) or phenolic-hydroxy groups, reducing the heteropoly acid (phosphomolybdate-phosphotungstic) in the Folin-Ciocalteau to a molybdenum-tungsten complex (Martono et al., 2019). Phenolic compounds react with the Folin-Ciocalteau reagent only in an alkaline environment to cause the dissociation of protons in phenolic compounds into phenolic ions (Carmona-Hernandez et al., 2021). This assay used 7% Na_2CO_3 to create alkaline conditions.

During the reaction, the hydroxyl group on the phenolic compound reacts with the Folin-Ciocalteau reagent. The blue color formed will be more concentrated, equivalent to the concentration of phenolic ions formed, meaning that the greater the concentration of phenolic compounds, the more phenolic ions will reduce heteropoly acid into a molybdenum-tungsten complex (Martono et al., 2019). In this study, incubation was carried out for 30 minutes at 25 – 27 °C to optimize the reaction between phenolic compounds and reagents.

The standard solution absorbances of the gallic acid and the ethanolic extract of VA leaves were started by running a suitable wavelength for the standard solution of gallic acid. The running results obtained are 765 nm, which is the maximum wavelength of the gallic acid standard. The absorbance of the standard solution of gallic acid was used to construct a linear regression curve, as shown in Figure 4.

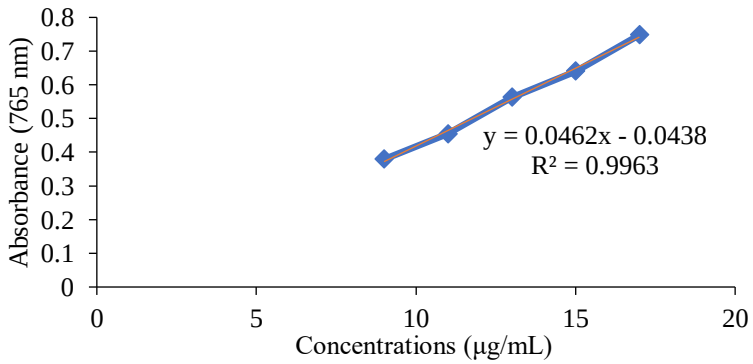

Figure 4. The Linear Regression Curve of Gallic Acid

Determination of the VA leaves extract's TPC was carried out based on the absorbance data of the sample obtained triplicate to ensure data accuracy. The absorbance data of the sample was then plotted

into a linear regression equation that was generated by measuring the standard solution of gallic acid. The TPC of the VA leaves extract was 21.47 mgGAE/g extract, meaning that each g contains 21.47 mg of phenolic, which is equivalent to gallic acid, as shown in Table 3. However, this result is lower than the previous study, which contains 54.61 ± 0.94 mgGAE/g (Harahap et al., 2021). The difference level of total phenolic might be due to the ethanol concentration and some physicals factor (Alara et al., 2020).

Table 3. Total phenolic content of VA leaves extract

Replication	Absorbance (y)	Initial Concentration (mg/L)	Total Flavonoid (mgGAE/g)	Average (mgGAE/g)
1	0.55	12.77	25.54	
2	0.54	12.55	25.10	25.37
3	0.55	12.74	25.48	

Challenges and Opportunities of Industries Based on VA Leaf

Based on data on the chemical components contained in VA leaf and its pharmacological activity, VA leaves extract has the potential to be developed as a standardized herbal anti-inflammatory drug. The data of this study is in accordance with previous research data, which rationalized the traditional use of this plant in several African regions. The potential of VA leaf is also supported by the availability of this plant in many parts of Indonesia. In addition, considering the Indonesian people's level of interest in herbal medicines, this is a great opportunity for commercialization. However, an in-depth market potential analysis is needed to support this product's readiness as a standardized herbal. The challenge is the possibility that VA leaf will become extinct, and hard to find if it has been used in mass production. For that, it still needs to be considered to conserve this plant to maintain its availability for a long time. In the future, the VA leaf plantations could be needed, which will become a new commodity for Indonesian farmers.

CONCLUSIONS

The ethanolic extract of VA leaf has potential anti-inflammatory activity with an IC_{50} 346.23 μg/mL. The anti-inflammatory activity of VA leaves extract might be due to the levels of polyphenol compounds. The total flavonoid and phenolic content of the ethanolic extract of VA leaves were 25.62 mgQE/g extracts and 21.47 mgGAE/g extract, respectively. Thus, the ethanolic extract of VA leaf is potential to be developed in herbal medicine as an anti-inflammatory agent. To further support these findings, anti-inflammatory evaluation utilizing various techniques will be required in the future, both in vitro and in vivo.

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