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Determination of Total Flavonoid Content in Ethanolic Leaf Extract of *Calotropis gigantea*: A Comparison Between Geothermal and Non-Geothermal Areas

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Abstract: *Calotropis gigantea* (L.), commonly known as biduri, is a plant recognized for its pharmacological properties, including antioxidant and anti-inflammatory activities, largely attributed to its secondary metabolites, particularly flavonoids. The concentration of these bioactive compounds is strongly influenced by the environmental conditions in which the plant grows. Geothermal manifestation areas, characterized by extreme abiotic conditions, can induce metabolic responses in plants, potentially enhancing flavonoid production. This study aimed to determine and compare the total flavonoid content in ethanolic leaf extracts of *C. gigantea* collected from geothermal and non-geothermal areas in Aceh Province. Extraction was conducted using 96% ethanol, and total flavonoid content was quantified using UV-Vis spectrophotometry via the aluminum chloride ($AlCl_3$) complexation method. Quercetin served as the standard, and flavonoid levels were expressed as milligrams of quercetin equivalents per gram of extract (mg QE/g). The calibration curve showed a linear relationship between quercetin concentration and absorbance, with a regression equation of $y = 0.0101x - 0.0098$ ($r = 0.9417$). The results demonstrated significant differences in flavonoid content between samples from geothermal and non-geothermal sites. *C. gigantea* plants from geothermal areas exhibited altered flavonoid levels compared to those from non-geothermal regions, indicating that environmental factors play a role in modulating the biosynthesis of these bioactive compounds.

Keywords: *Calotropis gigantea*; Flavonoid Total; Geothermal; Spectrophotometry.

Introduction

Plant biduri (*Calotropis Gigantea* L.) is a plant that is rich in chemical content, which is often used as medicine [1]. This plant is readily available as it grows wild in lowland areas and is commonly found among shrubs in tropical climates. It has been widely utilized by local communities as a traditional medicine, particularly for treating toothaches, colds, coughs, and asthma (Rajamohan et al., 2014) .

The quality plant drug is determined by secondary metabolites [3], secondary metabolites are the result of interactions between plants and their environment. The relationship between plants and environmental factors has a greater influence on the content of secondary metabolites than on primary plant metabolites [4], [5]. The content of secondary metabolites in a plant is strongly influenced by the plant's growing environment. This is due to the fact that each location has distinct environmental characteristics. Environmental factors such as soil, water availability, and climate play a crucial role in the biosynthesis of secondary metabolites in plants [6]. Plant species of the same type that grow under different environmental conditions often exhibit significant differences in the production and accumulation of secondary metabolites [7]. Moreover, metabolic profiling has become an important tool for understanding the systematic responses of organisms to changes in environmental conditions [8].

Geothermal manifestation areas are characterized by environmental conditions that differ significantly from those of non-geothermal regions. These areas are typically considered extreme environments due to the presence of high-temperature geothermal activity and elevated concentrations of various chemical elements. As a result, plant species that grow in geothermal manifestation zones must possess adaptations to withstand harsh abiotic stresses, which can significantly influence their growth and development (Baillie et al., 2018) , Consequently, not all plant species are capable of surviving in such environments.

Furthermore, geothermal sites themselves can vary in their chemical characteristics depending on the location. For instance, in the Seulawah Agam geothermal manifestation area in Aceh Province, different manifestation points exhibit dominant contents of distinct chemical compounds: bicarbonate-rich zones [10], chloride-dominant zones [11], and Ie Jue the more dominant content sulfate [12]. Previous reports and field observations indicate that *Calotropis gigantea* (Biduri) can grow in several of these geothermal manifestation zones. Therefore, this study aims to investigate and compare the *Calotropis gigantea* growing in geothermal manifestation areas with those growing in non-geothermal environments.

The development of traditional medicines, such as those derived from *Calotropis gigantea* (Biduri), is being pursued to align with modern pharmaceutical standards. In order for Biduri to be used as a raw material for standardized herbal medicines, it must meet established quality standards. The primary objective of quality standardization is to ensure consistent and reliable control over the raw materials used in the production of herbal medicines. Standardization is necessary because plants of the same species growing in different environmental conditions can exhibit variations in their chemical content. Therefore, before further testing is

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conducted, it is essential to perform preliminary characterization of the plant material to ensure that the extracts used as raw materials for standardized medicines meet specified quality standards. In addition to serving as a quality control measure, such characterization also helps to confirm the presence and concentration of key secondary metabolites, which are the most important compounds in medicinal plants. Among the secondary metabolites found in *Calotropis gigantea* leaves, flavonoids are a significant group [13].

Flavonoids are widely occurring phenolic compounds in plants, recognized for their diverse biological activities, including antioxidant, antibacterial, and anti-inflammatory effects [14]. The quantification of flavonoid content is commonly conducted using UV-Vis spectrophotometry [15]. However, this analytical approach is often time-consuming and involves several procedural steps, such as extraction, phytochemical screening, and compound identification. Moreover, it necessitates the use of considerable quantities of chemical reagents and laboratory materials.

The growing environment of plants plays a crucial role in the biosynthesis of secondary metabolites, including flavonoids [5]. Geothermal areas, characterized by elevated soil temperatures and the presence of volcanic gases, can significantly influence plant metabolism [16]. Previous studies have demonstrated that environmental stresses, such as high temperature and gas exposure, can stimulate the production of phenolic compounds and flavonoids as part of the plant's adaptive response. However, there remains a lack of comprehensive studies comparing the total flavonoid content in plants cultivated in geothermal versus non-geothermal regions.

Understanding the influence of the growing environment on the content of bioactive compounds is essential for the optimal development of medicinal plants. Therefore, this study aims to quantify the total flavonoid content in ethanol extracts of *Calotropis gigantea* (Biduri) leaves collected from both geothermal and non-geothermal areas, in order to elucidate the environmental impact on flavonoid biosynthesis. The findings of this research are expected to contribute to the sustainable utilization of natural resources as raw materials for the production of high-quality and efficacious herbal medicines.

Research Methods

Sampling

The leaves of *Calotropis gigantea* (biduri) were collected from both geothermal and non-geothermal areas. Four geothermal manifestation sites were selected as sampling locations: Ie Jue, Ie Suum, Ie Brôk (all three part of the Seulawah Agam geothermal system in Aceh Besar District, Aceh Province), and the Jaboi geothermal area in Sabang. As a comparison, samples from non-geothermal areas were collected from Lam Baro Village in Lhong Subdistrict, Aceh Besar District

Preparation Sample

A total of 2 kg of fresh leaves were collected, washed, and air-dried for 14 days. Drying was conducted to prevent spoilage and microbial decay, allowing the samples to be preserved for longer periods. Once dried, the leaves were ground into a fine powder using a blender

Extraction

The powdered leaves were macerated in 96% ethanol for 48 hours with occasional stirring. The mixture was then filtered, and the solvent was evaporated using a rotary evaporator at 78°C. The resulting ethanolic extract was collected and stored in a sealed bottle for further analysis..

Creation Solution Parent

A quercetin stock solution of 100 ppm was prepared by dissolving 10 mg of quercetin in 96% ethanol and diluting it to 100 mL in a volumetric flask.

Preparation of Standard Quercetin Solutions

A series of standard quercetin solutions (2, 4, 6, 8, and 10 ppm) was prepared by pipetting 0.2, 0.4, 0.6, 0.8, and 1.0 mL of the 100 ppm stock solution into separate 10 mL volumetric flasks. Each flask was filled to the mark with 96% ethanol.

Calibration Curve Preparation

To construct the calibration curve, 0.5 mL of each standard solution (2–10 ppm) was pipetted into 10 mL volumetric flasks. Each flask was then added with 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminium chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water. The mixtures were homogenized by shaking and incubated at room temperature for 30 minutes. Absorbance was then measured using a UV-Vis spectrophotometer at a wavelength of 435 nm.

Determining Flavonoid Content

A total of 10 mg of the ethanolic leaf extract was dissolved in 5 mL of 96% ethanol in a beaker, stirred until completely dissolved, and transferred into a 10 mL volumetric flask. The beaker was rinsed with ethanol and the rinsing solution was added to the same volumetric flask, which was then filled to the mark with 96% ethanol to obtain a 1000 ppm extract solution. A further dilution was performed by pipetting 1 mL of the 1000 ppm solution into a new 10 mL volumetric flask and diluting to the mark with ethanol to achieve a 100 ppm solution.

To determine the flavonoid content, 0.5 mL of the 100 ppm extract solution was pipetted into a 10 mL volumetric flask. Subsequently, 1.5 mL of 96% ethanol, 0.1 mL of 10% aluminum chloride, 0.1 mL of 1 M potassium acetate, and 2.8 mL of distilled water were added. The mixture was shaken to homogenize and incubated at room temperature for 30 minutes. The absorbance was measured using a UV-Vis spectrophotometer at the maximum wavelength. The total flavonoid content was then calculated using the method proposed by Chang et al. (2002).

Results and Discussion

The determination of flavonoid content in the ethanol extract of *Calotropis gigantea* leaves was carried out using the $AlCl_3$ method. Quercetin was used as the standard stock solution, as it is capable of forming a complex with $AlCl_3$ [17]. Following the preparation of the stock solution, a series of standard solutions at concentrations of 2, 4, 6, 8, and 10 ppm was prepared. The blank solution used in this study consisted of 96% ethanol, 10% $AlCl_3$, 1 M potassium acetate, and distilled water. The reaction mixtures were then incubated for 30 minutes in the dark to ensure complete reaction and to achieve optimal color intensity [18].

Aluminium chloride is added to form a complex with quercetin [19], and the addition of potassium acetate in this analysis serves to stabilize the formation of the complex between $AlCl_3$ and quercetin. Measurements were conducted using a UV-Vis spectrophotometer at a maximum wavelength of 435 nm. A preliminary reduction step was performed to construct a calibration curve. The purpose of the calibration curve measurement is to determine the linear equation. The calibration curve was generated using standard solutions with concentrations of 2, 4, 6, 8, and 10 ppm.

Table 1. Results of absorbance measurements for quercetin standard solutions measured at a wavelength of 435 nm.

Concentration (ppm)	Absorbance (y)	Line Equation
2	0.016	y = 0.0101x - 0.0098
4	0.026	
6	0.052	
8	0.060	
10	0.100	

Based on these measurements, it can be concluded that higher quercetin concentrations result in higher absorbance values. The absorbance data from the standard quercetin solutions were plotted against their concentrations to generate a calibration curve, yielding the linear equation y = 0.0101x - 0.0098 with a correlation coefficient (r) of 0.9417. This linear equation can be used to determine the total flavonoid content in the sample extract.

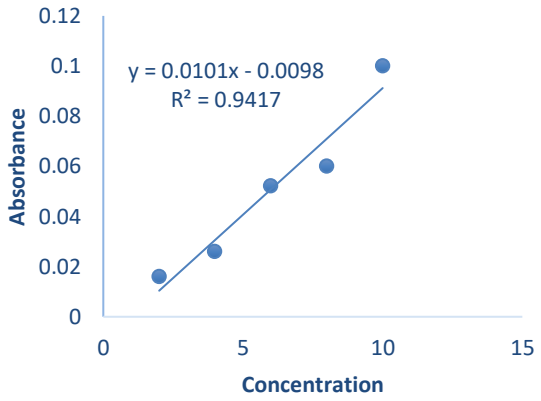

Figure 1. Standard calibration curve of quercetin measured at a maximum wavelength of 435 nm

In the determination of total flavonoid content in the ethanol extract of Biduri (Calotropis gigantea) leaves, the sample solution was added with $AlCl_3$, which forms a complex and causes a shift in the wavelength toward the visible region, indicated by the appearance of a yellow color. Potassium acetate was then added to help maintain the wavelength within the visible region [17]. An incubation period of 30 minutes prior to measurement was applied to allow the reaction to proceed completely, thereby maximizing the intensity of the resulting color [20]. The results of the total flavonoid content measurement of the ethanol extract of Biduri leaves are presented in Table 2.

Table 2. Total flavonoid content in Biduri (*Calotropis gigantea*) leaf extract (n = 2)

Sample	Absorbance	Initial Concentration (mg/L)	Total Flavonoid Content (mg QE/g extract)	Average Total Flavonoid Content (mg QE/g extract)	% Total Flavonoid
Ie ju	0.076	8.49505	84.9505	83.9604	8.39604
	0.074	8.29703	82.9703		
Ie Brook	0.032	4.1386	41.3861	43.36634	4.33663
	0.036	4.53465	45.3465		
Jaboi	0.011	2.05940	20.5940	20.09901	2.00990
	0.01	1.9603	19.6039		
Ie seum	0.052	6.11881	61.1881	58.21782	5.82178
	0.046	5.52475	55.2475		
Lam Baro	0.036	4.53465	45.3465	47.82178	4.78217
	0.041	5.02970	50.2970		
Lhong	0.041	5.02970	50.2970	54.25743	5.42574
	0.049	5.82178	58.2178		

The measurement results indicate that the total flavonoid content in *Calotropis gigantea* leaves from geothermal areas is generally higher than that in non-geothermal areas. The sample from Ie Ju (geothermal) exhibited the highest flavonoid content at 83.96 mg QE/g, followed by Ie Seum with 58.22 mg QE/g, while the Jaboi sample recorded the lowest content at 20.10 mg QE/g. In contrast, samples from non-geothermal areas, such as Lam Baro and Lhong, showed lower and relatively uniform flavonoid levels, at 47.82 mg QE/g and 54.26 mg QE/g, respectively. Statistical analysis using one-way ANOVA confirmed that these differences were significant ($F = 72.84$, $p < 0.001$), and subsequent Tukey's post-hoc test revealed that Ie Ju had significantly higher flavonoid levels compared to all other sites, whereas Jaboi consistently showed the lowest values. Meanwhile, non-geothermal areas such as

Lam Baro and Lhong did not differ significantly from each other or from Ie Seum.

The differences in total flavonoid content may be associated with the significantly distinct environmental conditions between the two zones, particularly with regard to heavy metal exposure, soil temperature, and osmotic pressure in geothermal areas. Geothermal environments often contain high concentrations of heavy metals and sulfur compounds, and they also experience extreme fluctuations in soil temperature and pH. These factors can induce abiotic stress, which in turn stimulates the biosynthesis of secondary metabolites such as flavonoids as part of the plant's defence mechanisms. Other studies have shown that plants exposed to cadmium (Cd) stress exhibit increased expression of genes related to flavonoid biosynthesis, including *PAL*, *CHS*, and *F3H* [21]. This suggests that plants in extreme environments tend to increase the production of antioxidant compounds as a protective response to oxidative stress.

These findings are consistent with previous studies reporting a total flavonoid content of 46.75 mg RE/g in ethanol extracts of *Calotropis gigantea* leaves, which also demonstrated strong antioxidant activity as assessed by DPPH, ABTS, and CUPRAC assays [22]. This value is comparable to the flavonoid content observed in non-geothermal areas such as Lam Baro (47.82 mg QE/g), indicating that flavonoid levels in non-geothermal environments tend to approximate previously reported standard values.

Interestingly, despite all sampling sites being part of geothermal systems, the flavonoid content in Jaboi (20.10 mg QE/g) was markedly lower than that in Ie Ju (83.96 mg QE/g). This finding suggests that geothermal environments do not exert a uniform influence on the accumulation of secondary metabolites. Site-specific factors such as the type of geothermal manifestation, root depth, microclimatic conditions, and variations in chemical pressure may contribute significantly. This observation is supported by previous studies reporting that the Jaboi geothermal field comprises two distinct fluid types: chloride (mature) and sulfate/bicarbonate (immature) [23].

Flavonoids are known to exhibit various biological activities, including antioxidant, anti-inflammatory, and anticancer properties [24]. Therefore, the results of this study reinforce the potential of *Calotropis gigantea* from geothermal areas as a candidate source of phytopharmaceutical compounds. In general, higher flavonoid content is associated with an increased potential for bioactive effects.

Conclusion

The observed significant variation in flavonoid content between *Calotropis gigantea* specimens collected from geothermal and non-geothermal sites is likely driven by differential environmental stressors that induce the biosynthesis of secondary metabolites. Among the geothermal locations, Ie Ju and Ie Seum, part of the Seulawah Agam geothermal system, exhibited the highest flavonoid concentrations, highlighting their potential as promising sources for the development of phytopharmaceuticals or natural antioxidant supplements. In contrast, the notably low flavonoid levels found in samples from Jaboi suggest that the geothermal influence on flavonoid accumulation is not homogeneous and may be modulated by site-specific environmental variables, such as soil composition, microclimatic conditions, or the intensity of geothermal activity.

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