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Total phenolic content, flavonoid content, and anthocyanin content in various of butterfly pea accessions in Bengkulu, Indonesia

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Abstract The results showed that the three accessions of Clitoria ternatea had phenotypic diversity of flowers and flower colors, namely dark blue-double petal (CTE_002), light blue-single petal (CTE_006), and light purple-single petal (CTE_013. In addition, the three accessions of Clitoria ternatea also had total monomeric anthocyanin content, total phenolic content, and total flavonoid content determined in the ethanol extract of the sample. Accession CTE_002 with dark blue-double petals had a total monomeric anthocyanin, phenolic, and flavonoid content of 56.76 ± 1.32 mg/l. 40.82±1.40mg GAE/g DW, and 4.76±0.72 mg QE/g DW. Variability in the anthocyanin content of the three accessions was only found in flowers with values ranging from 12.38±0.33 - 56.76±1.32 mg/l. The phenolic content of the three accessions ranged from 33.38 ± 0.37 - 40.82 ± 1.40 mg GAE/g DW. This shows that the three clitoria ternatea accessions in Bengkulu Province have high phenolic content and extract yield. Therefore, this accession can be used commercially in the food and cosmetics industry and shows high potential for medicinal plant breeding programs.

Keywords: Agro-morphology, Anthocyanins, Flavonoid, *Clitoria ternatea*, Secondary metabolites

Introduction

Butterfly pea (*Clitoria ternatea*) is a creeping herbaceous plant from the *Fabaceae* (*Leguminosae*) family, primarily found in tropical regions where it thrives under intense sunlight and shows resilience to environmental stress (Jamil *et al.*, 2018; Oguis *et al.*, 2019). It produces distinctive, solitary flowers that emerge from leaf axils, characterized by a papilionaceous structure with five petals: a central calyx, two wing petals, and two petals forming a keel (Bishoyi and Geetha, 2012). Like other leguminous plants, its roots develop nodules that facilitate nitrogen fixation, enriching the soil naturally (Oguis *et al.*, 2019). Originally cultivated as an ornamental plant (Mahmad *et al.*, 2016) and a

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protein-rich source of animal fodder (Abreu *et al*., 2014; Jamil *et al*., 2018), the butterfly pea is now gaining research attention for its bioactive compounds—particularly antioxidants—and protein derivatives that hold promise for developing natural pesticides (Oguis *et al.*, 2019; Nguyen *et al.*, 2011).

Clitoria ternatea, commonly referred to as butterfly pea, is recognized for its high anthocyanin content (Thuy *et al.*, 2021), alongside a diverse range of antioxidant compounds including phenolic acids, flavonoids, flavonol glycosides, and procyanidins (Jamil *et al.*, 2018). The anthocyanins extracted from its flowers are widely employed as natural dyes and antioxidant agents in the food and cosmetics industries (Oguis *et al.*, 2019). Beyond its antioxidant properties, the flower is also a rich reservoir of various bioactive constituents such as tannins, resins, steroids, saponins, triterpenoids, and xanthene pigments (Manjula *et al.*, 2013). Research has also reported various pharmacological activities attributed to the plant, including antimicrobial, antidiabetic, anticancer, anti-inflammatory, and cardioprotective effects (Al-snafi and Esmail, 2016; Esmail, 2016; Jamil and Pa'ee, 2018; Havananda and Luengwilai, 2019; Adwas, 2019; Alam *et al.*, 2021). Moreover, ethanol extracts derived from the roots have demonstrated potential antidepressant effects, possibly through modulation of the serotonergic and cholinergic pathways (Parvathi and Ravishankar, 2013). This content is an accumulation of secondary metabolite compounds produced by plants.

Secondary metabolites are biochemical compounds synthesized by plants primarily as defense mechanisms, and many of these substances exhibit important biological activities beneficial to human health (Thirumurugan *et al.*, 2018). The identification of these metabolites typically involves the use of specific solvents and advanced separation techniques. Although *hexane* is classified as a pollutant (Yara-Varon *et al.*, 2016), it is extensively used as a non-polar solvent because of its suitable polarity for extracting non-polar secondary metabolites (Ghazali and Yasin, 2016). These compounds are commonly analyzed and identified using Gas Chromatography–Mass Spectrometry (GC-MS) (Alp *et al.*, 2022). Interestingly, water and methanol extracts from butterfly pea (*Clitoria ternatea*) flowers have been shown to contain inositol as a significant component, which has exhibited notable anticancer activity against several types of cancer (Neda *et al.*, 2013).

In recent years, *Clitoria ternatea* has garnered substantial scientific and commercial attention due to its extensive applications. This species is notably rich in anthocyanins and various phytochemicals distributed throughout its plant organs, offering considerable potential for utilization within the food and nutraceutical sectors. However, no significant advances in crop improvement for this species have been documented. Understanding the genetic diversity among its genotypes is essential to support yield enhancement efforts. In Indonesia, butterfly pea is extensively cultivated and exhibits considerable intraspecific diversity, an important genetic asset requiring structured documentation. Preserving this diversity through sustainable practices—

balancing utilization with conservation—is essential to ensure its continued availability and utility. Clitoria ternatea is widely esteemed not only for its ornamental value and role as a natural dye but also for its cultural significance. Increasingly, it has attracted scientific interest for its prospective therapeutic applications and potential as a natural source of antioxidants. Nonetheless, comprehensive studies examining the antioxidant properties, total phenolic concentrations, and flavonoid content across different anatomical parts of the plant—namely its flowers, foliage, stems, and roots—are still limited.

This research is provided information regarding the total phenolic content. Total flavonoids and antioxidants in butterfly pea flowers (*Clitoria ternatea*). Apart from that, this research is also provided information regarding extracts from the parts of the Butterfly pea plant being the most potential as antioxidants.

Materials and methods

The study used three accessions of Clitoria ternatea in Bengkulu Province (Table 1, Figure 1). The butterfly pea plants were cultivated in the Experimental Garden of the Faculty of Agriculture at Bengkulu University, Indonesia. The three flower samples were planted, and their morphological characteristics were monitored until the plants reached 12 weeks of age. Observations included leaf length and width measurements, flower length and width, pod length and width, seed weight, total chlorophyll, phenol content, flavonoid content, total anthocyanin content, and antioxidant activity.

Table 1. *Clitoria ternatea* accessions used in this study, along with their corresponding codes, collection locations, and geographic coordinates

Accessions	Location	Latitude (N)	Longtitude (E)	Flower Color	Flower Type
CTE_002	Kota Bengkulu	-3.79744°	102.253828°	Dark Blue	Double petals
CTE_006	Kabupaten Mukomuko	-2.65303°	101.264601°	Light Blue	Single petals
CTE_013	Kota Bengkulu	-3.804903°	102.269496°	Light Purple	Single petals

Figure 1. Flower morphology of *Clitoria ternatea* accessions used in the research

Preparation of Clitoria ternatea flowers

Various parts of the plant—flowers, leaves, stems, and roots—were collected and separated. Their fresh weights were recorded using an analytical balance. The samples were then dried in an oven at 65°C for 72 hours. A portion of the dried material was reweighed to determine its dry weight, while the remaining samples were stored at room temperature (28 ± 2°C). All dried plant materials were ground into simplicia powder and passed through a 60-mesh sieve. For extraction, a solvent-to-dry-material ratio of 10:1 (v/w) using water was applied. Specifically, 1 gram of dried *Clitoria* flowers was combined with 10 ml of distilled water and extracted at 70°C. The resulting extract was centrifuged for 30 minutes (Nguyen *et al.*, 2021).

Total anthocyanin content

The total anthocyanin content was determined using the pH differential method. For the analysis, 10 mg of extract dissolved in 10 mL of 96% ethanol was mixed separately with 0.025 M potassium chloride (pH 1) and sodium acetate buffer (pH 4.5). Absorbance readings were taken at the same wavelength, using distilled water as the blank (Lee *et al*., 2005). The anthocyanin concentration was then calculated based on a specific formula and reported as milligrams of cyanidin-3-glucoside equivalents per liter (mg/l).

$$\text{Total monomeric anthocyanin } (mg/l \text{ c 3 g eq.}) = \frac{(Aabs * MWt * DF * 103)}{(e * l)}$$

Note:
Abs (Absorbance) = $(A_{520} - A_{700})_{ph}$ 1.0 – $(A_{520} - A_{700})_{ph}$ 4.5,
MWt (Molecular weight) of cyanidin-3-glucoside (Cy-3-glu) is 449.2 g/mol,
The dilution factor (DF) accounts for sample dilution before measurement,
The molar extinction coefficient (ε) for Cy-3-glu is 26,900 $L \cdot mol^{-1} \cdot cm^{-1}$,

The path length (l) of the cuvette used for absorbance measurement is expressed in centimeters. A factor of 10^3 is applied to convert grams to milligrams (g to mg).

Total phenolic content

The total phenolic content (TPC) of each extract was analyzed using a modified version of the *Folin–Ciocalteu* (FC) method outlined by Do *et al*., (2014). Extracts were dissolved in aqua pro injection (API) to a final concentration of 50 µg/mL. A calibration curve was prepared using gallic acid standards ranging from 50 to 600 ppm in API. For analysis, 1.6 mL of either diluted extract or gallic acid solution was combined with 0.2 mL of 5-fold diluted FC reagent and mixed for 3 minutes. Next, 0.2 mL of 10% (w/v) sodium carbonate solution was added, and the mixture was left to incubate at room temperature for 30 minutes. Absorbance was measured at 760 nm using a UV-Vis spectrophotometer. The TPC was calculated and expressed as milligrams of gallic acid equivalents per gram of sample (mg GAE/g). All experiments were performed in triplicate.

Chlorophyll content

300 mg of finely cut fresh leaves were taken and macerated with 80% acetone. Then this extract was centrifuged at 3000 rpm. The supernatant was transferred and made up to the volume of 25 ml using 80% acetone. The absorbance was measured at 645 and 663 nm using a spectrophotometer. The chlorophyll content of the samples was calculated using the formula mentioned in (Yoshida *et al*., 1971) and expressed as mg g-1 of fresh leaf.

Chlorophyll a = [12.7(A663) – 2.69 (A645)] *V/1000*W
Chlorophyll b = [22.9(A645) – 4.68 (A663)] *V/1000*W
Total Chlorophyll = [20.2 (A645) + 8.02(A663)] *V/1000*W
Chlorophyll a b ratio = Chlorophyll a / Chlorophyll b
Where:
A = Absorbance of specific wavelength
V = Final volume of Chlorophyll extract in 80% Acetone
W = Weight of leaf sample in gram.

Total flavonoid content

Total flavonoid content (TFC) in each extract was determined using a modified aluminum chloride colorimetric method based on Do *et al.*, (2014). An amount of 250 mg of the extract was mixed with 2.0 mL of 25% hydrochloric acid, 1.0 mL of 0.5% (w/v) hexamethylenetetramine (HMT), and 20 mL of acetone in an Erlenmeyer flask.

The mixture was shaken and subjected to reflux at 90 °C for 30 minutes. After cooling, a small volume of pro analysis-grade acetone was added, and the solution was filtered into a volumetric flask. From the filtrate, 25 mL was transferred into a separatory funnel, combined with 20 mL of distilled water and 15 mL of ethyl acetate. The resulting ethyl acetate layer was collected in a volumetric flask. Then, 1 mL of this fraction was mixed with 1 mL of 2% aluminum chloride solution and diluted to 25 mL using 5% (v/v) glacial acetic acid. The total flavonoid content was quantified by measuring absorbance at 426 nm using a UV-Vis spectrophotometer. Results were expressed as milligrams of quercetin equivalent per gram of sample (mg QE/g). All analyses were conducted in triplicate.

Results

Variation in morphological characteristics among three distinct accessions of Clitoria ternatea

The measurements of leaf and flower dimensions—specifically leaf length, leaf width, flower length, and flower width—for three *Clitoria ternatea* accessions is presented in Table 2. Accession CTE_013 recorded the longest leaf length at 4.25 cm, followed by CTE_006 at 3.85 cm, and CTE_002 at 2.90 cm. Similarly, CTE_013 exhibited the widest leaves at 3.20 cm, whereas CTE_002 had the narrowest, measuring 1.50 cm.

Table 2. Variability in leaf and flower morphology across three distinct accessions of *Clitoria ternatea*

Accessions	Leaf length (cm)	Leaf width (cm)	Flower length (cm)	Flower width (cm)
CTE_002	2.90	1.50	5.05	4.40
CTE_006	3.85	2.75	4.35	3.05
CTE_013	4.25	3.20	4.15	2.25
Mean	3.67	2.48	4.52	3.30
SE.d	0.40	0.51	0.27	0.58
CD (p=0.05)	0.023	0.042	0.621	0.404
	s	s	ns	s

Noted: s=significant, ns=non-significant

Data on pod length, pod width, pod weight, and seed weight for three mature accessions of *Clitoria ternatea* presented in Table 3. Accession CTE_002 exhibited the longest pods at 9.85 cm, followed by CTE_006 and CTE_013, with lengths of 8.60 cm and 8.30 cm, respectively. Pod width showed minimal variation among accessions, with

CTE_002 and CTE_013 both measuring 0.90 cm, while CTE_006 recorded a width of 1.30 cm. In terms of pod weight, CTE_013 had the highest at 0.86 g, followed by CTE_006 at 0.67 g, and CTE_002 at 0.43 g. A similar trend was observed for seed weight, where CTE_013 had the greatest value at 0.72 g, whereas CTE_002 had the lowest at 0.32 g.

Table 3. Variability in pod and seed morphology across three distinct accessions of *Clitoria ternatea*

Accessions	Pod length (cm)	Pod width (cm)	Pod weight (g)	Seed weight (g)
CTE_002	9.85	0.90	0.67	0.56
CTE_006	8.60	1.30	0.86	0.76
CTE_013	8.30	0.90	0.43	0.32
Mean	8.92	1.03	0.65	0.53
SE.d	0.47	0.13	0.12	0.12
CD (p=0.05)	0.048	0.075	0.024	0.039
	s	ns	s	s

Noted: s=significant, ns=non-significant

Chlorophyll content

The chlorophyll a, chlorophyll b, and total chlorophyll contents of three different *Clitoria ternatea* accessions are shown in Table 4. The three accessions were observed to contain the highest chlorophyll-A in accession CTE_002 (double petals) at 1.72 mg/g, followed by accession CTE_013 (single petal-light purple) and accession CTE_006 (single petal-light blue) each at 1.43 mg/g and 0.82 mg/g. Meanwhile, for chlorophyll b, the highest concentration was also found in accession CTE_002 (double petal, dark blue) at 0.80 mg/g, while the lowest was recorded in accession CTE_013 (single petal, light purple) at 0.55 mg/g.

Table 4. Chlorophyll-a, chlorophyll-b, total chlorophyll pigment quantity of 3-different accessions of *Clitoria ternatea*

Accessions	Chlorophyll a (mg/g tissue)	Chlorophyll b (mg/g tissue)	Total Chlorophyll (mg/g tissue)
CTE_002	1.72	0.80	2.67
CTE_006	0.82	0.65	2.34
CTE_013	1.43	0.55	2.23
Mean	1.32	0.67	2.41
SE.d	0.03	0.01	0.04
CD (p=0.05)	0.06	0.03	0.11
	s	s	s

Noted: s=significant, ns=non-significant

Total penolic content, total flavonoid, and total anthocyanin content

The total phenolic, flavonoid, and anthocyanin contents of three different *Clitoria ternatea* accessions are shown in Table 5. Among the three, accession CTE_002 (double petals, dark blue) showed the highest total phenolic content of 40.82±1.40 mg GAE/g DW, followed by CTE_006 (single petals, light blue) with 37.73±0.23 mg GAE/g DW and CTE_013 (single petals, light purple) with 33.38±0.37 mg GAE/g DW. Meanwhile, the highest total flavonoid content was found in accession CTE_002 (double petals, dark blue) at 4.76±0.72 mg QE/g DW, while the lowest was recorded in accession CTE_013 (single petals, light purple) at 1.45±0.17 mg QE/g DW. In addition to showing the highest total phenolic and total flavonoid content, accession CTE_002 (double petals, dark blue) also showed the highest total anthocyanin content of 56.76±1.32 mg/l, followed by CTE_006 (single petals, light blue) of 35.21±0.56 mg/l, and CTE_013 (single petals, light purple) of 12.38±0.33 mg/l.

Table 5. Total anthocyanin content, total phenolic content, and total flavonoid content in flowers of 3-different accession of *Clitoria ternatea*

Accessions	Total anthocyanin content (mg/l)	Total phenolic content (mg GAE/g DW)	Total flavonoid (mg QE/g DW)
CTE_002	56.76±1.32	40.82±1.40	4.76±0.72
CTE_006	35.21±0.56	37.73±0.23	3.26±0.52
CTE_013	12.38±0.33	33.38±0.37	1.45±0.17

Discussion

These agro morphology observations indicate the phenotypic diversity of Clitoria ternatea flowers between accessions in Bengkulu Province. This is to the research results of Suarna and Wijaya (2021) and Artiyani *et al*. (2023), except that the butterfly pea plants obtained in this study only had blue and purple flowers, even though some were white. Clitoria ternatea is a climbing monocot plant belonging to the Fabaceae family and is relatively resistant to abiotic stress, especially drought stress (Jamil *et al*., 2018; Oguis *et al*., 2019). The observed characters all influenced the diversity between accessions. The highest leaf length and width were found in the accession CTE_013 (light blue-single petal) at 4.25 cm and 3.20 cm, respectively. This aligns with the research results from Turos and Baladjay, 2021, regarding the Variety Characterization and Diversity Analysis of *Clitoria ternate* Blue. The leaf size in the lowlands is shorter and broader than in the plains and is longer and narrower in height. Results research by Muslim and Subositi (2020) revealed that the plant's leaves were sitting or *Desmodium triquetrum grown* in the Lowlands have higher air temperatures. Hence, it influences the

balance of plant hormones such as auxin, cytokinins, and gibberellins, which cause plant metabolic activity to increase, thereby supporting leaf growth and ultimately increasing the number and weight of leaves. Apart from environmental factors, the morphology of a plant is also influenced by genetic factors. According to Sufardi (2020) and Wang *et al.* (2019), factors in Genetics can influence the growth of plants, namely in the physiological processes that occur ultimately can influence the morphology of plants.

Meanwhile, the highest flower length and width were found in the accession CTE_002 (dark blue-double petal) at 5.05 cm and 4.40 cm. Reported by Karnati and Rao (2010), who stated that the length of the flower and the width of the flower will affect the weight of the flower because it is possible that the wider the flower area, the heavier the weight of the flower; this is due to the surface area of the flower, thickness and high water content. The size of pods butterfly peas in the lowlands is shorter and somewhat flatter, whereas, in the highlands, it is longer and denser or contains. The size of butterfly pea flower seeds in the plains highlands is more significant than in the lowlands. The research results of Qadry *et al.* (2017) show that the altitude of the planting location influences quality. The physical properties of Gayo Arabica coffee are that the higher the location planting, the better the size of the coffee beans will be. In line with the research results Supriadi *et al.* (2016) that quality improvement the physical characteristics of Arabica coffee are regular beans and weight 100 seeds as height increases planting place.

Determination of total flavonoid levels using the addition of $AlCl_3$ reagent can form a complex, so there is a shift in wavelength towards the visible, and the levels can be measured spectrophotometrically. Quercetin was chosen as a standard solution because quercetin is the most widely distributed compound in plants. Environmental factors such as sunlight intensity, temperature, humidity, soil pH, and altitude can affect plant growth and development and the total flavonoid content in plants (Sholekah, 2017). The study by Hadiyanti *et al*., (2018) shows that altitude affects the total flavonoid content of Physalis spp, where the flavonoid content in the lowlands is higher than in the highlands. Meanwhile, the soil's acidity can also affect the content of secondary metabolites and flavonoids in plants. Soil in the lowlands is included in the normal category compared to the highlands. Soil in lowlands with slightly acidic pH (pH 5.8) has the highest flavonoid content compared to other plains (Hadiyanti *et al.*, 2018; Safrina and Priyambodo, 2018; Yuliani *et al.*, 2019).

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Conflicts of interest

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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