Metana: Media Komunikasi Rekayasa Proses dan Teknologi Tepat Guna ISSN: 1858-2907
Juni 2026 Vol. 22(1):1-8 EISSN: 2549-9130

Acidified Ethanol Improves Extraction Yield while Modulating Antioxidant Activity in Butterfly Pea Extracts

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Abstract

Butterfly pea (*Clitoria ternatea* L.) flower is rich in bioactive compounds, particularly flavonoids and anthocyanins, which contribute to its antioxidant properties. This study evaluated the effect of ethanol and ethanol-citric acid extraction on extract yield, phytochemical composition, and antioxidant activity. Results showed that ethanol-citric acid extraction increased the yield from 30.14% to 44.58% but reduced the extract pH from 4.58 to 2.25. Phytochemical screening confirmed the presence of flavonoids, saponins, tannins, and triterpenoids in both extracts. Antioxidant activity analysis using DPPH revealed that ethanol extraction exhibited stronger antioxidant activity (IC_{50} = 98.74 ppm) than ethanol-citric acid extraction (IC_{50} = 146.46 ppm). These findings suggest that citric acid enhances extraction yield but may reduce antioxidant stability. Further research is needed to assess the impact of extraction conditions on the functional properties of butterfly pea flower extract.

Keywords: *Clitoria ternatea* L., phytochemical screening, antioxidant activity

INTRODUCTION

Butterfly pea (*Clitoria ternatea* L.) is a medicinal plant widely recognized for its vibrant blue flowers, which are rich in bioactive compounds, particularly anthocyanins and flavonoids. These compounds have been extensively studied for their strong antioxidant, anti-inflammatory, and neuroprotective properties, making butterfly pea flower extracts valuable for various applications in the food, pharmaceutical, and cosmetic industries (Rachma *et al.*, 2024). The extraction method used plays a crucial role in determining the yield and stability of these bioactive compounds, as solvent selection and processing conditions directly influence the composition and functionality of the extract (Cahyaningsih *et al.*, 2019). Therefore, investigating different extraction approaches is essential to understanding their effects on the chemical characteristics and biological activity of butterfly pea flower extracts.

Ethanol is widely used as an extraction solvent due to its effectiveness in isolating flavonoids and anthocyanins while being considered safe for food and pharmaceutical applications (Kusumanti *et al.*, 2023). However, Suseno *et al.* (2021) suggested that the addition of organic acids, such as citric acid, can enhance anthocyanin stability and improve extraction efficiency. Citric acid lowers the pH, stabilizing anthocyanins in their flavylium cation form and preventing their degradation (Pramitasari & Lim, 2022). While ethanol extraction has been well-documented, studies evaluating the effect of citric acid addition on butterfly pea flower extraction remain limited. Furthermore, the impact of this modification on the phytochemical profile and antioxidant activity of the extract has not been comprehensively explored.

Several studies have reported variations in extraction yield and bioactive compound stability based on solvent composition. For instance, Pramitasari & Lim (2022) found that the acidic environment created by citric acid improved anthocyanin retention in hibiscus flowers, yet its effect on flavonoids and other secondary

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DOI : 10.14710/metana.v22i1.71477
Diterima: 04-03-2025
Disetujui: 15-04-2026

metabolites in butterfly pea flowers remains unclear. Additionally, Andriani & Murtisiwi (2020) observed that solvent modifications influenced antioxidant activity in plant extracts, but the relationship between solvent acidity and antioxidant potential in butterfly pea extracts has not been fully established. Although ethanol-citric acid extraction has been explored for other plant-based extractions, a systematic comparison with ethanol extraction for butterfly pea flowers is lacking, leaving a gap in knowledge regarding its efficiency and effectiveness.

This study investigates the influence of ethanol and ethanol-citric acid extraction on the yield, phytochemical composition, and antioxidant activity of butterfly pea flower extracts. By comparing these extraction methods, this research aims to determine whether citric acid affects the stability and bioactivity of the extracted compounds. The findings will contribute to a better understanding of extraction techniques for butterfly pea flowers, supporting their potential applications in functional food and pharmaceutical industries while advancing sustainable extraction practices.

METHODS

The main material used in this study was butterfly pea flower (*Clitoria ternatea* L.) with a moisture content of 5.86±0.07% obtained from Serang, Banten. The chemicals used included technical grade ethanol (96%), citric acid, hydrochloric acid (HCl), sulfuric acid (H_2SO_4), acetic acid (CH_3COOH), Mayer's reagent, Magnesium powder, ferric chloride ($FeCl_3$), TLC silica gel 60 PF254 plate, ethanol, methanol, *n*-butanol, DPPH (2,2-diphenyl-1-picryhydrazyl), and vitamin C.

The extraction stages followed the procedures outlined by Dante *et al.* (2023) and Dewantoro *et al.* (2022) with slight modification to the solvents used. Chopped butterfly pea flowers were soaked in 96% ethanol and a combination of 96% ethanol with 3% citric acid at a solid-to-solvent ratio of 1:10 (w/v) for 24 h. After maceration, the extracts were filtered to remove solid residues, then concentrated on a rotary evaporator at 50°C for 30 min to obtain crude extracts. The extraction yield, residual solvent, and pH of the extracts were then determined, with each measurement performed in triplicate.

This assay was conducted to identify the secondary metabolite compound groups present in the test samples. The testing procedure followed the methods described by Cahyaningsih *et al.* (2019) and Azzahra *et al.* (2023) as detailed in Table 1.

Table 1. Phytochemical screening procedures.

Secondary metabolites	Reagents test and procedure	Indicators
Alkaloid	A 2 mL sample (a mixture of 0.5 g extract dissolved in 5 mL HCl) was prepared and reacted with 2 drop of Mayer's reagent.	A precipitate appears, or the solution becomes more turbid.
Flavonoid	A 2 mL sample (a mixture of 0.2 g extract, 0.1 g Mg powder, and 5 mL ethanol) was added with 1 mL HCl and then vigorously shaken.	The solution changes color to red, yellow, or orange.
Saponin	A mixture of 0.5 g extract with 2 drops of HCl and 10 mL of distilled water was shaken for 60 s.	The foam and bubbles formed remain stable for ±7 min.
Steroid and Triterpenoid	A mixture of 0.1 g extract with 10 drops of CH_3COOH and 3 drops of H_2SO_4.	A green color change indicates the presence of steroids, while a red color change indicates triterpenoids.
Tannin	A mixture of 0.2 g extract with 2 drops of $FeCl_3$ and 10 mL of ethanol.	A color change to dark blue or greenish-black.

Each crude extract sample was dissolved in ethanol and the spotted onto a TLC silica gel 60 PF254 plate (9×5 cm), following the procedure of Syarifah *et al.* (2019). The TLC plate was placed in a chromatography chamber pre-saturated with a mobile phase of *n*-butanol/CH_3COOH/distilled water (4:1:5). After development, the TLC plate was removed from the chamber and air-dried before observing the bands under a UV lamp at 254 nm. The retention factor (Rf) was calculated by dividing the distance traveled by the band by the total distance of the elution.

The antioxidant activity was determined using the DPPH scavenging assay, following the procedures of Andriani & Murtisiwi (2020) and Kusumanti *et al.* (2023). A 100 ppm DPPH solution was used as the stock solution and blank in the measurements, while vitamin C (2–10 ppm) was used as the control. Each BP flower extract (at 50–250 ppm) was taken at a volume of 2 mL and reacted with a 40 ppm DPPH solution at a ratio of 1:1 (v/v). The mixture was homogenized and incubated at room temperature for 30 min before being measured using a UV-Vis spectrophotometer at a wavelength of 517 nm. The inhibition values of the control and extract samples were then expressed as IC_{50}. All sample measurements were performed in triplicate.

RESULTS AND DISCUSSION

The addition of citric acid in the extraction process of butterfly pea flowers influenced the yield of the extract obtained. Figure 1 shows the color differences between the extracts obtained using ethanol and the ethanol-citric acid combination, which were blue and red, respectively. Furthermore, the extraction yield increased from 30.14±0.04% with ethanol to 44.58±0.06% with the ethanol-citric acid combination. Suseno *et al.* (2021) explained that citric acid enhances extraction yield due to its ability to denature cells, thereby facilitating the polar solvent, which enhances the solubility and stability of active compounds such as anthocyanins and flavonoids.

Table 2 presents the characterization results of butterfly pea flower extract, influencing its quality parameters such as residual solvent and pH. According to BPOM RI (2005), the residual solvent in an extract product should be lower than 1% to ensure and maintain its quality. The study showed that the residual solvent content of ethanol extraction and ethanol-citric acid combination extraction were 0.67±0.03% and 0.65±0.02%, respectively. These findings indicate that the addition of citric acid did not affect the residual solvent, thus maintaining the extraction quality even without citric acid addition. However, the pH of the extract decreased from 4.58±0.02 to 2.25±0.05 due ti the presence of citric acid combined with ethanol. This phenomenon occurred due to the naturally acidic nature of citric acid, which lowered the pH of the butterfly pea flower extract (Naimi *et al.*, 2024).

Table 2. Characteristics of butterfly pea flowers extract.

Solvent Extraction	Yield	Residual Solvent	pH
Ethanol	30.14±0.04%	0.67±0.03%	4.58±0.02
Ethanol–Citric acid	44.58±0.06%	0.65±0.02%	2.25±0.05

Figure 1. Visual appearance of butterfly flower extract using ethanol (blue color) and a combination of ethanol-citric acid (red color).

The results of the qualitative analysis in the phytochemical screening of fresh materials and extracts are presented in Table 3. Alkaloids, which are found in some medicinal plants due to their pharmacological activities such as analgesic and anticancer effects, were not detected in the butterfly pea flower or its extract. This finding is consistent with the results reported by Cahyaningsih *et al.* (2019), which also showed the absence of alkaloids in the butterfly pea flower used in their study. However, the absence of alkaloids in the butterfly pea flower does not negate its pharmacological activities, as the flower and its extract contain flavonoids (Rachma *et al.*, 2024).

Table 3. Phytochemical screening results of fresh materials and the extracts of butterfly pea flowers.

Phytochemical compounds	Fresh materials	Ethanol extracts	Ethanol–Citric acid extracts
Alkaloid	 no precipitate (-)	 no precipitate (-)	 no precipitate (-)
Flavonoid	 deep red color changes (+)	 deep red color changes (+)	 deep red color changes (+)
Saponin	 stable foam for 7 mins (++)	 stable foam for 7 mins (++)	 stable foam for 7 mins (++)
Triterpenoid	 deep red color changes (+)	 deep red color changes (+)	 deep red color changes (+)
Tannin	 deep blue color changes (+)	 greenish color changes (+)	 deep purple color changes (+)

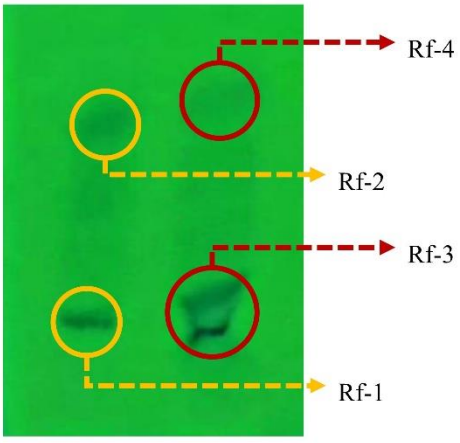
Flavonoids were detected with positive results, indicated by the color change of the solution to deep red. Flavonoids are one of the main compounds in butterfly pea flowers, known for their roles in antioxidant and anti-inflammatory activities (Dewantoro *et al.*, 2022). The presence of these flavonoids serves to protect cells from oxidative damage (Speisky *et al.*, 2022). Another compound, saponin, was detected in the fresh materials and both types of extracts with positive intensity. This presence demonstrates the consistent occurrence of saponins in butterfly pea flowers and their extracts. Identified triterpenoids contribute to anti-inflammatory and hepatoprotective activities while supporting antioxidant effects (Yin *et al.*, 2019). Triterpenoids are frequently found in flowering plants as part of their defense mechanism against environmental stress.

Tannin were also detected through the color change of the solution to deep blue. Tannins are known for their astringent and antioxidant properties (Pratama *et al.*, 2019). Their presence in butterfly pea flower contributes to its protective effects against oxidative stress and provides antimicrobial protection. Kováč *et al.* (2023) stated tannins are often used in herbal products due to their protein-binding abilities, which help reduce infections and inflammation.

The extraction samples tested positive for flavonoids, indicated by the appearance yellow-brown spots under visible light. Under UV 366 nm, blue spots appeared against a dark plate, while under 254 nm, the dark spots were observed on a green plate background (Rizkita *et al.*, 2023). Figure 2 shows a green TLC plate with dark spots. The consistent spot patterns in the extracts suggest the presence of flavonoids (Rizkita *et al.*, 2023). The distance and Rf values of the spots showed minimal variation. Similar Rf values, such as for spot 1 and spot 2, indicate that the flavonoids detected in butterfly pea flower extract remain present. This suggests the potential stability of the active components within the extract.

Figure 3 shows the linear regression curves of the concentration of vitamin C and the two butterfly pea flower extracts against their antioxidant inhibition. Vitamin C, as a control, demonstrated excellent antioxidant activity, achieving 10.21% inhibition at a low concentration of 2 ppm. This confirms the high antioxidant potency of vitamin C even at low concentrations. Meanwhile, the ethanol extract of butterfly pea flower showed significant inhibition at 50 ppm, reaching 31.37%, indicating its antioxidant capacity, although weaker than that of vitamin C. The addition of citric acid in the extraction solvent affected the stability and interactions of active compounds, leading to reduced antioxidant activity. This is evidenced by the fact that the ethanol-citric acid extract exhibited the lowest inhibition percentage compared to the ethanol extract and vitamin C.

The linear regression curves in Figure 3 were used to determine the IC_{50} values of the tested samples. According to Table 4, vitamin C has an IC_{50} value of 13.51±0.16 ppm, categorized as highly strong activity. 'Aisy *et al.* (2022) explained a low IC_{50} value indicates a high antioxidant capacity to scavenge free radicals. The ethanol extract of butterfly pea flower has an IC_{50} value of 98.74±0.07 ppm, indicating strong antioxidant activity.



Annotated Rf	Corresponding bands	Rf calculated
Ethanol extracts		
Rf-1	1.10	0.22
Rf-2	2.30	0.46
Ethanol–Citric acid extracts		
Rf-3	0.90	0.18
Rf-4	2.50	0.50

Figure 2. TLC chromatograms of flavonoids on extract results as antioxidant agents.

This suggests that the butterfly pea flower extract has good antioxidant potential, although it is not as potent as vitamin C. However, the addition of citric acid did not enhance the antioxidant performance of the butterfly pea flower, as the IC_{50} value decreased to 146.46±0.06 ppm. This phenomenon occurred because citric acid affects the stability of anthocyanin color under acidic conditions, leading to the protonation of the flavylium cation and a subsequent decrease in antioxidant capacity (Pramitasari & Lim, 2022).

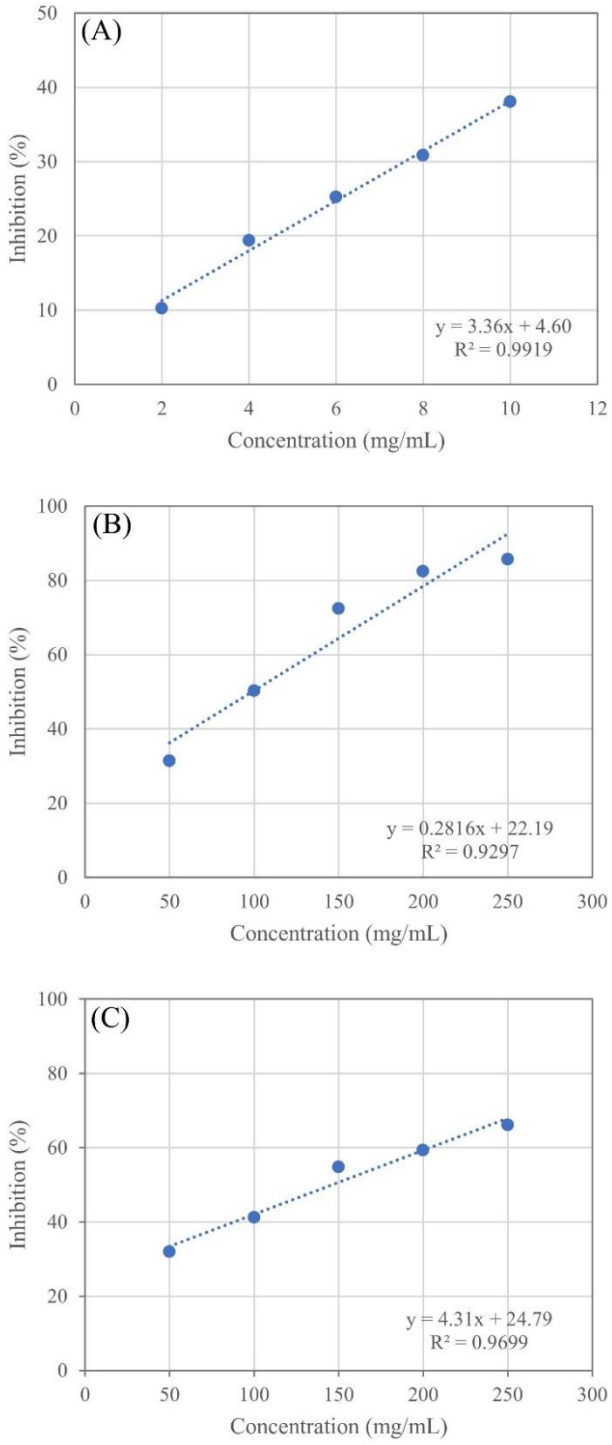


Figure 3. Sample concentration-to-antioxidant inhibition plot: (A) Vitamin C; (B) Ethanol extracts; and (C) Ethanol-Citric acid extracts.

Table 4. Antioxidant activity of Clitoria ternatea extracts.

Samples	IC_{50} value	Activities category
Ethanol extracts	98.74±0.07 ppm	Strong
Ethanol–Citric acid extracts	146.46±0.06 ppm	Moderate
Vitamin C	13.51±0.16 ppm	Highly strong

Anthocyanins, which are in the flavonoid class of phenolic compounds, are widely recognized for their high antioxidant activity due to their capacity to donate hydrogen atoms or electrons and stabilize free radicals through resonance within their aromatic structure (Patra *et al.*, 2022). These compounds contribute greatly to the total antioxidant activity of butterfly pea extract, since their hydroxyl groups contribute to radical scavenging processes and metal ion chelation. This occurs because citric acid alters the stability of anthocyanins under acidic environments, causing the flavylium cation form to dominate (Kilel *et al.* (2019) and Zaidan *et al.,* 2024). Although this protonated form is responsible for the characteristic red coloration, the high acidity can disrupt the structural equilibrium of anthocyanins, thereby limiting their capacity to operate as efficient radical scavengers (Teixera *et al.*, 2023). Consequently, the presence of citric acid may enhance extraction yield while simultaneously modifying anthocyanin structure and lowering the measured antioxidant capacity.

CONCLUSION

This study demonstrated that ethanol-citric acid extraction increased the yield of butterfly pea (*Clitoria ternatea* L.) flower extract from 30.14±0.04% to 44.58±0.06%, while maintaining similar residual solvent content. However, it significantly reduced extract pH from 4.58 ± 0.02 to 2.25 ± 0.05. Phytochemical screening confirmed the presence of flavonoids, saponins, tannins, and triterpenoids in both extracts. Antioxidant activity analysis revealed that ethanol extraction had a stronger effect (IC_{50} = 98.74±0.07 ppm) than ethanol-citric acid extraction (IC_{50} = 146.46±0.06 ppm), indicating possible alterations in compound stability. These findings suggest that citric acid improves extraction yield but may reduce antioxidant capacity, warranting further research on its impact on bioactive compound stability and functional applications.

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