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Effect of Butterfly Pea (*Clitoria ternatea*) Flower Fraction on the Total Leukocyte Count of Common Carp (*Cyprinus carpio*)

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Abstract

Intensive common carp (*Cyprinus carpio*) farming is prone to bacterial infections such as Aeromonas hydrophila, and while antibiotics are effective, their overuse raises concerns about resistance and environmental impact. This study aimed to identify the fractions of butterfly pea flower (*Clitoria ternatea*) extract through phytochemical tests, UV-Vis, and FTIR spectrophotometry, and evaluate their immunostimulant potential by assessing total leukocyte counts in common carp (*C. carpio*). The research was conducted from December 2024 to February 2025 at the Fish Disease and Health Laboratory, Brawijaya University. Fifty Majalaya strain common carp were divided into treatment and control groups, receiving 25 ppm extract fractions. Extraction was performed using ethanol, ethyl acetate, and n-hexane, followed by fractionation via thin-layer chromatography (TLC). Leukocyte count analysis was conducted from day 0 to day 3 using microscopy. Results showed that 96% yielded the highest extract recovery (22.16%), while ethyl acetate contained the best bioactive compounds. The optimal TLC eluent was chloroform: methanol (9:1), producing distinct light green and pink spots. Fraction 5 showed the highest leukocyte increase from day 0 (0.73×10^4 cells/mm³) to day 2 (0.29×10^4 cells/mm³) and the lowest decrease on day 3 (0.13×10^4 cells/mm³). UV-Vis spectrophotometry revealed a peak at 659 nm, indicating the presence of phenolics, flavonoids, and alkaloids. FTIR spectroscopy confirmed the presence of key functional groups, including O-H, C-H, C≡N, C=O, and C=C. These findings suggest fractionated butterfly pea flower extract as a promising natural immunostimulant for sustainable aquaculture.

INTRODUCTION

Intensive aquaculture of common carp (*Cyprinus carpio*) faces challenges from bacterial infections, particularly those caused by *Aeromonas hydrophila*, the

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bacterium responsible for Motile Aeromonas Septicemia (MAS). Infected fish show symptoms like anorexia, skin ulceration, pale gills, abdominal swelling, abnormal swimming, and fin damage (Kari *et al*., 2022; Debnath *et al*., 2024). Although antibiotics are commonly used for disease control due to their effectiveness and ease, their prolonged use raises concerns about bacterial resistance and environmental contamination. Therefore, alternative strategies, such as immunostimulants, are being explored to enhance fish health without these drawbacks (Lulijwa *et al*., 2020; Barathan *et al*., 2024).

Immunostimulants are substances or actions that enhance the nonspecific immune response in fish. One commonly used immunostimulant in aquaculture is beta-glucan, typically sourced from yeast (*Saccharomyces cerevisiae*). As a Pathogen-Associated Molecular Pattern (PAMP), beta-glucan is readily recognized by immune and intestinal cells, triggering an immune response when administered through feed. However, prolonged use of beta-glucan may reduce fish immunity by impacting the expression of genes related to macrophage production and antibacterial activity. (Cornet *et al*., 2021; Thépot *et al*., 2021).

The butterfly pea (*Clitoria ternatea*) flower, a plant abundant in Indonesia, represents a promising alternative source of immunostimulants. It contains various bioactive compounds, including tannins, saponins, terpenoids, phenols, flavonoids, alkaloids, and steroids, which exhibit pharmacological activities such as antioxidant effects that neutralize free radicals and enhance phagocytic activity in fish (Kong *et al*., 2022). Additionally, *C. ternatea* compounds have demonstrated antibacterial activity against pathogens such as *Pseudomonas aeruginosa*, *A. hydrophila*, *A. formicans*, *Streptococcus agalactiae*, *Escherichia coli*, and *Bacillus subtilis*. Furthermore, studies have shown the immunomodulatory effects of *C. ternatea* in mammals, particularly its ability to influence immune cell populations and enhance phagocytic function (Rashid *et al*., 2021; Safhi *et al*., 2022).

Despite the potential of *C. ternatea* for fish disease management, their application has been limited to crude extracts. Although *C. ternatea* shows potential for fish disease management, its application has so far been limited to crude extracts. According to a previous study by Andriani *et al*. (2020), leaf extract of *C. ternatea* can be used to treat koi fish (*C. carpio*) infected with Aeromonas hydrophila, exhibiting symptoms such as hemorrhage, pale coloration, and ulceration, at a concentration of 300 ppm, with a rapid recovery period of nine days. However, no studies have explored the use of *C. ternatea* flowers for disease prevention, particularly in *C. carpio*, a species highly susceptible to pathogens. This is noteworthy considering that the flower contains numerous bioactive compounds that may play a role in disease prevention. To maximize its effectiveness, it is essential to identify and characterize the active fractions of butterfly pea flowers to evaluate their potential in enhancing fish immunity. The leukocyte count in blood serves as a key parameter for assessing immune response, as leukocytes are crucial for pathogen defense, inflammation regulation, and the facilitation of adaptive immunity. Monitoring leukocyte activity provides critical insights into fish health and their ability to respond to environmental stressors.

According to Pratiwi (2022), various parts of the butterfly pea (*C. ternatea*), including leaves, flowers, and seeds, contain nutrients such as protein, lipids, fiber, and carbohydrates, with seeds having the highest protein content (40.59%). These components have been used as feed ingredients in tilapia (*Oreochromis niloticus*), African catfish (*Clarias gariepinus*), and swordtail fish (*Xiphophorus helleri*) without negatively affecting growth, highlighting their potential as alternative protein sources in aquaculture. Building on this potential, this study aims to identify the fractions of *C. ternatea* flower extract through phytochemical tests and UV-Vis and FTIR spectrophotometry. Additionally, it seeks to

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evaluate its potential as an immunostimulant by assessing total leukocyte counts in common carp (*C. carpio*) in vivo. The findings are expected to provide insights into the bioactive compounds in *C. ternatea* flower extract fractions and their potential as a safer and more effective alternative immunostimulant for sustainable aquaculture practices.

METHODOLOGY

Ethical Approval

All experimental animals were handled ethically and with care throughout the study. Proper procedures were followed to ensure optimal environmental conditions, high water quality, and adequate feed availability, with no harm or mistreatment inflicted on the fish.

Place and Time

This study was conducted from December 2024 to February 2025 at the Fish Disease and Health Laboratory, Faculty of Fisheries and Marine Sciences, Brawijaya University.

Research Materials

This study uses equipments such as an evaporator (RV 10, IKA, Germany), chamber (Daihan Scientific, South Korea), column tube (Pyrex®, USA), vial bottles (Mulia Indah Packaging, Indonesia), rearing containers (SHINPO, Indonesia), syringe 1 cc (Terumo, Japan), EDTA tube (OneMed, Indonesia), toma leukocyte pipette (Hecht Assistent, Germany), haemocytometer (Marienfeld, Germany), microscope (CX33, Olympus Company, Beijing), UV-Vis spectrophotometer (UV-1900i, Shimadzu Corporation, Japan), FTIR spectrophotometer (IRSpirit, Shimadzu Corporation, Japan), test tube (Pyrex®, USA), hotplate stirrer (C-MAG HS7, IKA, Germany), and micropipet (Eppendorf Research Plus, Eppendorf SE, Germany).

This study uses the main materials in the form of common carp (*C. carpio*) Majalaya strain measuring 10-12 cm with a body weight of 25-40 g obtained from fish farmers in Canggu Village, Pare District, Kediri City, East Java and fresh butterfly pea flowers (*C. ternatea*) were obtained from the North Maluku Agricultural Instrument Standards Implementation Center. Also, this study uses supporting materials such as an ethanol 96% (Merck & Co, Germany), ethyl acetate (Merck & Co, Germany), n-hexane (Merck & Co, Germany), TLC plate silica gel 60 F254 (Merck & Co, Germany), filter paper (Whatman 40, Cytiva, USA), capillary tube (NESCO, Indonesia), silica gel G60 (Merck & Co, Germany), turk's solution (Indo Reagen, Indonesia), and microtip (OneMed, Indonesia). In addition, other materials used in the biochemical test include aquadest, methanol (Pro Analys, Merck & Co, Germany), $AlCl_3$ (Merck & Co, Germany), magnesium powder (Merck & Co, Germany), HCl (Merck & Co, Germany), $FeCl_3$ (Merck & Co, Germany), Lieberman-Burchad reagent, Mayer reagent, Bouchardat reagent, Dragendorf reagent, chloroform (Merck & Co, Germany), acetic anhydride (Merck & Co, Germany), and H_2SO_4 (Merck & Co, Germany).

Research Design

This study utilized 10 treatment groups, consisting of 9 different butterfly pea (*C. ternatea*) flower fractions at a concentration of 25 ppm and 1 control group. The fractions varied in color and polarity, with fraction 1 exhibiting a brown color, fraction 2 dark brownish-black, fraction 3 dark green, fraction 4 green, and fraction 5 bright green. The subsequent fractions included yellowish-green (fraction 6), yellow (fraction 7), bright yellow (fraction 8), and yellowish-white (fraction 9). These color variations reflect a polarity gradient, where fraction 9, located in the topmost layer, is the most polar, and fraction 1 is the most nonpolar. Each treatment was tested on five common carp (*C. carpio*) that were obtained from fish farmers in Canggu village, Kediri district, East Java, housed in 25-liter rearing containers, and all treatments were conducted in triplicate to ensure data reliability.

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Work Procedure

Extraction of Butterfly Pea Flower (*C. ternatea*)

The extraction and phytochemical analysis methods in this study were adapted from previous research by Ambrin *et al*. (2024) and Rather *et al*. (2023), with slight modifications. A total of 2500 g of fresh butterfly pea flowers were sun-dried to obtain 513 g of dried material. Extraction was performed via maceration using ethanol 96%, ethyl acetate, and n-hexane (1:100 w/v) for three 24-hour cycles. The resulting macerates were concentrated using a rotary evaporator (RV 10, IKA, Germany) at 45°C and 121 rpm to yield crude extracts in paste form. Phytochemical screening (tannins, flavonoids, alkaloids, saponins, steroids, triterpenoids) was conducted to identify active compounds, while yield (Rend) and dry weight (DW) were calculated using standard formulas adapted from Lee *et al*. (2022) in Eqs 1 and 2.

$$\text{DW}\ (\%) = \frac{W_d}{W_w} \text{x}100\% \quad (1)$$

$$\text{Rend}\ (\%) = \frac{W_{RE}}{W_S} \text{x}100\%. \quad (2)$$

In this equation, Wd is the dry weight of the sample (g), Ww is the wet weight of the sample (g), and Ws is the weight of simplicia (g).

Biochemical Test

Fractionation of butterfly pea flower extract was performed using the Thin Layer Chromatography (TLC) method, modified from Raj (2022). A TLC plate (8 × 2 cm) was used as the stationary phase, with boundaries at 0.5 cm (top) and 1 cm (bottom). The eluent chamber was saturated with filter paper for 1 minute. A 10 mg crude extract was dissolved in 1 mL of extraction solvent and spotted onto the plate, which was then placed vertically into the chamber. The eluent was allowed to move upward, and spots were observed under UV light at 254 nm and 366 nm. The Rf value was calculated using the equation adapted from Xu *et al*. (2025) in Eq. 3 to isolate the compounds for column chromatography.

$$\text{Rf} = \frac{\text{the distance traveled by the analyte}}{\text{the distance traveled by the eluent}} \quad (3)$$

Column chromatography was performed following TLC using silica gel G60 as the stationary phase and the optimal eluent from TLC as the mobile phase, based on a modified method from Jolin *et al*. (2021). The column was first plugged with cotton and filled with n-hexane to a height of 20 cm. Silica gel was dissolved in the eluent and added to the column, allowing it to settle for 24 hours. A 15 g crude extract of *C. ternatea* flower was dissolved in 5–10 drops of eluent and loaded onto the column. Elution was conducted using a gradient method, and fractions were collected in vial bottles based on color differences.

Maintenance and Handling of Fish

Common carp (*C. carpio*) measuring 10–12 cm in length were used as test subjects in this study to evaluate the total leukocyte response to the administration of *C. ternatea* flower fraction, which was obtained from farmers in Canggu Village, Pare Subdistrict, Kediri City. Fish were maintained for a total of 10 days, consisting of a 7-day acclimatization period followed by 3 days of *C. ternatea* flower fraction treatment, at a stocking density of one fish per 2 liters of water. Feeding was carried out three times daily at 08.00, 12.00, and 16.00 Western Indonesian Time (WIB), at a rate of 4% of the fish biomass. To maintain optimal water quality, siphoning of the culture medium was performed once daily in the afternoon. Total leukocyte counts were measured by removing the fish from the rearing medium and placing them on a tray with the head covered using a soft cotton cloth to minimize stress during blood sampling. Before blood sampling, the fish's skin surface was aseptically cleaned using 70% alcohol. Blood was then drawn using a 1 cc syringe, with the needle inserted at a 45 degree angle into the lateral line region. After sampling, the fish were returned to the rearing container.

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Total Leukocyte Test in Common Carp (*Cyprinus carpio*) Against Butterfly Pea Flower Fractions

The test was conducted following a modified procedure from Yousefi *et al*. (2020), using five common carp in jars with 10 liters of water. The fish were exposed to 25 ppm fractions for 3 days, and behavioral observations were made. Fish showing signs of distress were transferred to an untreated container. The treatment using *C. ternatea* flower was administered through immersion by dissolving 0.25 mL (equivalent to 250 μL) of the *C. ternatea* flower fraction into 10 liters of water used as the rearing medium. Leukocyte counts were measured from day 0 to day 3 by collecting blood from the linea lateralis using a syringe and storing it in an EDTA tube. The blood was diluted with Turk's solution, discarding the first three drops before placing the sample onto a haemocytometer. Leukocyte counts were observed under a trinocular microscope (CX33, Olympus) at 40x magnification, following Yanuhar *et al*. (2021) method, following the equation in Eq. 4

$$\text{Leukocyte (cell/mm}^3\text{)} = \Sigma n \times 50 \quad (4)$$

In this equation, Σn is the total leukocyte count in four squares, and 50 is the dilution factor.

Analysis of Butterfly Pea Flower (*Clitoria ternatea*) Fraction

The identification of the butterfly pea flower fraction was performed using UV-Vis spectrophotometry with a UV-1900i spectrophotometer (Shimadzu, Japan), analyzing a 3 mL sample within a 200–800 nm wavelength range. Results were interpreted based on the observed maximum wavelength, following a method adapted from Winkler *et al*. (2020). Additionally, FTIR analysis of the *C. ternatea* flower fraction involved mixing 0.5 mg of the fraction with 180 mg of Potassium Bromide (KBr), pressing the mixture at 80 torr for 10 minutes to form a pellet, and analyzing it with an IRSpirit FTIR spectrophotometer (Shimadzu, Japan) in the 4000–400 cm^{-1} range. Peaks were identified to characterize the compound, following a method adapted from Ordoudi *et al*. (2023). Phytochemical testing of *C. ternatea* fraction was performed using a modified method from Shaikh and Patil (2020). For the flavonoid test, 1 gram of extract was boiled with 10 mL of hot distilled water, filtered, and treated with magnesium powder and concentrated HCl. An orange or red color indicated a positive result. Tannins were tested by mixing 0.5 grams of the sample with distilled water and $FeCl_3$, yielding a dark blue or blackish-green color if positive. For saponins, 0.5 grams of the sample were mixed with distilled water, heated, cooled, and shaken with 2N HCl, with persistent foam signaling a positive result. Alkaloids were tested by adding Mayer, Bouchardat, and Dragendorf reagents to a 0.5-gram sample, with precipitate or color change indicating a positive result. Steroids and triterpenoids were identified by dissolving the sample in chloroform, adding acetic anhydride and concentrated H_2SO_4, with a greenish-blue color indicating steroids and a brown or violet color indicating triterpenoids.

Data Analysis

The data from this study were analyzed quantitatively using analysis of variance (ANOVA) with a 95% confidence interval to assess the effect of butterfly pea flower fraction (*C. ternatea*) on the total leukocyte count in common carp (*C. carpio*). Data processing and analysis were conducted using the SPSS software.

RESULTS AND DISCUSSIONS

Butterfly Pea Flower (*C. ternatea*) Extraction

Fresh butterfly pea flowers (2500 g) were sun-dried, resulting in 513 g of dried material with a dry weight of 20.52%. The crude extract, obtained as a paste, was produced using three different solvents. Yield analysis showed that 96% ethanol produced the highest yield at 22.16%, followed by ethyl acetate (10.38%) and n-hexane (7.01%) (Figure 1). The best solvent was determined based on both yield and the

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range of compounds extracted, as identified through phytochemical tests.

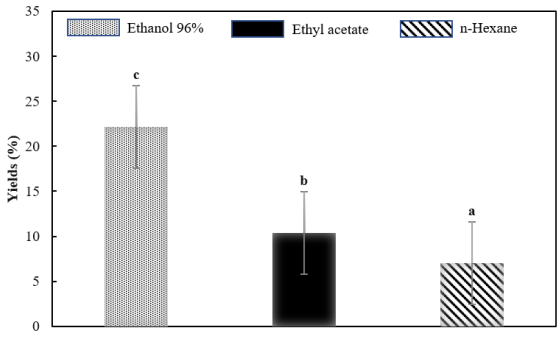

Figure 1. Yield of butterfly pea (*C. ternatea*) flower extraction.

Phytochemical tests showed that ethyl acetate was the most effective solvent, revealing a higher presence of active compounds than other solvents. However, the yield was not directly proportional to the number of active compounds in the butterfly pea flower extract. Flavonoids were soluble in solvents with varying polarities (polar, semipolar, nonpolar), while steroid compounds dissolved only in n-hexane due to their nonpolar nature. Triterpenoids, although semipolar to nonpolar, were best dissolved by ethyl acetate. (Table 1).

Table 1. Phytochemical test results of butterfly pea (*C. ternatea*) flower extract.

Active Compound	Result		
	Ethanol 96%	Ethyl Acetate	n-Hexane
Tannin	+	+	-
Alkaloid	+	+	-
Flavonoid	+	+	+
Saponin	-	+	-
Steroid	-	-	+
Triterpenoid	-	+	-

Description: (+) Detected, (-) Not detected.

The dry weight yield obtained in this study was 20.52%, which is consistent with the findings of Fauzi *et al*. (2022), who reported that the dry weight of *C. ternatea* flowers ranges between 9.44% and 20.55%, influenced by factors like humidity and drying conditions. Water content is crucial as it affects extraction efficiency and serves as a benchmark for comparing materials. Ethanol yields the highest extract due to its ability to dissolve both polar (e.g., flavonoids, alkaloids) and nonpolar compounds. Ethyl acetate yields less, as it is selective for slightly polar compounds like terpenoids and flavonoids. N-hexane yields the lowest, as it effectively dissolves nonpolar compounds but is less efficient for polar ones. (Ismail *et al*., 2020; Ushie *et al*., 2022).

Butterfly Pea Flower (*C. ternatea*) Fractionation

In this study, Thin Layer Chromatography (TLC) was conducted using eluents and spotters selected based on the phytochemical profile of the crude butterfly pea flower extract. Spot formation, observed under UV light at 366 nm (Figure 2a), was used for result interpretation. The chloroform: methanol (9:1) combination with Liebermann-Burchard reagent produced the most distinct spots, indicating optimal separation and strong interaction with the mobile phase, particularly for saponins. This eluent was then applied in column chromatography for fractionation. The movement of spots was measured to calculate Rf (retardation factor) values in

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centimeters (Figure 2b).

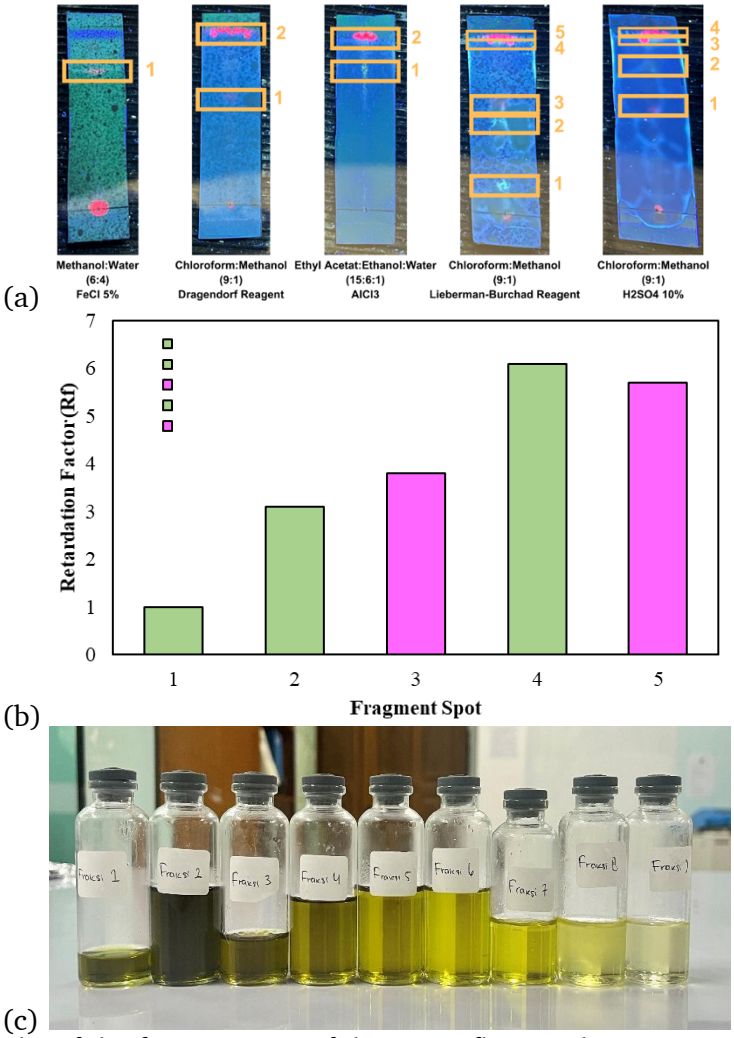

Figure 2. The results of the fractionation of the Butterfly Pea Flower Extract (*C. ternatea*).
Description: (a) thin layer chromatography spots, (b) the best Rf values, (c) column chromatography fractions.

The chloroform: methanol (9:1) eluent, chosen for optimal TLC separation, yielded nine fractions in column chromatography, distinguished by color at a flow rate of 2–3 drops per second (Figure 2c). Fraction 1 was brown, followed by dark brownish-black (2), dark green (3), green (4), bright green (5), yellowish-green (6), yellow (7), bright yellow (8), and yellowish-white (9). The fraction order indicates polarity, with Fraction 9 being the most polar and Fraction 1 the most nonpolar.

Thin Layer Chromatography (TLC) is a widely used method for analysis, isolation, and determination of compounds in column chromatography. Silica plates serve as the stationary phase due to their polarity, while organic solvents act as the mobile phase. The fluorescence bands on the TLC plate, indicated by spot colors, reveal the characteristics of the compounds. In this study, a light green spot, characteristic of flavonoids, and a pink spot, indicative of terpenoids, were observed. (Guggenberger *et al*., 2021; Móricz *et al*., 2020).

Column chromatography separates mixture components based on their solubility in a specific solvent and interaction with the stationary phase. Polar fractions move less with the mobile phase, while less polar fractions in the lower layers travel more easily. The movement of fractions is

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influenced by time, volume, and the polarity of the mobile phase. This technique is commonly used in compound synthesis and isolation, especially in drug development, as shown in this study for isolating fish immunostimulants. (Fan *et al.*, 2021; Susanti *et al.*, 2024).

Characterization of Butterfly Pea Flower Fraction (*C. ternatea*)

The compound analysis of the best butterfly pea flower fraction showed seven absorption bands at 295 nm, 354 nm (3.907 absorbance), 415 nm (3.664 absorbance), 537 nm (3.724 absorbance), 558 nm (0.294 absorbance), 606 nm (0.459 absorbance), and 659 nm (1.746 absorbance) (Figure 3a). FT-IR analysis of the fifth fraction revealed 13 frequency regions corresponding to various bond types. (Figure 3b).

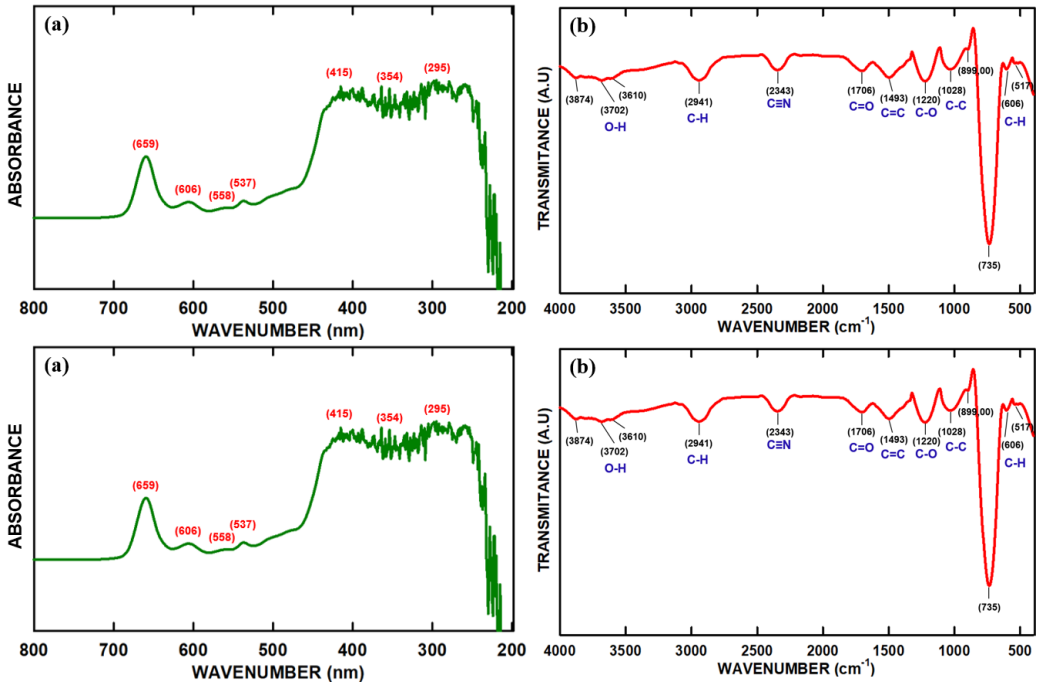

Figure 3. Characterization results of butterfly pea flower fraction (*C. ternatea*).
Description: (a) light absorbance waves, (b) maximum lambda

The identification of active compounds in the best fraction of the butterfly pea flower extract was conducted through qualitative phytochemical testing. The tests for flavonoids, alkaloids, tannins, steroids, triterpenoids, and saponins revealed that the fifth fraction of the butterfly pea flower extract tested positive for alkaloids and triterpenoids, as indicated by a brown color change (Figure 4).

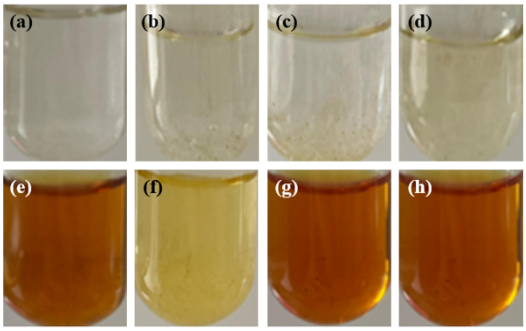

Figure 4. Phytochemical test result.
Description: (a) Saponin, (b) Flavonoid, (c) Alkaloid (Mayer Reagent), (d) Alkaloid (Drafendrof Reagent), (e) Alkaloid (Bouchardat Reagent), (f) Tannin, (g) Triterpenoid, (h) Steroid

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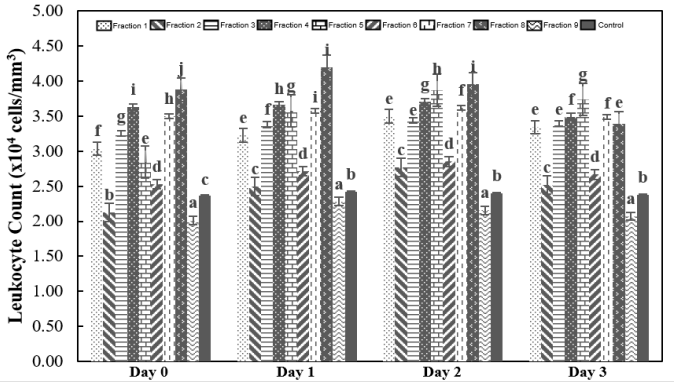
The fifth fraction of the butterfly pea flower extract showed a maximum wavelength at 659 nm, indicating the presence of phenolic compounds, flavonoids, and alkaloids (Figure 3a). This is attributed to the use of methanol: chloroform (9:1) as the mobile phase. Phenolic compounds are polar due to hydroxyl (-OH) groups, enabling them to form hydrogen bonds with polar solvents like methanol. Flavonoids, soluble in both polar solvents (ethanol, methanol) and semi-polar solvents (chloroform), are polyphenolic compounds. Alkaloids, with varying polarity, can dissolve in both polar and semi-polar solvents (Tamayo-Ramos *et al*., 2022; Souza *et al*., 2021). FT-IR spectrophotometric analysis of the fraction revealed characteristic peaks at 3874.62 cm^{-1} and 3702.29 cm^{-1} (O–H stretching of free groups) and 3610.38 cm^{-1} (O–H stretching in amines, alcohols, or phenols). The peak at 2941.15 cm^{-1} indicated C–H stretching from alkyl groups, while 2343.73 cm^{-1} corresponded to C≡N stretching of nitrile compounds. A strong peak at 1706.10 cm^{-1} was attributed to C=O stretching (carboxylates, esters, or ketones), and 1493.55 cm^{-1} reflected C=C stretching in alkenes. Peaks at 1220.69 cm^{-1} indicated C–O stretching, while 1028.25 cm^{-1} and 899.00 cm^{-1} corresponded to C–C vibrations. Lower peaks at 735.29 cm^{-1}, 606.04 cm^{-1}, and 517.00 cm^{-1} were associated with C–H bending of aromatic alkyl compounds. (Angel *et al*., 2024).

Alkaloids and triterpenoids enhance leukocyte levels in common carp (*C. carpio*) by acting as immunostimulants, increasing the phagocytic activity of neutrophils and macrophages, and accelerating leukocyte production and mobilization. They stimulate hematopoiesis in organs such as the kidney and spleen by activating key pathways like Nuclear Factor Kappa B (NF-κB) and Mitogen-Activated Protein Kinase (MAPK), which regulate immune cell proliferation and gene expression. These compounds also upregulate proinflammatory cytokines (IL-1β, IL-6, TNF-α), boosting leukocyte production, while their antioxidant properties help maintain immune homeostasis in fish. (Ghafarifarsani *et al*., 2022; Khanzadeh *et al*., 2023; Mohammadi *et al*., 2020).

Total Leukocyte Test of Common carp (*C. carpio*) on Butterfly Pea Flower Fraction

The total leukocyte test in common carp (*C. carpio*) using the butterfly pea flower fraction was conducted to determine the most effective fraction for increasing leukocyte levels. Additionally, this test aimed to evaluate the duration of effectiveness of the fraction as an immunostimulant in common carp. Therefore, blood samples were collected daily from day 0 to day 3 (Figure 5).

(a)



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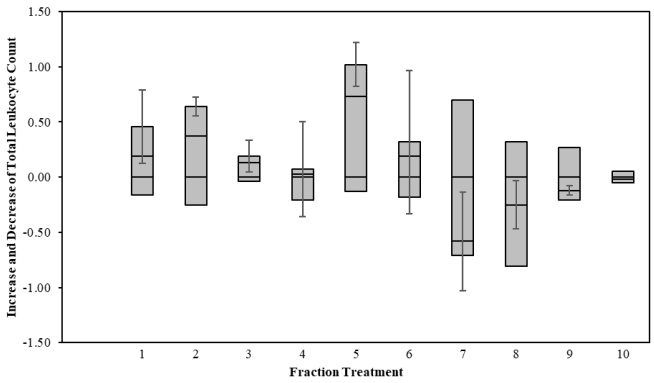

(b)

Figure 5. Results of the total leukocyte test in common carp (*C. carpio*).
Description: (a) total leukocyte count, (b) increase and decrease in leukocyte count from day 0 to day 3

The administration of the butterfly pea flower fraction significantly affected the total leukocyte count in common carp on each observation day ($p < 0.05$), indicating different immune responses across fractions (Figure 5a). On day 0, before administration, leukocyte counts varied significantly among treatments, with the highest count in fraction 8 (3.88×10^4 cells/mm³) and the lowest in fraction 9 (2.01×10^4 cells/mm³), suggesting a varying immune status but within the normal range. On day 1 after fraction administration, fractions 7 and 8 showed a significant increase in leukocyte counts (4.20×10^4 cells/mm³) without significant differences between them, while fraction 9 and the control group had notably lower counts. On day 2, fractions 8 (3.96×10^4 cells/mm³) and 5 (3.87×10^4 cells/mm³) exhibited the highest leukocyte levels, followed by fractions 1 (3.50×10^4 cells/mm³) and 3 (3.44×10^4 cells/mm³), which showed no significant differences. By day 3, fractions 1 (3.34×10^4 cells/mm³), 3 (3.40×10^4 cells/mm³), and 8 (3.39×10^4 cells/mm³) maintained similar leukocyte counts, as did fractions 4 (3.49×10^4 cells/mm³) and 7 (3.49×10^4 cells/mm³), indicating consistent immunostimulatory effects across these treatments.

Based on the trend observed in the total leukocyte count, fraction 5 demonstrated the most promising immunostimulatory effect among all treatments. It showed the highest leukocyte increase on day 0 (0.73×10^4 cells/mm³) and day 2 (0.29×10^4 cells/mm³), indicating an early immune activation following treatment. Additionally, fraction 5 experienced the smallest decline in leukocyte count on day 3 (0.13×10^4 cells/mm³), suggesting a more sustained immune response. These findings highlight fraction 5 as the most effective among the tested fractions in enhancing and maintaining leukocyte levels in common carp (*C. carpio*) (Figure 5b).

The increase in leukocyte count indicates that the *C. ternatea* flower fraction activates the nonspecific immune response in common carp (*C. carpio*), stimulating macrophages and neutrophils. Flavonoid compounds act as antioxidants that can stimulate the activation and proliferation of leukocytes such as neutrophils, monocytes, and lymphocytes, as well as protect immune cells from oxidative stress-induced damage. Additionally, flavonoids promote the activation and differentiation of monocytes into macrophages and enhance the expression of proinflammatory cytokines such as IL-1 and TNF-α (Li *et al*., 2019; Roshni *et al*., 2023). Meanwhile, alkaloids function as immunomodulators by promoting leukocyte formation in hematopoietic organs such as the anterior kidney and enhancing phagocytic activity. They also stimulate neutrophil activation

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and regulate neutrophil mobilization Ye *et al*., 2019; Nurhalisa *et al*., 2022), thereby increasing leukocyte production in hematopoietic tissues. The decrease in leukocyte levels observed on days 2 and 3 may be attributed to environmental stressors or the gradual degradation of the extract over time, which could reduce its efficacy. Additionally, immune downregulation may occur as part of the organism's homeostatic response. (Shekarabi *et al*., 2021; Sheikhzadeh *et al*., 2022).

CONCLUSION

This study demonstrated that 96% ethanol produced the highest extract yield (22.16%) from butterfly pea flowers, while ethyl acetate yielded more bioactive compounds. Chloroform: methanol (9:1) was the optimal eluent, producing distinct TLC and chromatography spots. Notably, Fraction 5 elicited the strongest immunomodulatory response in common carp (*C. carpio*), marked by the highest leukocyte count increase on days 0 (0.73×10^4 cells/mm^3) and 2 (0.29×10^4), and the smallest decline on day 3 (0.13×10^4). UV-Vis (λmax 659 nm) and FTIR confirmed the presence of phenolics, flavonoids, alkaloids, and key functional groups, highlighting significant therapeutic potential.

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest, whether financial, personal, or professional, with any individuals or organizations that could have influenced the content or interpretation of the material presented in this manuscript.

AUTHOR CONTRIBUTION

All authors have contributed to the final manuscript. The contribution of each author is as follows: REP: collected the data, drafted the manuscript, and designed the figures; AS and SSPR: devised the main conceptual ideas and provided critical revision of the article. All authors discussed the results and contributed to the final manuscript.

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