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Histopathological Analysis of Liver Damage in *Rasbora lateristriata* Fish Induced by extract Butterfly Pea flower (*Clitoria ternatea*)

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ABSTRACT

Butterfly pea flower (*Clitoria ternatea*) is an Indonesian native plant known for its medicinal properties due to its rich content of anthocyanins, flavonoids, and ternatins. Although widely recognized for its health benefits, the long-term effects of its bioactive compounds require careful evaluation. This study aimed to investigate the potential hepatotoxic effects of butterfly pea flower extract on the liver of *Rasbora lateristriata* fish. Fish were exposed to 0 (control), 50, 75, and 100 ppm of butterfly pea extract for four days, with four fish allocated per group. Histological markers, including necrosis, vacuolization, pyknosis, and hemorrhage, were assessed in liver tissues. Data were analyzed using the Kruskal-Wallis test and Mann-Whitney U test at a 95% confidence level. Results indicated that butterfly pea flower extract caused significant hepatic damage, with increased severity at higher concentrations. The most pronounced damage was observed at 75 ppm, suggesting an optimal dose for inducing oxidative stress. This study highlights the potential hepatotoxicity of butterfly pea flower extract in aquatic species and underscores the need for further research to evaluate its safety for therapeutic use.

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INTRODUCTION

Indonesia is renowned for its rich biodiversity, ranking second globally after Brazil. This biodiversity encompasses a wide range of wild and cultivated plants, including medicinal herbs commonly used by the communiy (Jumiarni & Komalasari, 2017; Muawanah *et al*., 2023). Herbal medicines remain a popular alternative for addressing various health issues. According to WHO data, approximately 75% of the global population relies on herbal medicines for treating diseases (Mahajan *et al*., 2022). Among Indonesia's native plants, the butterfly pea flower (*Clitoria ternatea*) has gained attention for its medicinal potential (Kurniawati *et al*., 2024; Taufik & Ainiyah, 2021). The butterfly pea flower, a member of the Fabaceae family, is predominantly found in tropical areas and is easily recognizable by its blue flowers, rich in anthocyanin pigments. These anthocyanins acts as antioxidant, promoting skin health by slowing aging and protecting against oxidative stress (Dhangar *et al*., 2023; Mahapatra *et al*., 2024). Additional antioxidant compounds in *C. ternatea* L., such as kaempferol, p-coumaric acid, and delphinidin-3,5-glucoside, further enhance its therapeutic potential (Mahapatra *et al*., 2024).

All parts of the *C. ternatea*, including its roots, stems, leaves, flowers, and seeds are useful. Secondary metabolites such as ternatin offer various benefits, including antioxidative, antidiabetic, anti-obesity, anti-inflammatory, anticancer, antihyperlipidemic, and antiasthmatic properties. Based on Mahapatra *et al*. (2024), phytochemicals like flavonoids and anthocyanins in *C. ternatea* can be used as antimicrobials by controlling and promoting a balanced microbiome. These compounds also act as anticancer by inhibiting tumor growth and inducing apoptosis in cancer cells. Despite these benefits, the safety of natural substances requires careful consideration, particularly when used as medical alternatives, as their chemical compounds can affect various biological processes. According to Chandra *et al*., (2019), prolonged use of certain herbal medicines may impair liver function. The liver plays a key role in detoxification, and the metabolism of certain compounds can generate active metabolites that cause oxidative stress, leading to cellular and tissues damage. Thus, understanding the safety profile of a natural substances is critical to avoid potential adverse effects associated with long-term use.

Previous studies have highlighted the hepatoprotective effects of butterfly pea flower extract in white rats induced with paracetamol, as well as its nephroprotective effects in the kidneys of rats exposed to MSG (Pebiansyah *et al*., 2022; Dewi *et al*., 2024). Flavonoids and anthocyanins in the butterfly pea flower extract have demonstrated protective effects against chemical-induced organ damage. However, research into potential toxicity or adverse effects of butterfly pea flower extract remains limited. To address this gap, this study explores the potential toxicity of butterfly pea flower extract using *Rasbora lateristriata,* a freshwater fish native to Indonesia, as a research model. The liver of *R. lateristriata* is highly sensitive to chemical exposure and oxidative stress. *R. lateristriata* is an endemic species distributed throughout Sumatra, Kalimantan, Java, Bali, Sumbawa, and Lombok, and thrives in clear freshwater rivers. This fish is recognized for its scientific relevance and economic potential (Retnoaji *et al*., 2017). The fish's small body size, transparent larvae, rapid embryogenesis, sustainable egg production under simple maintenance, and close resemblance to zebrafish—a widely used research model—make it highly suitable for toxicological studies (Retnoaji *et al*., 2023).

The liver is a critical organ in fish, responsible for nutrient metabolism, detoxification, and digestive enzyme production. Its high sensitivity to environmental changes makes it a reliable indicator for assessing the bioactive compounds from natural substances, such as butterfly pea flower extract. This study investigates the potential toxicity of butterfly pea flower extract on the liver of *R. lateristriata* by analyzing histological markers of damage, including vacuolization, hemorrhage, necrosis, and pyknosis.

MATERIALS AND METHOD

Fish collection and acclimation

Fish maintenance. Yellow rasbora (*Rasbora lateristriata*) were obtained from the Laboratory of Animal Structure and Development, Faculty of Biology, Universitas Gadjah Mada, Indonesia. Two-month-old male and female fish (mean weight 1.13 g) were randomly selected and maintained in 30 × 25 × 15 cm aquaria (five fish per aquarium). Fish were acclimated for four days under controlled conditions with a water temperature of 27.1 °C, total dissolved solids (TDS) of 298 mg/L, and pH 8.51, under a 12:12 h light/dark photoperiod. Aquaria were covered with nets to prevent fish from jumping.

Extraction and experimental treatment

Dried butterfly pea flowers (*Clitoria ternatea*) were ground and extracted at concentrations of 50 ppm (0.25 g/5 L), 75 ppm (0.375 g/5 L), and 100 ppm (0.5 g/5 L) by boiling for 15 minutes at a maximum temperature of 90 °C. A control group without extract was included. Fish were divided into four groups (control, 50 ppm, 75 ppm, and 100 ppm), each consisting of six fish. The extract was directly added to the aquarium water, and fish were continuously exposed for four days. Fish body weight was measured prior to treatment.

Hepatic tissue preparation

Liver histological slides were prepared using the paraffin method. Tissues were fixed in 10% neutral-buffered formalin overnight, dehydrated through graded ethanol, cleared in toluene, and embedded in paraffin. Sections (5 µm) were cut using a microtome, mounted on albumin-coated slides, dried at 40 °C, deparaffinized, rehydrated, stained with hematoxylin–eosin, cleared in xylene, and mounted with entellan for microscopic observation. The quantification of normal hepatocytes, as well as those exhibiting pyknosis, necrosis, and vacuolization, was performed. The levels of hepatic histopathological damage were assessed based on Table 1.

Table 1. Levels of hepatic histopathological damage

Level of damage	Description	Score
Normal	Clear nucleus, round shape	0
Mild	Hemorrhage +, vacuolization +, necrosis +, pyknosis +	1
Moderate	Hemorrhage ++, vacuolization ++, necrosis ++, pyknosis ++	2
Severe	Hemorrhage +++, vacuolization +++, necrosis +++, pyknosis +++	3

Description:
- : Normal
\+ : Damage percentage 0.0001-0.25% per field of view.
++ : Damage percentage 0.26-0.5% per field of view.
+++ : Damage percentage > 0.5% per field of view.

Statistical analysis

Image visualization was performed using a Leica microscope connected to a computer at 40X magnification. The number of normal hepatocytes, hepatocytes with pyknosis, necrosis, and vacuolization, and the hemorrhagic areas were counted from 12 fields of view per group using the ImageJ 1.5k software. Data were analyzed using the non-parametric Kruskall-Wallis test and the Mann-Whitney U test for inter-group comparison using IBM SPSS Statistic 25 software. A significance threshold of $p < 0.05$ was considered statistically significant.

RESULTS AND DISCUSSION

The liver is a large organ connected to the vertebrate digestive system, and its size, shape, and volume are determined by the space available among other visceral organs. The liver performs various functions in vertebrates, including regulating anabolism and catabolism, storing metabolites, and producing most plasma proteins (Faccioli *et al*., 2014). Fish exhibit a slightly different organ system than mammals due to their aquatic environment (Abusrer & Shtewi, 2023). In fish, the liver regulates anabolic and catabolic processes (Lall & Kaushik, 2021). In addition, the liver plays a role in metabolite storage and produces most plasma proteins. The liver also breaks down old red blood cells, producing by-products that are excreted through bile into the duodenum (Abusrer & Shtewi, 2023). Every drug that enters the body undergoes pharmacokinetic processes, including absorption, distribution, metabolism, and excretion. The liver is the primary organ responsible for drug metabolism. It converts lipophilic drugs into hydrophilic forms, facilitating their excretion through urine or bile. However, this process can lead to the accumulation of xenobiotics in the liver, potentially causing hepatotoxic effects (Nofrian & Wijayahadi, 2017).

Butterfly pea flower (*C. ternatea*) is a wild climber plant from the Fabaceae. It is widely recognized for its medicinal properties, with every part of the plant, from roots to flowers, believed to have therapeutic properties and improve organ function (Putri & Baharza, 2023). Butterfly pea blossoms have been extensively studied and show great potential in improving human health. They are rich in polyphenols, which act as potent antioxidants, benefitting overall health (Yurisna *et al*., 2022). These flowers have also been reported to help manage diabetes, reduce inflammation, serve as antimicrobial agents, combat obesity, prevent cancer, and protect liver health. The roots contain various bioactive compounds, including steroids, saponins, flavonoids, and glycosides, while the flowers are abundant in phytochemicals with therapeutic potential (Putri & Baharza, 2023; Yurisna *et al*., 2022).

In **Figure 1A**, the controls group provides a basic overview of the histological condition of the liver in *R. lateristriata* not exposed to butterfly pea flower extract. According to Septriani *et al*., (2023), normal liver cells are characterized by a round, clear cell nucleus surrounded by evenly distributed basophilic cytoplasm. This structure forms a typical lobular pattern around the central vein, indicating the optimal physiological function of liver tissue. Additionally, normal hepatocytes exhibit no signs of damage, such as vacuolization, pyknosis, hemorrhage, or necrosis. In this study, histological analysis revealed that the number of normal hepatocyte cells in the control group varied between 403 and 486 cells per field of view. However, mild damage, such as vacuolization, pyknosis, and hemorrhage, was observed, likely due to liver's natural detoxification processes. In contrast, the liver tissues of fish treated with butterfly pea flower extract showed increasing levels of histopathological damage, including vacuolization, pyknosis, necrosis, and hemorrhage, as the concentration increased from 50 to 100 ppm **(Fig. 1B-D)**. Quantitative analysis **(Fig. 2)** confirmed that the highest histological damage scores were observed at 75 and 100 ppm, indicating significant dose-dependent hepatic toxicity. The toxic and genotoxic activities of plant compounds may vary depending on interactions between mutagenic and protective chemicals, which may result in a variable response pattern depending on the drug's production method and the physiological situation (Liu *et al*., 2011).

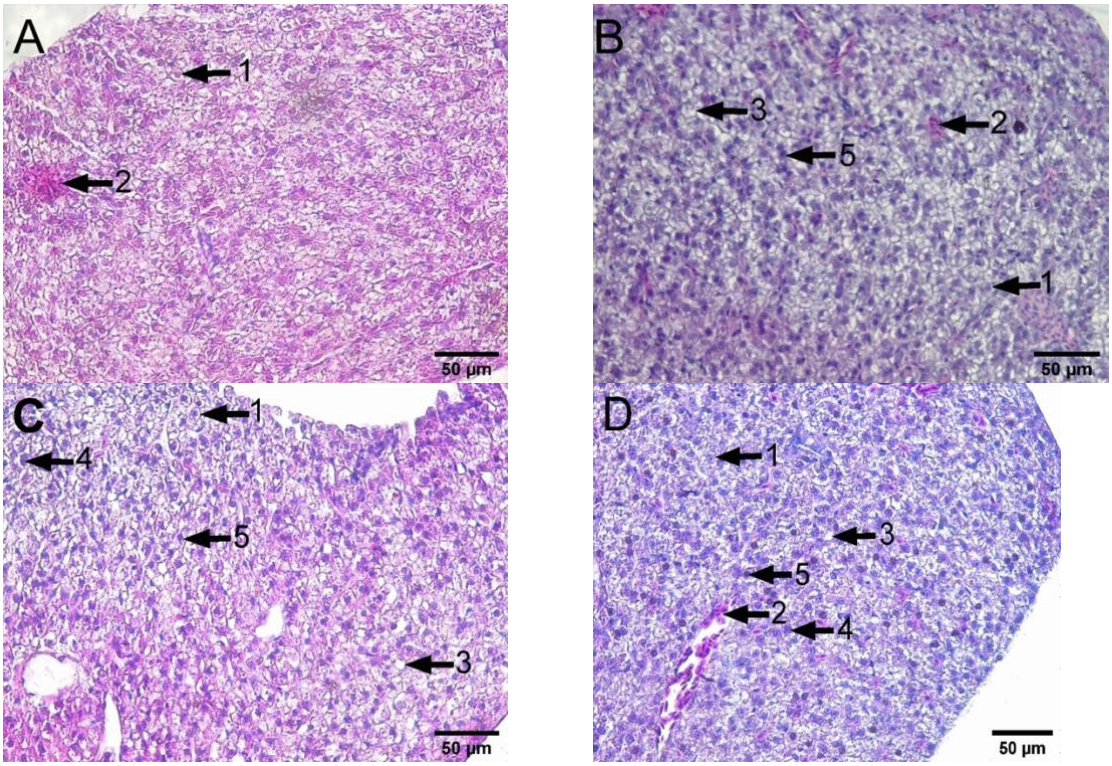

Fig 1. Histological examination of hepatic tissue from *Rasbora lateristriata* fish exposed to butterfly pea flower extract. The histopathological findings are labeled as follows: 1) normal cell morphology, 2) hemorrhage, 3) vacuolization, 4) necrosis, and 5) pyknosis. A: control; B: 50 ppm; C: 75 ppm; and D: 100 ppm of butterfly pea flower extract.

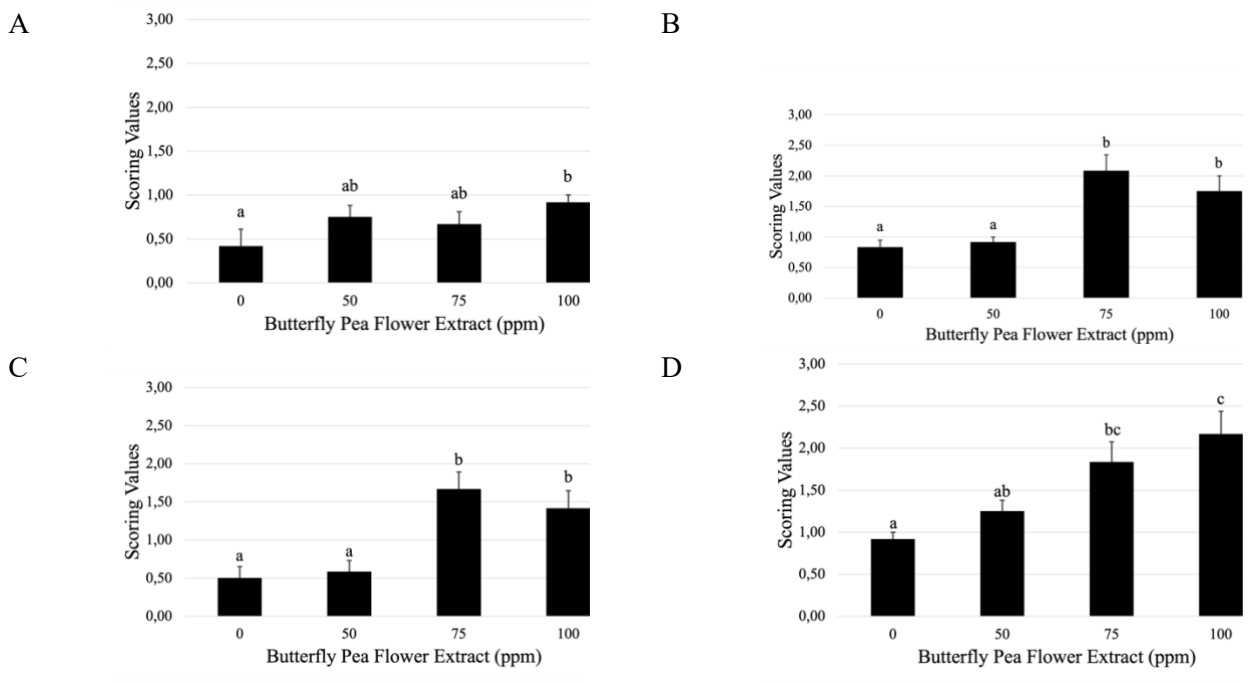

Fig 2. Effect of butterfly pea flower extract on hemorrhage (A), vacuolization (B), necrosis (C), and pyknosis (D) of hepatocytes in *Rasbora lateristriata* fish. Different letters denote statistically significant differences. 0: control group

Histological analysis of the liver **(Fig. 2)** in *R. lateristriata* exposed to butterfly pea flower extract at doses of 50, 75, and 100 ppm revealed damage to hepatic tissue, indicated by pyknosis, necrosis, vacuolization, and hemorrhage. In Khatib *et al*., (2024), toxicity testing of butterfly pea flowers on hepatic tissue in mice showed damage characterized by expanded inflammatory cells infiltration in the pericentral region, necrotic cells, and pyknotic nuclei. In acute and subacute liver injuries, non-lipid cytoplasmic vacuolation and swelling are typically observed in evenly distributed hepatocytes.

According to Berata *et al*. (2015), hemorrhage is characterized by the presence of blood vessels or tissues with blood infiltration. Hepatocytes with vacuolization exhibit a broad, white, concave cytoplasm. These cells are larger in volume, with a centrally located solitary nucleus, and show evidence of vacuolization. Hepatocytes undergoing pyknosis appear darker and exhibit shrunken, blackened nuclei, without fragmentation (Fajariyah *et al*., 2010). Necrosis begins with morphological changes in the nucleus, including pyknosis. Necrosis cells are characterized by plasma membrane rupture and nuclear shrinkage. This release of toxic cellular components, such as cytokines and fragmented DNA, triggers inflammation and cell death. Necrosis regulation systems differ according to cell type and stress situation (Liu *et al*., 2023). Necrosis is long-term cell death caused by more advanced molecular mechanisms such as autophagy and apoptosis. Recent studies using flow cytometry have detected both apoptosis and necrosis in response to various cytotoxic agents (Zulfikri *et al*., 2024).

In this study, necrosis was absent in the control group, but present in the treatment group **(Fig. 1).** This aligns with findings that certain doses of herbal medicine can disrupt liver function (Navarro *et al*., 2017). According to Kolo *et al*. (2024), this effect may result from active compounds, such as flavonoids, alkaloids, steroids/terpenoids, tannins, and phenolics in the extract. Interestingly, the hemorrhage scores (Fig. 2A) peaked at 100 ppm, suggesting potential damage to blood vessels caused by oxidative stress. These findings highlight the dual nature of butterfly pea flower extract, offering therapeutic benefits at lower doses but significant toxicity at higher concentrations. Yumnamcha *et al*. (2015) reported that saponins, alkaloids, phenolic compounds, and triterpenoids in aqueous extracts of *Millettia pachycarpa* cause hemolytic activity in zebrafish, leading to immediate membrane death. Saponin combinations and alkaloids can also cause DNA damage (Liu *et al*., 2011; Yumnamcha *et al*., 2015).

Exposure to 75 ppm of butterfly pea flower extract likely induces significant oxidative stress, leading to lipid peroxidation of hepatopancreatic cell membranes. This disruption compromises intracellular enzyme activity, disrupt cellular metabolism, and culminate in necrosis (Ammendolia *et al*., 2021, Febrianti *et al*., 2022). Interestingly, 75 ppm extract exhibited more pronounced cell membrane damage compared to 100 ppm,

suggesting an optimal dose for inducing cellular damage. The observation that hepatic damage was more severe at 75 ppm than at 100 ppm suggests a non-linear dose response relationship, potentially indicating a hormetic effect. At higher concentrations, certain phytochemicals may undergo aggregation or precipitation in water, reducing bioavailability and cellular uptake. Alternatively, exposure to 100 ppm may activate compensatory cellular defense mechanisms, such as upregulation of antioxidant enzymes or detoxification pathways, partially mitigating tissue damage. Hormesis describes a non-monotonic dose–response relationship in which plant-derived xenobiotics may exert greater biological effects at moderate concentrations than at higher doses, a pattern that has been consistently documented across diverse toxicological models and endpoints (Calabrese & Baldwin, 2003).

These findings also raise ecological concerns regarding the discharge of herbal-based products into freshwater ecosystems. Although often perceived as environmentally safe, plant-derived extracts may exert toxic effects on non-target aquatic organisms. Therefore, the widespread use of *C. ternatea* in food, cosmetic, and medicinal products should consider potential environmental exposure and species-specific sensitivity, particularly in endemic freshwater fish. The findings underscore the need for further research into the molecular mechanisms of butterfly pea flower extract toxicity, particularly in aquatic models, to establish safe therapeutic thresholds.

CONCLUSION

Butterfly pea flower (*Clitoria ternatea*) extract caused significant hepatic damage in *Rasbora lateristriata* fish, including necrosis, vacuolization, pyknosis, and hemorrhage. The severity increased with concentration, with 75 ppm showing the most pronounced effects. These findings raise concerns about its safety for prolonged or high-dose use. Further studies should investigate the molecular mechanisms of hepatotoxicity, long-term effects, and species-specific responses. Exploring optimal dosages and refining extraction methods can enhance therapeutic potential while minimizing risks.

AUTHORS CONTRIBUTION

A.E.P. contributed to data collection, laboratory experiments, and histopathological analysis, S.I. assisted with sample preparation, processing liver tissues, and performing microscopy. I.S. focused on maintaining experimental fish cultures and monitoring exposure treatments. S.R. Involved in statistical data analysis and compiling experimental results. P.S.R. participated in conducting the experimental setup and preparation of butterfly pea extract. F.M.P. contributed to writing the manuscript and reviewing relevant literature. S.S. assisted with experiment documentation and and controlled the concentration accuracy butterfly pea extract. A. supported the experimental procedures and contributed to quality control in histological processes. N.I.S. (corresponding author) supervised for the research, contributed to conceptualizing the study, designing experiments, analyzing data, reviewing the manuscript, and overseeing the research's scientific rigor. B.R. contributed to histopathological evaluation, providing expertise in microscopy and biological analysis, and supporting manuscript preparation. F.S. helped in data analysis and drafting sections of the manuscript. A.N. contributed to experimental protocols and manuscript preparation, focusing on theoretical insights. N.W. involved in guiding methodology development and providing feedback on experimental techniques. H.T.S.S.G.S contributed to data interpretation and the final manuscript review. Z.R. assisted with data interpretation and manuscript editing. S.W. contributed conceptual inputs and methodological design. D.E.P.E assisted in the manuscript's revision process and providing context for the implications of the study. W.A. provided critical feedback on manuscript drafts and supported statistical analysis.

CONFLICT OF INTEREST

The authors declare no conflicts of interest and take full responsibility for the content of the article, including any implications of AI-generated art.

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