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Research Article

Chronic toxicity assessment of nano-formulated *Bischofia javanica* leaves: Implications for pharmacological use

Cheryl Grace Pratiwi Rumahorbo[1], Syafruddin Ilyas[1], Salomo Hutahaean[1], Cut Fatimah Zuhra[1], Putri Cahaya Situmorang[1]

1 *Universitas Sumatera Utara Medan, North Sumatera, Indonesia*

Corresponding author: Syafruddin Ilyas (syafruddin6@usu.ac.id)

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Abstract

This study explores the chronic toxicity of nano-formulated *Bischofia javanica* leaves, a plant with pharmacological significance in Indonesia, following OECD guidelines 452. Graded doses (2, 4, 6, and 8 g/kg BW) of nano-formulated *Bischofia javanica* were administered to the treatment groups for 60 days, while the control group (K-) received only water. On the 61st day, the mice were euthanized, and samples for biochemical, hematological, and histopathological analysis were collected. Statistical analysis involved one-way ANOVA and Tukey's post hoc tests. Graded doses of nano-formulated *Bischofia javanica* leaves caused significant alterations in liver function. Doses between 2 and 4 g/kg BW improved liver histology and physiological markers. Higher doses (6 to 8 g/kg BW) led to liver dysfunction and histological degeneration, characterized by substantial fatty alterations, intracellular fat accumulation in hepatocytes, and compromised central blood vessels and sinusoids. The lungs showed signs of alveolar inflammation, epithelial exfoliation, debris, and the accumulation of inflammatory cells in alveolar spaces. Lower doses showed uniformly distributed cardiac blood vessels, while higher doses resulted in heart hemorrhages and amorphous exudates. Except for high doses, which significantly elevated specific liver damage indicators, no other levels of *Bischofia javanica* caused treatment-related mortality or significant alterations in hematological and biochemical parameters. Blood sugar levels remained stable across the dose range. In conclusion, moderate doses of nano-formulated *Bischofia javanica* leaf extracts can enhance physiological functions positively. However, caution is necessary when contemplating high doses, as they can cause dysfunction or damage vital organ systems.

Keywords

Bischofia javanica, toxicity, biochemical, hematological, microanatomy, proper dosage

Introduction

Traditional medicinal therapies are widely practiced worldwide, especially in middle- and low-income countries. They offer several advantages over modern medicine, primarily due to their lower likelihood of causing harmful effects when used appropriately. When utilizing herbal therapies, it is crucial to consider the correct dosage, timing, administration method, ingredient selection, comprehensive information review, and appropriate indications. Traditional medicines often contain multiple chemical compounds, enabling complementary or synergistic effects to achieve treatment goals. Additionally, traditional medicine offers

flexibility in administration, with options ranging from brewing and infusion to incorporation into food. This highlights the versatility and potential benefits of traditional medicine as a complementary or alternative approach to healthcare.

Pharmaceutical research into herbal medicines has gained recent prominence due to several factors, as outlined above. An essential initial step in developing these medicinal plants is the investigation of their toxic properties. Toxicity tests conducted on test animals provide crucial supporting evidence for the safety of these preparations. The choice of tests depends on the substance's intended use and the potential risks associated with human exposure. One medicinal plant has garnered significant attention from researchers regarding its efficacy is *Bischofia javanica*. Several advanced studies have explored its pharmacological effects. For instance, locomotor activity has been assessed using different tests, including the Open Field (OP) test (Kulkarni and Reddy 1996) and the Hole Cross (HC) test (Takagi et al. 1971). Additionally, the soothing effect of *Bischofia javanica* leaf extract was evaluated through thiopental sodium-induced deep sleep experiment (Ferrini et al. 1974), while its anxiolytic properties were tested by Lister (1987) and Sonavane et al. (2002) using different methods. The alpha-amylase inhibitory assay explored the plant's anti-diabetic potential (Hansawasdi et al. 2000). Furthermore, the phytochemical content of *Bischofia javanica*, including essential phytoconstituents in pharmaceuticals, has undergone extensive examination for various activities such as Thrombolytic-Preventing Activity (Prasad et al. 2006), Cancer-Preventing Activity (Lingadurai et al. 2011), Antioxidant Properties (Lingadurai et al. 2009), Activity Against Inflammation (Andersson et al. 1997), Anti-Allergic Activity (Florin et al. 2010), Anti-diabetic activity (Hutahaean et al. 2021), and Anti H. Pylori Activity (Wang and Huang 2005). All these pharmacological properties of *Bischofia javanica* have been assessed through oral administration of the plant extract to experimental animals.

While previous studies on *Bischofia javanica* toxicity have primarily focused on the cytotoxic test LC50 method (Chowdhury et al. 2020; Rumahorbo et al. 2023a) and the acute toxicity test LD50 method (Rumahorbo et al. 2023a), it is essential to note that other types of toxicity assessments are equally significant. Surprisingly, a review of previous research on *Bischofia javanica* toxicity has not revealed any investigations into its chronic toxicity. Thus, this study aims to fill this gap by examining the chronic effects of repeatedly administering nano-formulated *Bischofia javanica* leaves to test animals over three months. Additionally, this study will comprehensively analyze the impact of prolonged nano-formulated *Bischofia javanica* leaf administration on the physiological factors of growing mice used as test subjects. The physiological factors under assessment include complete blood profiles, end products of liver and kidney function, and histological examinations of vital organs such as the liver, kidneys, heart, and lungs. This study will detail the materials and methods employed for these assessments.

Materials and methods

Preparation of *Bischofia javanica* leaf nano-formulated

The production of nano-formulated *Bischofia javanica* leaf involves High Energy Milling (HEM). The process commenced with preparing 2 kg of fresh *Bischofia javanica* leaves, which were meticulously washed with running water and dried in a shaded room for one week. Afterward, a grinding machine was utilized to grind the dried *Bischofia javanica* leaves into a coarse powder. The coarse powder was then placed in a grinding container, along with alumina grinding balls, in a ratio of 1:20 (powder mass to grinding ball mass). The grinding process was initiated at a speed of 350 rpm and followed a specific time variation pattern. This pattern encompassed grinding for 3 hours, a subsequent 1-hour pause, another grinding session lasting 6 hours, and another 1-hour pause. This sequence continued until a final grinding session of 9 hours was completed, resulting in the nano-formulation of *Bischofia javanica* leaves. The diameter of the particles was assessed using a Particle Size Analyzer to confirm their nano-sized range, certifying the suitability of the herbal material for various applications.

Animal handling and the treatment

The study employed forty healthy adult male *Mus musculus* weighing between 20 and 40 g. To facilitate identification and data collection, each rodent received individual labeling and was distributed into five groups, each consisting of six mice (n=6). These groups were housed separately, and an initial ten-day acclimatization period in a laboratory environment was provided for all mice before the experiment. During this period, the animals were maintained at consistent room temperature and humidity levels, adhering to a 12-hour light and 12-hour dark cycle. They were supplied with standard pellet food and had access to water ad libitum. Among these groups, four received graded doses of nano-formulated *Bischofia javanica* leaves, while the fifth group, serving as the control, received only clean water and standard feed. Weekly weight measurements of the mice were recorded before dosing with nano-formulated *Bischofia javanica* leaves. On the 60^{th} day of the experiment, the animals were humanely euthanized under mild chloroform anesthesia.

For this investigation, four categories of nano-formulated *Bischofia javanica* leaves were designated as T1 (2 g/kg BW), T2 (4 g/kg BW), T3 (6 g/kg BW), and T4 (8 g/kg BW). These doses were selected based on our previous research, utilizing the LD50 method for nano-formulated *Bischofia javanica* leaves, which determined the LD50 dose to be 12.6 g/kg BW. Throughout the 60-day study period, the mice were orally administered daily doses of nano-formulated *Bischofia javanica* leaves and sterile drinking water. The living conditions, food, animal dosages, and daily observations strictly adhered to the recommendations outlined in the Organization for Economic Co-operation and Development (OECD) guideline 452. The allocation of mice to each

group adhered to the principles of the 3R concept, emphasizing the reduction, refinement, and replacement of animal use. Furthermore, international principles and standards were strictly followed to prevent suffering or distress among the test animals. The guidelines for Animal Research: Reporting of In Vivo Experiments (ARRIVE) and the Helsinki Declaration (updated in 2013) were also adhered to, ensuring the ethical and scientific integrity of the study.

General studies

Daily monitoring of the animals was conducted to detect any potential adverse effects of nano-formulated *Bischofia javanica* leaves following dosing throughout the study period. This monitoring encompassed observations of rat feeding behavior, fur color, self-isolation signs, pain indications, and any instances of mortality.

Hematological parameter evaluation

Blood samples were obtained from the tail vein, incubated with anticoagulants, and analyzed utilizing automated hematology (Mindray BC-2800 Auto Hematology Analyzer). The following parameters were assessed through the Automatic Hematology Analyzer BF-6800: White Blood Cell (WBC) count, Red Blood Cell (RBC) count, hemoglobin (HGB) levels, hematocrit (HCT) levels, Mixed Cell Count (MXD), Mean Corpuscular Volume (MCV), Mean Corpuscular Hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count, Red Cell Distribution Width-Standard Deviation (RDW-SD), lymphocyte count, MXD, Neutrophil count, Lymphocyte count, Neutrophil count, Red Cell Distribution Width-Coefficient Variation (RDW-CV), Platelet Distribution Width (PDW), mean platelet volume (MPV), and Platelet-Large Cell Ratio (P-LCR).

The biochemical assessment

Mice were weighed 12 hours before euthanasia with chloroform, and blood specimens were collected through a cardiac incision. Five milliliters of blood were collected in gel separator tubes, allowed to clot, and then centrifuged for 15 minutes at 3000 rpm. The serum was separated and stored at -20 °C. Biochemical parameters were determined using the Automatic Biochemistry Analyzer NEUES480 (Brand: MedGroup). These parameters included Albumin, Globulin, Total Protein, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), Indirect Bilirubin, Direct Bilirubin, Total Bilirubin, Low-Density Lipoprotein (LDL), High-Density Lipoprotein (HDL), Very Low-Density Lipoprotein (VLDL), Total cholesterol, Triglycerides, Creatinine, Uric acid, Blood sugar, Gamma-glutamyl transferase (GGT), and Blood Urea Nitrogen (BUN).

Histology

The vital organs, including the liver, heart, kidneys, and lungs, were carefully removed, weighed, and preserved in 10% neutral buffered formalin. Subsequently, these organs underwent histological processing. A small section from each organ was precisely sliced, dehydrated in graded alcohol, and embedded in paraffin. Following preparation, the sections, ranging from 4 to 10 µm in thickness, were fixed using a neutral DPX medium, stained with hematoxylin and eosin, and then mounted. Images were magnified at 40×, 100×, and 400× using a light microscope.

Data analysis

The findings were subjected to a one-tailed analysis of variance (ANOVA) with a 95% confidence level. Subsequently, Tukey's post hoc analysis was applied to the data utilizing SPSS version 21, and the results were presented in tables and graphs. Graphical analysis was additionally conducted using GraphPad Prism version 8.0.

Results

Macroscopic evaluation

Regular checks on drinking, eating, physical appearance, and activity were crucial in toxicology investigations. Macroscopic studies of the mice involved in this investigation revealed no significant changes in feeding, exploration, or drinking habits during treatment. Furthermore, we assessed incisor height, fur color, and overall appearance to maintain standards.

Body weight

Certain xenobiotic substances can disrupt eating, drinking, and digestive patterns, leading to hormonal and enzymatic issues. Reduced appetite often leads to weight loss and delayed growth. This study examined the impact of graded nano-formulated *Bischofia javanica* leaf doses on mouse body weight over eight weeks. All groups showed increased body weight (Fig. 1), with no statistical difference between the control and comparison groups.

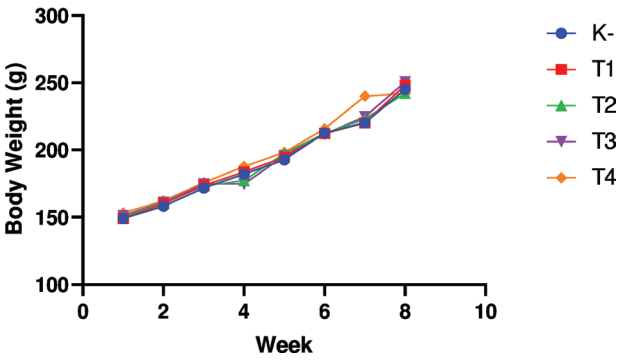

Figure 1. Weekly changes in body weight. Values are shown as mean ± SEM. K- (Control), T1 (2 g/kg BW), T2 (4 g/kg BW), T3 (6 g/kg BW), and T4 (8 g/kg BW).

Hematological function parameters

Medicinal substances may adversely affect immune function, hormone and enzyme activity, and hematopoiesis (Lubran 1989). Bioactive substances can disrupt hematopoiesis, leading to various blood disorders. Xenobiotics increase white blood cell (WBC) production as part of the body's defense mechanism (Wang et al. 2021; Han et al. 2016). Thus, this study evaluated hematological parameters to assess the toxicity of *Bischofia javanica* concerning hematological functions.

WBCs increased from 8.94 ± 0.56 in the negative control to 12.10 ± 0.31 in the T3 group (6 g/kg BW dose) (Fig. 2). The percentage of mixed cells (MXD), consisting of monocytes, eosinophils, and basophils, significantly increased from 14.43 ± 0.93 in the negative control to 19.29 ± 2.24 in group T1 (2 g/kg BW dose) and 16.62 ± 0.84 in group T4 (8 g/kg BW). Among all measured hematological parameters, only those related to neutrophils showed significant differences with the control group. Neutrophils increased in percentage from 8.83 ± 0.62 in the negative control group to 20.42 ± 1.20 in the T1 group, 15.57 ± 1.84 in the T3 group, and 14.22 ± 3.13 in the T4 group. Meanwhile, the percentage of neutrophils decreased in the T2 group (7.30 ± 0.29), showing no significant difference compared to the negative control group. Additionally, the number of neutrophils ($\times 10^3$) exhibited significant differences between the negative control group and the T1, T3, and T4 groups, with a decrease observed in the T2 group (although not statistically significant from the negative control value).

Biochemical function parameters

AST is typically found in the cytoplasm and mitochondria of the heart, liver, and skeletal muscle (Upur et al. 2009), while ALT resides mainly in the cytosol of liver cells. These liver enzymes enter the bloodstream during necrosis, liver injury, or changes in hepatocellular permeability, making their blood levels useful markers for hepatotoxicity, reflecting hepatocyte degeneration (Wong et al. 2017). Indirect Bilirubin, a product of HGB and red blood cell breakdown, is converted to direct Bilirubin in the liver and excreted via bile. In bioactive toxicity studies, bilirubin assays are crucial for evaluating red blood cell hemolysis and liver catabolic function. The kidneys handle urea, Bilirubin, and other waste substances excretion. The liver also produces body triglycerides and proteins, while approximately 80% of cholesterol in the body is endogenously synthesized. Liver cholesterol contributes significantly to transportation and other functions. Hence, liver cholesterol levels can describe the liver's synthetic capacity after herbal administration.

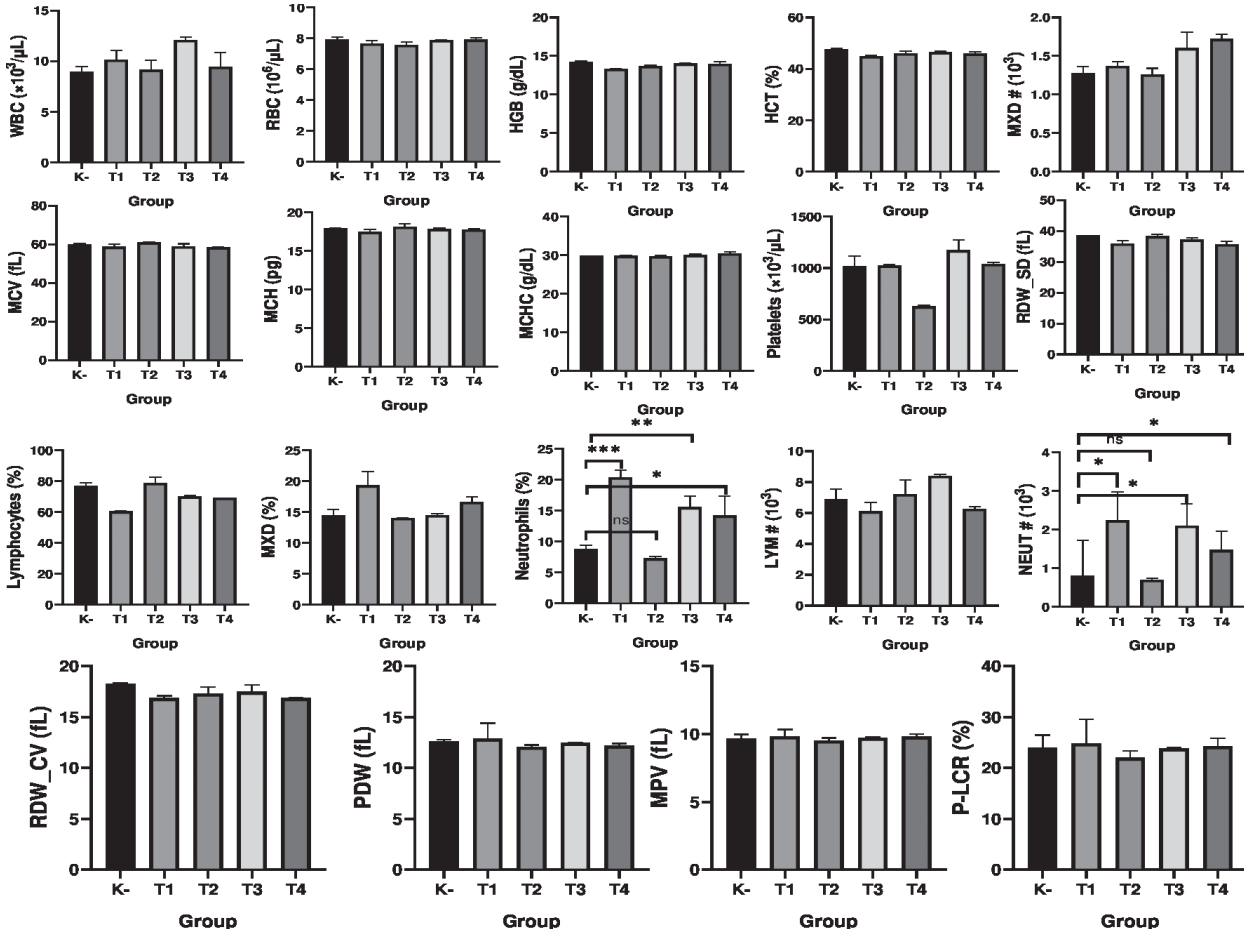

Figure 2. The impact of nano-formulated *Bischofia javanica* leaf extract on hematological parameters. Values are shown as mean ± SEM. K- (Control), T1 (2 g/kg BW), T2 (4 g/kg BW), T3 (6 g/kg BW), and T4 (8 g/kg BW).

Serum creatinine and blood urea nitrogen levels are vital indicators of renal function because they help eliminate creatinine, a byproduct of protein metabolism (Kluwe 1981). Bilirubin levels showed no immediate increase with T1, T2, or T3 dosing but significantly rose at T4. Albumin levels were similar to those in group K. Total Bilirubin and direct bilirubin levels increased in the T3 and T4 groups, while AST levels increased across all treatment groups. HDL levels decreased in all treatment groups, while LDL and total cholesterol increased. Notably, LDL levels significantly increased in groups T2, T3, and T4 compared to the control (Fig. 3). Most lipid parameters in the treatment groups were not significantly different from the control. Blood sugar levels did not significantly differ in any treatment group compared to the control, remaining within the normal range (Fig. 3).

Organ weight

Certain bioactive substances can induce inflammation in various tissues and organs, potentially leading to body and organ weight changes. Organ and body weight comparisons between treated and control groups serve as a crucial measure of the toxic effects of these substances. In general toxicity studies, the Society of Toxicologic Pathology regards organ weight evaluation as a vital screening tool for assessing the toxicity of bioactive substances (Michael et al. 2007; Simanjuntak and Rumahorbo 2022; Rumahorbo et al. 2023b). This study assessed the heart, liver, kidney, spleen, and testes. Most organs decreased in the treatment group, but it is noteworthy that specific organs, including the liver, kidney, and heart, showed significant reductions in the negative control group (Fig. 4).

Histologic evaluation

Microscopic analysis of vital tissues and organs is crucial to assess the safety of bioactive substances, revealing asymptomatic toxic effects often missed in biochemical studies (Mensah et al. 2020). This study exposed experimental animals' hearts, lungs, livers, kidneys, and brains to nano-formulated *Bischofia javanica* leaf extract for histological evaluation. These organs were also examined in the control group for comparison.

Liver

In the control group, there was moderate to severe central venous congestion (Fig. 5). In contrast, the *Bischofia javanica*-treated group displayed subcapsular hemorrhage and congestion in central veins, sinusoids, and prominent veins beneath the capsule (Fig. 5). The T1 dose group exhibited areas of necrosis without signs of inflammation. The T3 and T4 groups also had scattered hepatocytes containing

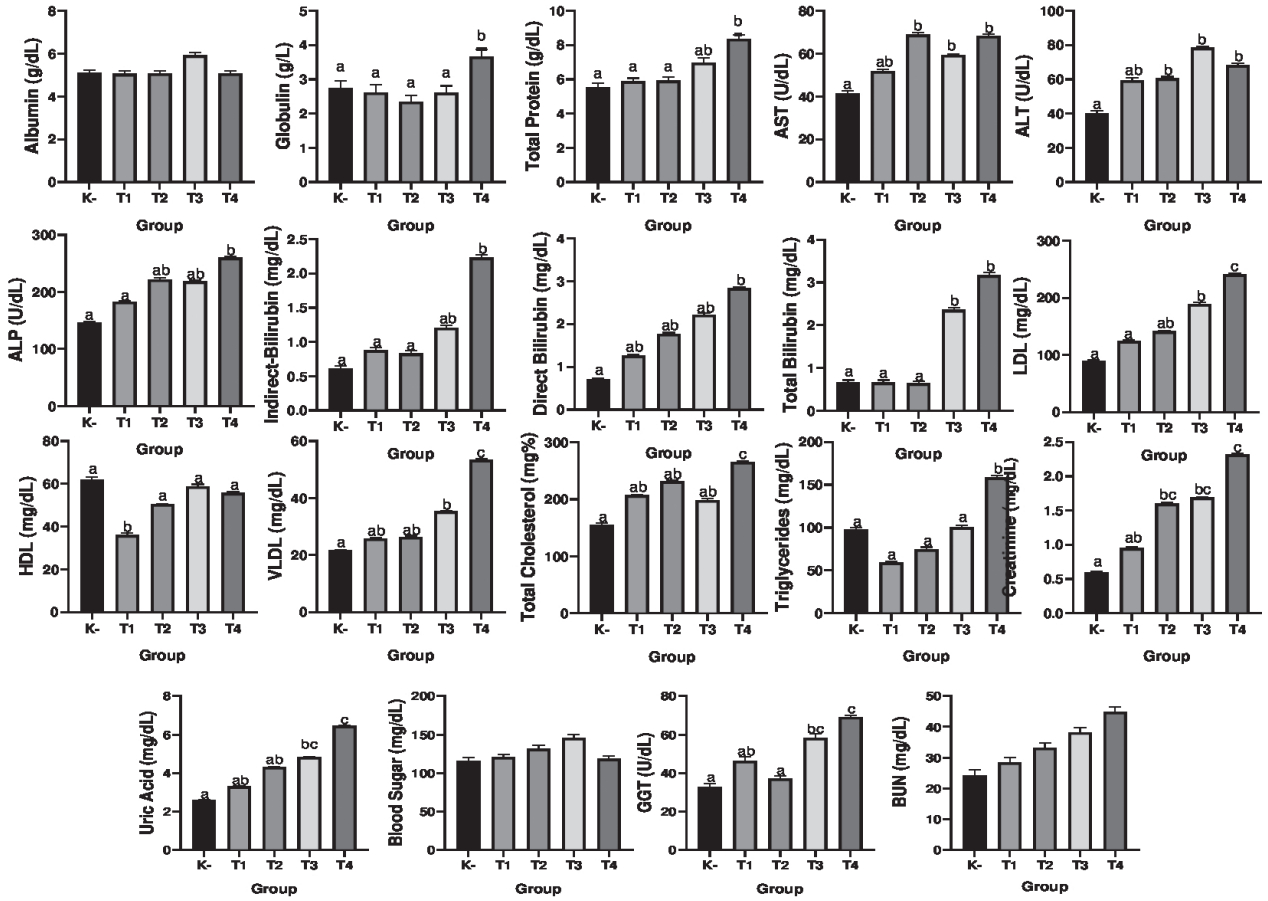

Figure 3. The impact of nano-formulated *Bischofia javanica* leaf extract on the parameters of biochemical function. Values are shown as mean ± SEM. K- (Control), T1 (2 g/kg BW), T2 (4 g/kg BW), T3 (6 g/kg BW), and T4 (8 g/kg BW).

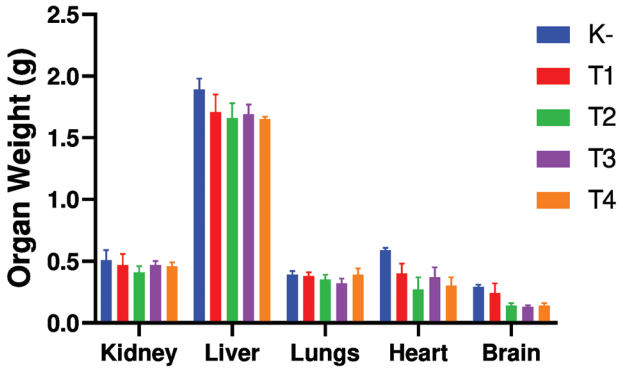

Figure 4. Organ weights relative to treatment with nano-formulated *Bischofia javanica* leaves. Values are shown as mean ± SEM. K- (Control), T1 (2 g/kg BW), T2 (4 g/kg BW), T3 (6 g/kg BW), and T4 (8 g/kg BW).

intracellular fat globules. Central venous congestion was evident in the livers of T1, T2, and T3-treated mice (Fig. 5). The hepatocyte cells in the T2 group displayed microvesicular conformation with noticeable areas of fatty change. Fig. 5 illustrates more pronounced liver conditions in the T2, T3, and T4 treatment groups, highlighting congestion in various liver regions, including under the capsule, sinusoids, large blood vessels, and central veins. Furthermore, the T4 group exhibited foamy hepatocytes.

Kidney

In control and T1 mice, renal architecture appeared normal, showing typical glomeruli, renal tubules, and collecting ducts with minimal stromal congestion (Fig. 5). However, all treatment groups, except for T1, exhibited severe stromal congestion and congestion in glomeruli, renal tubules, and collecting ducts (Fig. 5). Notably, the T4 group, receiving the highest dose of nano-formulated *Bischofia javanica* leaves, displayed persistent inflammation (Fig. 5).

Heart

Dispersed, severely clogged cardiac arteries were observed at all nano-formulated *Bischofia javanica* dose levels, with no signs of inflammation, infarction, or fibrosis (Fig. 5). In the cardiac sections of mice in the T4 group, we observed highly eosinophilic muscle fibers, hemorrhage with accompanying amorphous exudates in isolated muscle fibers, and scattered, highly congested arteries (Fig. 5).

Lungs

The control group exhibited airway epithelial sloughing in the lungs, while the T4 group displayed chronic inflammation with inflammatory cell clusters in the alveolar spaces (Fig. 5). In the T3 group, the lungs showed consistent inflammatory changes characterized by lymphocytes and macrophages, along with epithelial cell sloughing and debris covering the alveolar spaces (Fig. 5). Mice administered 8 g/kg BW of nano-formulated *Bischofia javanica* displayed moderate chronic inflammatory changes dominated by lymphocytes and macrophages, with some areas showing epithelial cell exfoliation in the lungs. Fig. 5

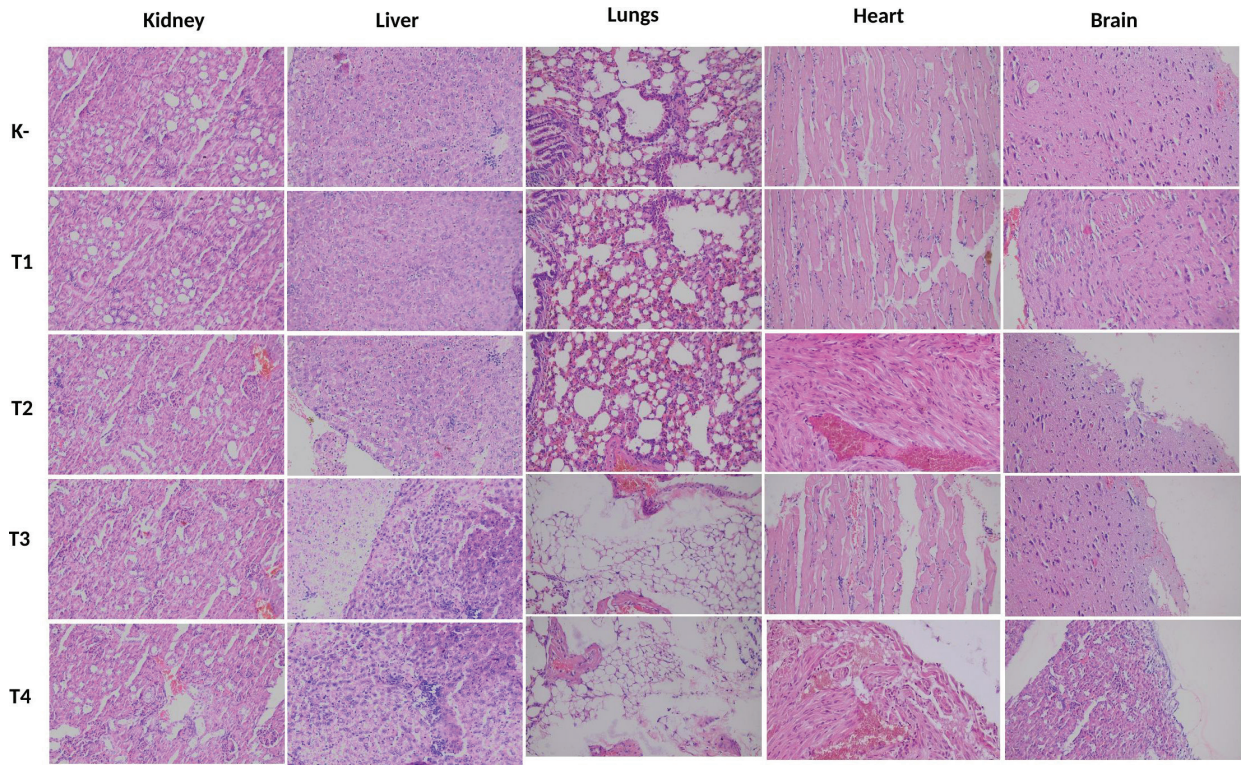

Figure 5. Microscopic images of vital *Mus musculus* organs under chronic nano-formulated *Bischofia javanica* leaf treatment. K- (Control), T1 (2 g/kg BW), T2 (4 g/kg BW), T3 (6 g/kg BW), and T4 (8 g/kg BW).

illustrates debris and inflammatory cells covering the alveolar space. In group T2, some areas in the alveoli exhibited chronic inflammation with clusters of inflammatory cells and airway exfoliation (Fig. 5). The T1 group showed minor inflammation in certain areas, with stable clusters of inflammatory cells filling the space (Fig. 5).

Brain

The brain histology of the adverse control group (K-) remained unchanged, showing no edema or congestion (Fig. 5). In the T1 group, mild edema and congestion were observed, along with focal congestion and edema lesions (Fig. 5). In contrast, the brain tissue in T2 exhibited more severe congestion lesions after 60 days of exposure to nano-formulated *Bischofia javanica* leaves compared to T1 (Fig. 5). Similar conditions were observed in the T3 group. Meanwhile, the T4 group displayed a high formation level of focal congestion lesions and severe edema. Nearly every neuron cell in this group's cerebrum of the brain tissue exhibited similar changes (Fig. 5). This suggests that administering high doses of nano-formulated *Bischofia javanica* leaves for an extended duration strongly impacts rat brain histology.

Discussion

Bischofia javanica, a commonly used herbal plant in Indonesia for its potential therapeutic benefits, has been the focus of this study to evaluate its safety, particularly in the nano form. The limited scientific data on its potential harm prompted this investigation into chronic toxicology. The results obtained from the test groups did not reveal significant deterioration in hematological function or any treatment-related mortality. All groups that received graded doses of nano *Bischofia javanica* leaves exhibited no signs of macrocytic or microcytic anemia, as evidenced by red blood cell and WBC counts. Although some groups showed a slight increase in red blood cell counts, these differences were statistically insignificant compared to the control group and were considered non-toxicologically relevant. In practical terms, medium and high doses of *Bischofia javanica* led to increased WBC counts, which were also statistically similar to the control group. However, upon euthanasia and examination, mice receiving a high dose of nano *Bischofia javanica* leaves (T4) exhibited slight ulceration in the small intestine during macroscopic digestive tract inspection.

Our results suggest high doses of nano-formulated *Bischofia javanica* leave significantly elevated AST levels in mice, indicating potential long-term toxicity or hepatocyte cell damage. This finding aligns with previous phytochemical screenings that have suggested moderate toxicity for these plants, emphasizing the importance of appropriate dosages (Rumahorbo et al. 2023c). While prior studies have suggested hepatoprotective properties of *Bischofia javanica*'s active ingredients, such as ursolic acid and betulinic acid (Li et al. 2015; Rumahorbo et al. 2021; Rumahorbo et al. 2023c), our histological findings imply potential liver toxicity at high doses. High-dose nano-formulated *Bischofia javanica* led to sinusoidal congestion beneath the capsule and within large vessels. In the T4 group, we observed congestion in major blood vessels, hepatocytes containing intracellular fat globules, and a prevalence of hepatocytes with fatty changes and microvesicular conformation. The T3 group exhibited foamy hepatocytes, while T2 showed liver synthetic capacity demonstrated by fat globules and a decreased albumin level. Compared to the control, the significantly lower albumin/globulin ratio in the T2 group supports using *Bischofia javanica* herbs as medicine with appropriate dosing. The high oil content in *Bischofia javanica* seeds, previously deemed inedible, provides a compelling reason to avoid high doses (Ameen et al. 2023).

Mice treated with a moderate dose of nano-formulated *Bischofia javanica* (T3; 6 g/kg BW) exhibited elevated blood sugar levels compared to the control group, potentially signaling impaired glucose metabolism, pancreatic injury, or a predisposition to diabetes mellitus. Previous studies have extensively explored hyperglycemia and diabetes mellitus (Richter et al. 2018). Long-term exposure to nano-formulated *Bischofia javanica* leaves at doses exceeding 2 g/kg BW (the recommended human equivalent dose) significantly reduced blood glucose levels in mice, maintaining them within the normal range. However, exposure beyond this dosage threshold can lead to diabetes mellitus (Rumahorbo et al. 2023c). Furthermore, in mice across all dose levels of nano-formulated *Bischofia javanica* leaves, lung histology revealed signs of inflammation, congestion, and the presence of chronic inflammatory cells, suggesting moderate lung toxicity. The lung weight in the T4 group greatly exceeded that of the control group. It is essential to note that many toxic substances enter the body through the lungs via absorption, distribution, and excretion processes, potentially leading to lung toxicity when exposed to very high nano-formulated *Bischofia javanica* leaf doses (8 mg/kg BW).

This study revealed that mice treated with nano-formulated *Bischofia javanica* leaves experienced gastrointestinal (GIT) issues, particularly in the small intestine. Their lung morphology exhibited similar changes, with alveolar inflammation dominated by lymphocytes and macrophages and alveolar spaces filled with debris and chronic inflammatory cells. These effects are attributed to high doses of herbal *Bischofia javanica* leaves exceeding 8 g/kg BW. Drugs, administered through various routes, undergo absorption, distribution, and binding to exert their effects. With its high metabolic rate, the brain demands a continuous supply of calories and oxygen, receiving approximately 20% of the cardiac output, equivalent to around 750 ml of blood per minute. Our findings demonstrate that administering nano-formulated *Bischofia javanica* leaves induces histological changes in the cerebrum of white mice (*Mus musculus*), evident in congestion and perivascular edema lesions. While such pathological conditions are expected, even in control mice, differences lie

in lesion distribution and treatment object health status. Experimental animals not bred specifically as pathogen-free (SPF) may exhibit unexpected changes (Tsatsakis et al. 2018). Moreover, administering graded doses of nano-formulated *Bischofia javanica* leaves over extended periods, known to contain high sulfur substances, can have toxic effects on various body cells, including the brain. The brain's heightened oxygen-carrying blood absorption sets it apart from other organs.

Mice given low doses of nano-formulated *Bischofia javanica* leaves (T1) displayed mild kidney toxicity, while those receiving 4 g/kg BW showed moderate kidney toxicity. This was characterized by capillary blockages in the glomerular system, kidney tubules, and collecting duct blood vessels. Additionally, chronic inflammation was observed in mice given 6 g/kg BW, and the kidney weight in the 8 g/kg BW group was notably lower than in the control group. This decreased kidney weight in T4 might be attributed to chronic inflammation and potential atrophy (Kluwe 1981). This study's foci of chronic inflammation could be linked to the high doses and prolonged administration of nano-formulated *Bischofia javanica*.

Consequently, administering this nano-formulated leaf only for 60 days is advisable. Mice regularly receiving nano-formulated *Bischofia javanica* leaves showed minor cardiac damage, characterized by dispersed yet heavily clogged blood vessels. Rodents exposed to this herb also exhibited highly eosinophilic muscle fibers, hemorrhages containing unstructured excrement, isolated muscle tissue, and congested veins. These effects may be attributed to the high concentration and prolonged exposure to *Bischofia javanica* leaf nano herbs. Toxicity is influenced by toxin concentration, exposure duration and frequency, composition, and environmental conditions (Sexton and Hattis 2007). Chronic cardiac toxicity is typically associated with inflammatory changes and blood vessel blockages (Wang et al. 2019). This herbal preparation's high dose and extended exposure duration likely contributed to the observed effects, even though the therapeutic botanical elements of *Bischofia javanica* nano-herbal leaves examined in this study may be connected to the mice's toxic experiences.

Conclusion

Our study indicates that prolonged exposure to high doses (> 4 g/kg BW) of nano-formulated *Bischofia javanica* leaves for ≥ 60 days may potentially damage critical organ systems. Conversely, at doses below 4 g/kg, these herbs could be considered for therapeutic purposes, showing positive effects on liver, blood, kidney, and heart functions, with minimal impact on the brain and lungs. We advise caution when using nano-formulated *Bischofia javanica* leaves, and we recommend further safety assessments, particularly in teratogenic toxicity models, especially for pregnant individuals. To ensure the quality of the plants, we advocate for improved processing and safe agricultural practices to prevent contamination with medicinal plant pesticides.

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