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Research Article

Oral chronic toxicity test of nano herbal *Phaleria macrocarpa*

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Abstract

Extracts of *Phaleria macrocarpa* have been used for years in traditional medicine and have been evaluated scientifically as well. This study aimed to evaluate the chronic toxicity level of nano herbal *Phaleria macrocarpa* and its effect on the changes in hematology, biochemistry, electrolytes, and organ histopathology. High Energy Milling (HEM) produced nano herbal *Phaleria macrocarpa*. By giving nano herbal *Phaleria macrocarpa* at doses of 300, 600, and 900 mg/kg BW for 56 days, the mice's blood was collected for the hematological and electrolyte parameters. The kidney and liver were assessed for biochemical parameters. As a result, the nano herbal *Phaleria macrocarpa* affected the histopathological organs, hematological, biochemical, and electrolyte parameters at an appropriate dose of 300–600 mg/kg BW. The maximum period of the administration this herbal medicine is one month. Therefore, with the correct dose and period of administration, this plant can be used as herbal medicine in the future.

Keywords

Nanoherbal, *Phaleria macrocarpa*, Chronic toxicity

Introduction

As a tropical country, Indonesia has high biodiversity, including plants that can be used for medicine. *Phaleria macrocarpa*, commonly known as God's crown or Mahkota Dewa, is one of the Indonesian medicinal plants with a million benefits. This native Indonesian plant that grows in Papua is believed to be able to treat various diseases. The extract of *Phaleria macrocarpa*'s fruit has been proven to have anti-cancer, anti-diabetic, anti-gout, and anti-hepatitis properties. The main content of *Phaleria macrocarpa* is phalerin (content up to 9.52%) (Ali et al. 2012). In 32 g of fruit pulp paste that was analyzed for its phytoconstituents, there was 9.1% Mahkoside A, 0.21% kaempferol, and 0.1% mangiferin, while 60% of this coarse paste consisted of sucrose (Zhang et al. 2006). With different methods, the percentage of essential ingredients in the *Phaleria macrocarpa* flesh fruit is usually different; for example, with pressurized hot water as the solvent extraction method, a 2.1% yield of mangiferin is obtained (Kim et al. 2010).

The effectiveness of the *Phaleria macrocarpa* extract is also influenced by the size of the herbal particles that penetrate the cell membrane. Therefore, modifying particle size to nano size is expected to increase the effectiveness of the herbal. In addition to particle size, the accuracy of the dose is also one of the effectiveness determinants of herbal medicine. The acute toxicity test of the LD50 nano herbal *Phaleria macrocarpa* method has been carried out in previous studies, which found that the LD 50 nano herbal *Phaleria macrocarpa* was 1g/kg BW ± 0.075 (Simanjuntak and Ru-

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mahorbo 2022). A chronic toxicity test determines hazardous reactions that show up after multiple administrations of the experimental preparation for most of the test animal's life. The test preparation period is at least two months for a generally safe test substance and six months for completely natural substances or samples with hazardous potential (OECD 2018). The objective of the chronic oral toxicity test is; to obtain information on the presence of a toxic effect of a substance that was not detected in the acute toxicity test, information on the possibility of toxic effects after repeated exposure to the test preparation within a certain period; information on doses that do not cause toxic effects (No Observed Adverse Effect Level / NOAEL); and study the cumulative effects and reversibility effects of these substances. Therefore, in this study, a chronic toxicity test was carried out on the flesh fruit *Phaleria macrocarpa* as a continuation of the acute toxicity test in previous studies.

Materials and methods

Materials

5 kg of *Phaleria macrocarpa* fresh fruit were purchased from the traditional market of Medan City, Indonesia. First, the fresh *Phaleria macrocarpa* was washed using flowing water. Then, the *Phaleria macrocarpa* was air-dried in a room without exposure to direct sunlight for three weeks. Next, the dried *Phaleria macrocarpa* was ground to obtain a coarse powder. Finally, 2.5 kg of *Phaleria macrocarpa* coarse powder was processed into nanoparticles using a high-energy milling tool with HCl 2 M activator solution (Tokyo, Japan). The milling was done with a mass ratio of 1:20 (powder to ball milling mass) and milling times of 3, 6, and 9 hours until producing the nano herbal flesh fruit *Phaleria macrocarpa* (Rumahorbo et al. 2023).

Animals

Twenty-four male mice (*Mus musculus*) aged between 2.5–3 months with an average body weight of 25–30 g and without anatomical defects were obtained from animal cages at the Faculty of Mathematics and Science, Universitas Sumatera Utara, Indonesia. They were divided into four groups consisting of 3 test groups (T1, T2, T3) and one control group (C). Before starting the experiment, all mice were acclimatized for seven days to accustom the animals to the experimental environment. Food and drink are provided in moderation. The mice used were healthy and did not experience a change in body weight of more than 10%, and visually showed normal behavior. The dose of the test preparation given to the test animals was determined based on previous research, which showed that the LD50 value was 1 g/kg BW. Therefore, the doses of the test preparations given to the test animals were 300 (T1), 600 (T2), and 900 (T3) mg/kg BW. The test preparations were given orally with a frequency of once a day for 56 days (OECD 2018). The test preparations were given in nanopowder and CMC with a 1:1 ratio and homogenized with a vortex mixer. For the control group, only given CMC-NA solution and standard feed. Liver and kidney assessment and blood electrolyte examinations were conducted on days 0, 14, 28, 42, and 56. The blood count and histopathological analysis were completed on the 56th day, and the mice were euthanized.

Physiological analysis

The hematological analysis was conducted using Hematology Analyzer Merk Wiener Lab at North Sumatra Regional Health Lab. Complete blood counts were tested to measure the physiological effect of the herbal. Liver and kidney function were analyzed by COBAS 6000, and blood electrolytes were analyzed by Cobas b 221 POC system.

Histopathology

The liver, lungs, kidney, and heart specimens were covered in paraffin wax over two hours at 60–700 degrees Celsius. The paraffin blocks were cut with a microtome about 5–7 μm thickness, molded, and then frozen. The organ cuts were then placed on a heating surface between 56–58 °C for about ten seconds to stretch and adhere to the slide. Adjustments were made to avoid folded tissues. Further, hematoxylin and eosin stains were carried out. The tissue was soaked for twelve minutes in a xylene solution. After five minutes of immersion in 70%, 80%, 90%, and 100% ethanol, the tissue was dehydrated by washing in flowing water. After 5 minutes in the hematoxylin solution, the samples were washed under the water flow, stained with eosin, and then buried in 70%, 80%, 90%, and 100% ethanol for 10 minutes. In the last step, the samples were soaked in xylene for 12 minutes before being examined at 40× magnification under the microscope.

Data analysis

The statistical, qualitative, and intervention analyses use Microsoft Excel 2021 and SPSS 25. Analysis of variance (ANOVA) was used to determine whether there were any significant differences in histopathological assessment and physiological parameters of the blood, kidney, and liver at various concentrations of nano herbal flesh fruit *Phaleria macrocarpa*. The data were tested at a 95% confidence interval, with the result considered significant if the $p < 0.05$. The least significant difference (LSD) test was used to determine which treatment was statistically significant compared to the control.

Result and discussion

Physiological of the liver

Fig. 1 shows the result of liver biochemical parameters. This study assessed three enzymes that play a role in the

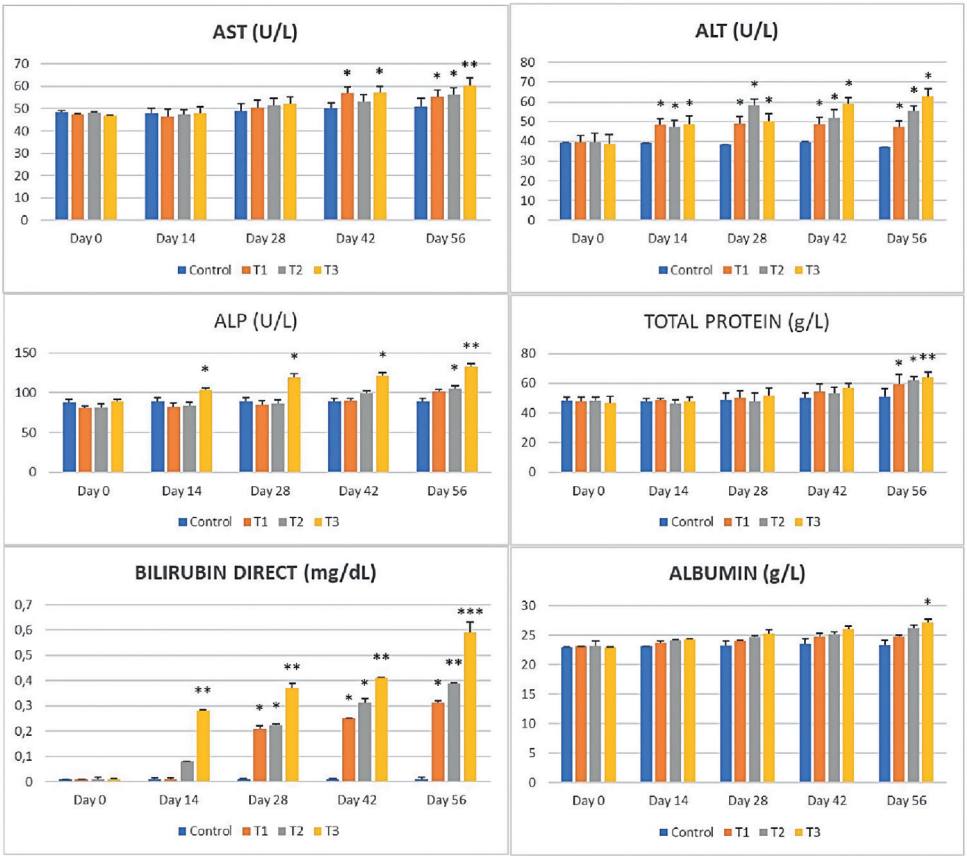

Figure 1. Liver biochemical parameters.

physiological function of the liver as a parameter due to exposure to nano herbal *Phaleria macrocarpa*. Significant differences between the control and treatment groups (T1, T2, T3) occurred on the 14th day, whereas ALT and direct bilirubin parameters increased significantly on the day of the 56th. ALT helps convert protein into energy for liver cells. When the liver is damaged, ALT is released into the bloodstream, and its levels increase (Kim et al. 2008). Significant liver damage occurred on day 56th in the T3, whereas in the direct bilirubin parameter, only T4 had a significant effect, followed by day 28th to day 56th; T1, T2, and T3 showed significant differences. A significant increase occurred on day 56th in the T3 group. An increase in bilirubin usually occurs due to an abnormality of erythrocytes in the body. The incidence of increased direct bilirubin values aligns with the relatively low erythrocyte values in the hematologic parameters.

Meanwhile, other parameters such as AST, ALP, total protein, and serum albumin showed a significant increase in the last days of herbal administration or at high doses, as in the ALP parameter. High ALP values only occurred at T3 from day 14th to day 56th, while the other treatment groups did not increase significantly. Based on this finding, it can be concluded that administering nano herbal *Phaleria macrocarpa* can increase the values of several biochemical parameters in the liver. That value is still within the normal range. However, it is recommended that the administration of these nano-herbs be at most one month. This finding is supported by a literature review conducted by Ballotin et al. 2021 regarding Herb-induced liver injury (HILI). They concluded that on average herbal medicines can cure various types of liver disease with average healing of 85–90% of cases depending on the duration of administration and dosage of herbs used. The most effective dose of the nano herbal *Phaleria macrocarpa* in nourishing the liver is a dose of 600 mg/kg BW. Giving this dose within one month can affect changes in the values of several liver biochemical parameters, which are still within the normal range.

Hematological analysis

Fig. 2 shows the result of the blood parameters assessment. The administration of nano-herbal *Phaleria macrocarpa* significantly affects the MCV. A high MCV value in the control group indicates that the size of the red blood cells is more significant than it should be. A high MCV is usually a sign of anemia due to vitamin B12 or folic acid deficiency in the blood (Aslinia et al. 2006). These findings are synchronous with increased direct bilirubin values due to relatively low erythrocyte values in the control group. By administering nano-herbal *Phaleria macrocarpa*, the MCV value in the treatment group can be lowered, and the value is still within the normal range. The increase in the amount of direct bilirubin in the treatment group could be overcome by administering nano herbal *Phaleria macrocarpa* with a long duration of administration of 56 days.

Other blood test results demonstrated no significant difference between the treatment and control groups.

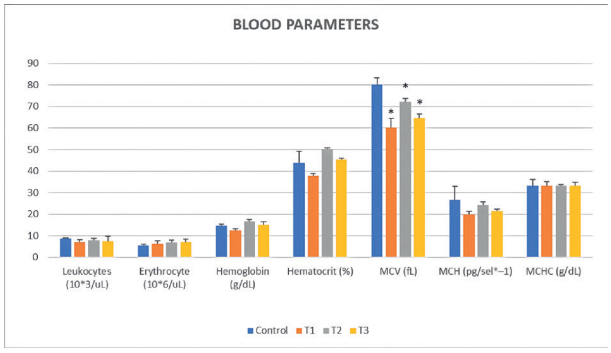

Figure 2. Hematological analysis results.

Although insignificant, there was a change in the value of all types of blood parameters in the administration of nano-herbal *Phaleria macrocarpa*. Through this discovery, it can be concluded that the administration of nano-herbal *Phaleria macrocarpa* did not significantly affect the physiological changes in the blood of the animal test up to day 56th of exposure. This finding was confirmed by previous findings by Verna and Estuningtyas 2022 found that the administration of the *Phaleria macrocarpa* fruit extract did not provide a significant change in the hematologic profile induced by excess iron, and this is the reason why changes in the hematology profile in this study were not significantly different from the controls (Verna and Estuningtyas 2022).

Blood electrolyte parameters

Fig. 3 shows the blood electrolyte parameters. The normal potassium range in the blood is 3.5–5.1 mmol/L, while the sodium is 136–145 mmol/L, and the chloride is 96–106 mmol/L (Pfortmueller et al. 2018). On the day 14th of nano herbal *Phaleria macrocarpa* administration, the potassium level differed significantly compared to the control group. While sodium only showed a significant effect starting on day 42 in the T3 group, followed by T2 on day 56th. Chlorine levels differed significantly from the control group at T2 and T3. The body needs electrolytes to maintain the organ's function. In this study, only 600–900 mg/kg BW of nano herbal *Phaleria macrocarpa* impacted blood electrolyte balances. Calcium, phosphorus, potassium, sodium, iron, zinc, copper, and magnesium are rich in P. macrocarpa (Altaf et al. 2013). When administered to the body, the nano-sized drugs prepared from the nano herbal fruit *Phaleria macrocarpa* improved the drug's solubility, bioactivity, and consistency. However, in high doses, these electrolytes flow into the blood. *Phaleria macrocarpa* enhances medicinal qualities, improves macrophages in the tissue, and improves durability against chemical and physical damage (Ahmad et al. 2023).

Kidney biochemical parameters

Fig. 4 shows kidney biochemical parameters. UREA/BUN average level is 16–30 mmol/L. This increase that exceeds normal limits occurs at 900 mg/kg BW on day 56th. However, this increase is still within normal limits. The standard value of serum creatinine levels in mice is 0.578–1.128 mg/dl. The increase in creatinine levels towards abnormal began on day 42nd in all treatment groups. Day 56th shows that the value indicates loss of kidney function. The same thing happened to the uric acid parameter, where an abnormal increase in uric acid values had also started to occur on day 42nd. Even though the measurement on day 28th had shown a significant increase, this value was still categorized as usual. Blood uric acid levels in mice ranged from 1.2–5.0 mg/dL. The levels of urea, blood-urea-nitrogen (BUN), creatinine, and ammonia in the kidneys are closely linked to the amount quality of eat and drink consumed (Chen et al. 2020), and this might be caused by the metabolizing process of nano herbal *Phaleria macrocarpa* in the cells, as proven by waste products filtered out by the urinary system.

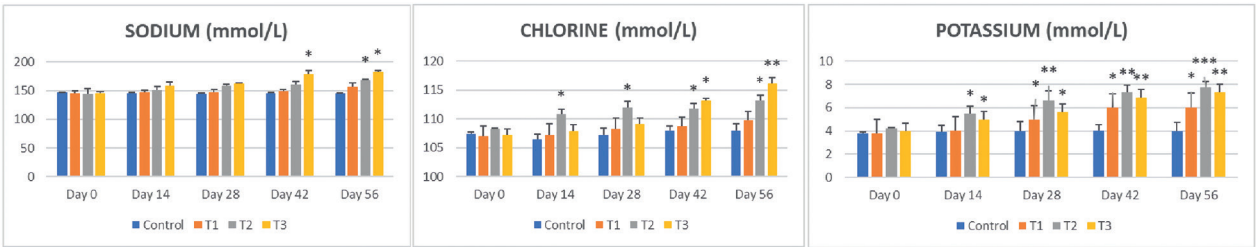

Figure 3. Electrolyte parameters.

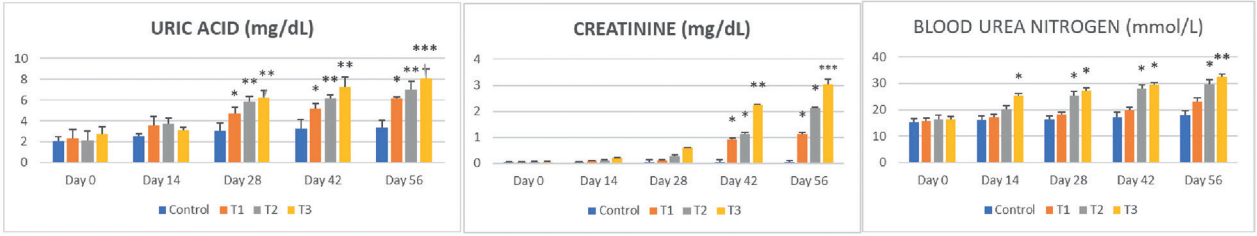

Figure 4. Kidney biochemical results.

Histology of the organs after giving nano herbal *Phaleria macrocarpa*

Liver histopathology

In Table 1, hepatocyte cells, parenchymatic degeneration, hydropic degeneration, and necrosis indicated a significant difference. A higher dose affects the average amount of hepatocyte cells after 56 days of exposure to nano herbal *Phaleria macrocarpa*, as shown in Fig. 5 (A1–A2). Meanwhile, other parameters, such as liver tissue damage parameters characterized by parenchymal degeneration, hydropic degeneration, and the number of necrotic cells, only occur at T2 and T3. The T1 group worked very well to increase the number of normal hepatocyte cells, reduce other mild symptoms of liver damage, such as parenchymal and hydropic degeneration, and reduce the necrotic cells (Table 1). Degeneration is reversible, so the 300 mg/kg BW dose of nano herbal *Phaleria macrocarpa* improved the histological picture of the liver of *Mus musculus* better than the control group. Degeneration can result from aging and caused by disease. The aging process can occur as a result of exposure to free radicals. Other causes that can cause degeneration are injuries, reduced blood supply, poisoning such as certain substances, and accumulation of exposure to high doses of substances (Lobo et al. 2010).

Table 1. Degree in liver histology.

Group	Hepatocytes (400×)	Degeneration of the parenchyma (%)	Hydropic degeneration (%)	Necrosis (%)
Control	376.23±4.24	22.32±2.44	21.12±1.25	2.82±2.44
T1	382.07±7.21	20.24±2.71	16.61±2.72	2.72±1.07
T2	111.57±6.23*	44.34±2.63*	45.22±3.11*	8.77±3.80*
T3	78.17±9.22**	66.73±2.92*	52.11±1.21*	12.12±3.62*

Data: Mean ± SEM.
*Significantly different from the control.

Lung histopathology

Lung histology evaluates three criteria: inflammation, parenchymal degeneration, and the lumen narrowing of the alveolar. The level of inflammation was: average/0, light/1, medium/2, severe/3. Parenchymal degeneration level was: average/0, 0–30% damage/1, 31–60% damage/2, >61% damaged/3. Narrowing of the alveolar lumen level was 0; average/0, 0–30% narrowing/1, 31–60% narrowing/2, > 61% narrowing/3. Level 1 inflammation, level 1 parenchymal damage, and level 2 alveolar lumen narrowing occurred in the control group, as shown in Fig. 5 (A1). Administration of a dose of 300 mg/kg BW (T1) reduced the level of inflammation to level 0, the level of parenchymal damage to level 0, and the narrowing of the alveolar lumen to level 1, which can be seen in Fig. 5 (B2). The histological features of the lungs on T2 and T3 have similarities in inflammatory conditions and parenchymal damage, both at level 2. The difference lies in the narrowing of the alveolar lumen, where the highest level of narrowing occurs in the group given a dose of 600 mg/kg BW (T2).

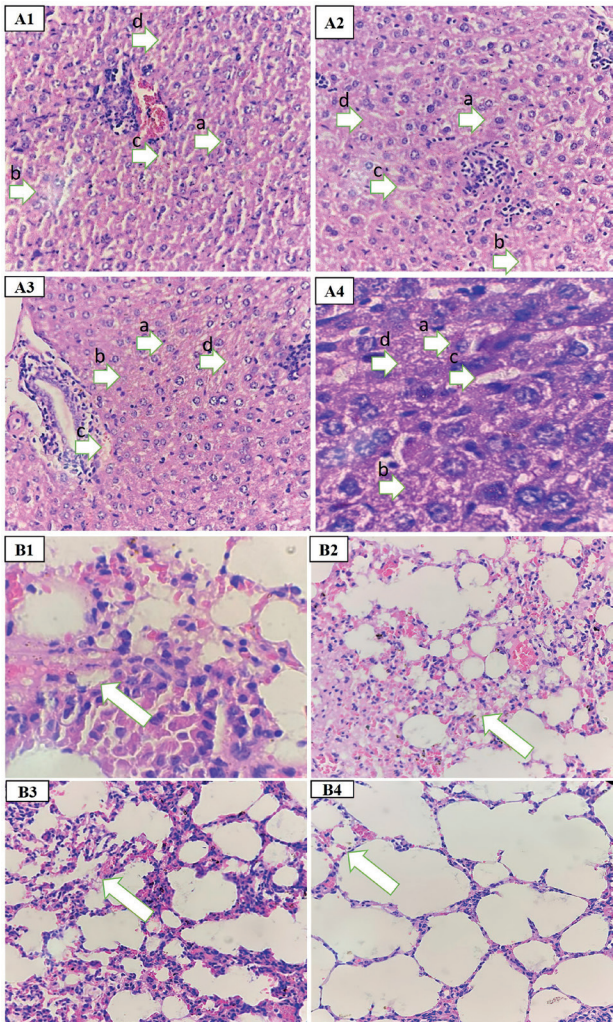

Figure 5. Histology of liver and lung. **A** for Liver; (A1) Control, (A2) T1, (A3) T2, (A4) T3 **B** for Lung; (B1) Control, (B2) T1, (B3) T2, (B4) T3. a. normal hepatocyte, b. parenchymal degeneration, c. hydropic degeneration, d. necrotic cells (40×).

The inflammation structure was linked to the fact that the terminal of bronchioles, alveolar tubes, and adjacent alveoli were the locations of the most significant inhaled tiny particle (Hogg et al. 2017). According to the correlation among alveoli in the control group, the matrix surrounding cells comprises damaged collagen and elastin fibers. In addition, the alveolar lumen appeared distinctly, and injury to both endothelial and epithelial cells caused damage to the structure of the pulmonary microanatomy.

Macrophages, neutrophils, and eosinophils detoxify free radicals. Therefore, an excessive rise in the airway might lead to the migration of neutrophils, macrophages, and eosinophils, triggering an inflammatory response and increasing death cells (Caliri et al. 2021). Compared to the LD50 acute toxicity test of the nano herbal *Phaleria macrocarpa* by Simanjuntak and Rumahorbo 2022, the dose-induced variation initiated significant differences in the pulmonary anatomic characteristics between the three experimental groups. That finding stated that there was no significant effect between the administration of nano herbal *Phaleria macrocarpa* on changes in lung histology,

Table 2. Lungs damages degree.

Treatments	Alveolar inflammation	Parenchymal damage (%)	Narrowing of the lumen alveolar
Control	1 ± 0.00	1 ± 0.00	2 ± 0.00
T1	0 ± 0.00	0 ± 0.00	1 ± 0.00
T2	2 ± 0.00	2 ± 0.00	3 ± 0.00
T3	1 ± 0.00	2 ± 0.00	2 ± 0.00

closely related to the duration of herbal administration (Simanjuntak and Rumahorbo 2022). In the acute toxicity test, the administration took 30 days, while in the chronic toxicity test, the administration took 56 days. So, the effect is more pronounced.

Kidney histopathology

The histopathological characteristics of the renal system were evaluated using the following assessment method.

According to Table 4, serious injury was discovered in T2's kidneys relating to Table 3. The control group had mild kidney abnormalities in the glomerulus, endothelium, and tubulointerstitial level 1, as shown in Fig. 2 (C1). However, the degree of abnormality was reduced in the T1 group after exposure to 300 mg/kg BW nano-herbal fruit flesh for 56 days, as shown in Fig. 6 C2. Therefore, it is highly not recommended to give doses of more than 600 mg/kg BW nano-herbal crown fruit pulp for ≤56 days because it causes inflammation of the kidney tubules and increases the number of necrotic cells, as seen in Fig. 6 (C3 and C4).

Table 3. The evaluation method of kidney assessment.

Assessed tissue	Description	Score
Tubular	No damage	0
	<25% loss of the brush border, basement membrane intact.	1
	>25% loss of the brush border, basement membrane thickening	2
	<60% necrotic tubular cells	3
	>60% necrotic tubular cells	4
Endothelium	Normal	0
	Endothelial cell swelling	1
	Endothelial dysfunction	2
	Loss of endothelial	3
Glomerulus	Normal	0
	Thickening of capsule Bowman	1
	The juxtaglomerular apparatus retracts	2
	Glomerular fibrosis	3
Tubulointerstitial	Normal	0
	Inflammation (<25% tissue bleedings)	1
	Necrosis (25% tissue bleeding)	2
	60% Necrosis	3
	>60% Necrosis	4

Table 4. Kidney damage degree.

Treatments	Tubular	Glomerulus	Endothelium	Tubulointerstitial
Control	0	1	1	1
T1	0	0	0	1
T2	2	2	1	2
T3	1	1	2	1

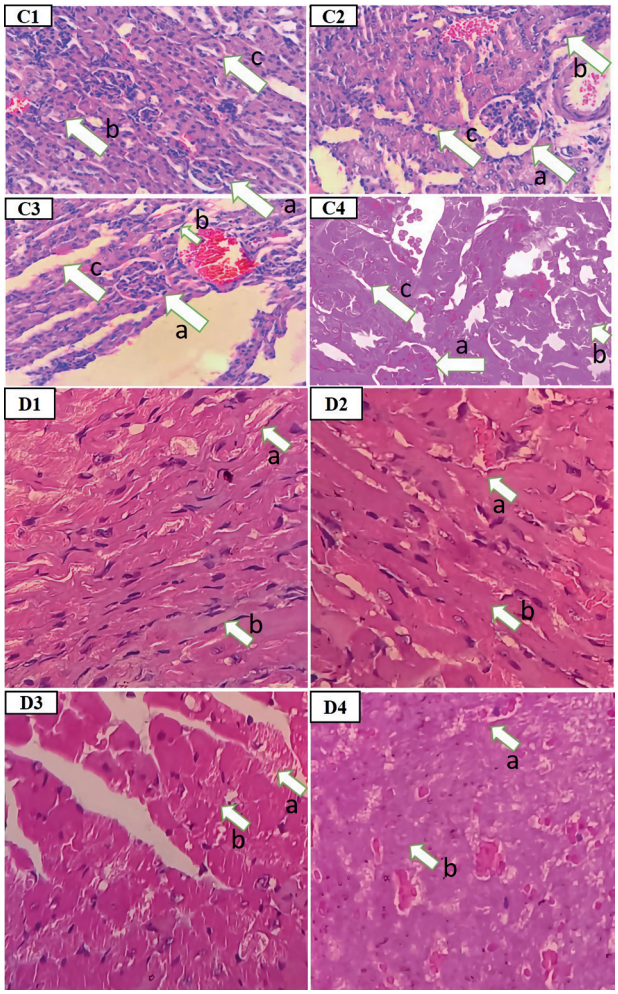

Figure 6. Histology of Cardiac and Kidney. **C** for Kidney; (C1) Control, (C2) T1, (C3) T2, (C4) T3 (a. tubular, b. endothelium+-glomerulus, c. tubule-interstitial). **D** for Cardiac; (D1) Control, (D2) T1, (D3) T2, (D4) T3 (a. parenchymal and vacuolar degeneration, b. necrosis) (40×).

The renal system plays a vital role in sustaining body stability by managing the body's fluids, ions, and acidic substances by conducting filtering blood (Shioji et al. 2016). Death cell is a type of toxicity that frequently occurs in the renal. Death cells might be triggered by several factors, such as solid contaminants (e.g., phosphorus, poisonous mushrooms such as arsenic, and others), metabolic disorders, and infection by viruses leading to the aggressive form, also known as a cancerous virus (Beaumier et al. 2019). Given the moderate toxicity of the nano herbal flesh fruit *Phaleria macrocarpa*, long-term tested dose exposure may cause damage to tissues.

Cardiac histopathology

Table 5 shows cardiac damage after giving the nano herbal *Phaleria macrocarpa*. In all cardiac histology parameters, there were significantly differed between the control group and the T3 group. In the same conditions as some of the previous parameters, the T1 group also improved the histological picture of the heart of the *Mus musculus* exposed to nano herbal *Phaleria macrocarpa* during 56

Table 5. The cardiac damages level.

Group	Normal cardiocytes	Parenchymal degeneration (%)	Vacuolar degeneration (%)	Necrosis (%)
Control	56.78±5.41	28.91±8.21	27.23±4.91	12.34±3.72
T1	55.17±7.14	12.23±9.43*	17.94±5.13	16.23±2.73
T2	25.98±7.54*	36.13±4.98	30.21±6.52	47.24±6.74*
T3	15.21±8.95**	61.13±11.56*	56.21±7.22*	32.22±9.32*

days of administration. Histology of cardiac cells, as seen in Fig. 6 (D1-D4), shows that at T2 and T3, the pattern of parenchymal and hydropic degeneration and the death cell case turned uncertain, and cell density decreased. Autoimmune inflammation targets the heart muscle. Age, thymoma, and anti-CV1 antibodies are hazards that may lead to cardiac disease being detected. With the correct amount and terms of drug administration, the compounds of this herb could potentially be used as an anti-oxidant by inhibiting the production of reactive oxygen species (ROS), which cause malignancies and different kinds of inflammation (Bracamonte-Baran and ihákova 2017).

The nano herbal *Phaleria macrocarpa* can improve the physiology and the histology of the animal test, referring to the phytochemical content of this plant and the improvisation of the particle size to be a nano size. *Phaleria macrocarpa* fruit chemical composition revealed that the seed shell and flesh fruit of *Phaleria macrocarpa* obtained flavonoid substances, phenols, saponins, tannins, and sterols/terpenes from herbal extracts of hexane, ethyl acetate, and methanol (Altaf et al. 2013). Isolation is used to obtain lignans, a class of polyphenols with recognized structures believed to be toxic to cells. Other particular bioactive compounds derived from *Phaleria macrocarpa* include Phalerin, the acid gallic, Icaricide C, mangiferin, mahkoside A, dodecanoic acid, acid palmitic, desacetylflavicordin-A, flavicordin-A, flavicordin-D, flavicordin-A glucoside, ethyl stearate, and others. The nano size is expected to overcome the kinetics of the material's entry into the cell membrane. Nano herbs have a higher loading capacity and a smoother surface, enabling them to be administered in higher quantities (Ajazuddin and Saraf 2010).

Further, based on our review of the literature, the crude extracts of *Phaleria macrocarpa* fruit that have been evaluated preclinically in previous studies, obtained by the process of extraction that passed by an excessive heating stage, reached temperatures of more than 60 °C. These techniques can harm the bioactive substances found within them, reducing their effectiveness. These issues can be solved using nanotechnology, which does not use any heating process. Medicinal plants are also suggested because they are less likely to trigger side effects than synthetic medications (Karimi et al. 2015).

Conclusion

Administration of nano herbal *Phaleria macrocarpa* dose on chronic toxicity improves the liver, kidneys, lungs, and heart histology. In addition, it improves the body's physiological function through the parameters of complete blood count, liver and kidney biochemical, and blood electrolytes. The range of doses that do not cause symptoms of chronic damage is 300–600 mg/Kg BW, and the duration of administration does not exceed a month.

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