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Full Length Article

Chronic Oral Exposure to *V. gracilis* Nanoherbs: Toxicity Assessment

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Abstract

The nanoherb *Vitis gracilis* (Guill. & Perr.) Baker has traditionally been used by farmers to increase their stamina and has recently begun to be scientifically researched by researchers. Here, we analyze the chronic toxicity of *V. gracilis* nanoherbs and examine their effects on hematological, biochemical, and histopathological conditions of organs. *V. gracilis* nanoherbs are made with High Energy Milling (HEM) technology. Research treatment with doses of *V. gracilis* nanoherbs at 100, 200, and 300 mg/kg body weight for 56 days. Mouse blood was then collected to determine mouse blood parameters (hematology), as well as liver and kidney biochemistry associated with various organs, such as histopathology of the liver, kidneys, heart, brain, and lungs. We found that *V. gracilis* nanoherbs affected histopathological, hematological, and biochemical parameters of organs, at an optimum dose of 100–200 mg/kg body weight. Duration of the grant limitation of this nanoherb is one month. We conclude that the *V. gracilis* plant may be able to be used as a medicinal herb in the form of nanoherbs in the future, with a maximum dose and period of time of 100–200 mg/kg body weight per day and one month, respectively.

Keywords: Chronic toxicity; Histopathology; Nanoherbs*; Vitis gracilis*
Abbreviations: BCL-2; B cell lymphoma family protein 2, MCH; Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, Hb: Hemoglobin, RBC; red blood cell

Introduction

Indonesia has a heterogeneous distribution of plants and is rich in medicinal plants. One such plant is *Vitis gracilis*, known locally as "Gagatan Harimau," which has been empirically shown to benefit bodily health. “Gagatan harimau" grows in the karo land of North Sumatra Province, Indonesia and is believed to treat various diseases, being used as an energy enhancer, stomach pain medicine, diarrhea medicine, malaria drug, propping up hunger, and diabetes treatment. Scientific research has not proven much effectiveness of tiger gagatan leaves as medicine. The main ingredients of *V. gracilis* are diphyllin (arylnapthalene, 16.49%), dysolenticin B (triterpenoid, 10.48%), 2-[2-(2-butoxyethoxy) ethoxy] ethanol, 2- (2-methoxypropoxy)propan-1-ol (alcohols, 7.31%), ethyl-4-hydroxybenzoate, ethylparaben (hydroxybenzoic acid, 6.70%), isomasticadienonalic acid (triterpenoids; 5.97%), kinoprene (fatty acids, 5.02%), linolenic acid (fatty acids, 4.92%), terpinyl isobutyrate (monoterpenoids, 4.62%), scortechinone B (xanthones, 4.43%), and 1-monolinolenoyl-rac-glycerol (glycerolipids, 4.23%) (Ilyas *et al.* 2023).

In addition to the influence of active substances on plants, the effectiveness of *V. gracilis* can also be determined based on the size of herbal particles, which facilitate entry of herbal material into cells through cell membranes. Thus, reducing particle size to the size of nanomaterials can accelerate the effect of herbs. Aside from controlling size of the material, the determination of appropriate dosages should be another major target in ensuring the effectiveness of herbal remedies.

Toxic effects have sometimes appeared following chronic toxicity test treatment, indicating evidence of toxic activity shown after a set time of research trial (i.e., more than one month) using experimental animals as models. OECD (2018) stated that the adaptation period of research is

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at least two months for test materials that are considered generally safe, and as long as six months for samples with harmful effects. The main targets of chronic oral toxicity testing are: to obtain concrete evidence of the toxic effects of a test material that is not clearly detected in acute toxicity tests; evaluate the possibility of toxic effects after repetition of treatment during the adaptation study period in a certain period at doses that do not cause toxic effects (No Observed Adverse Effect Level/NOAEL); and to deepen the cumulative influence and reversibility influence of these materials. Therefore, in this study, chronic toxicity tests were carried out with nanomaterials made from *V. gracilis* leaves.

Materials and Methods

Materials

V. gracilis plants were collected from Tangkahan in Batang Serangan Regency, North Sumatra Province, Indonesia (coordinates X = 395928.28, Y = 408308.48). The plant was identified by the Herbarium team of the Plant Systematics Laboratory of the University of North Sumatra (USU; Voucher number 201/MEDA/2023), verifying that the collected plants were *V. gracilis* (Ilyas *et al.* 2023). First, fresh *V. gracilis* plant was washed with running water. Then, it was dried and aerated with no direct sunlight for one week. Next, the dried *V. gracilis* was ground to a coarse powder. In the activator solution, the activator HCl 2 M was ground with a high-energy round (Tokyo, Japan). For grinding, the ratio for mass was 20:1 (balls in machine: powder mill) with grinding periods ranging from 3, 6 and 9 hours from the end of the sign of the presence of *V. gracilis* nanoherbal leaf flesh (Santoso *et al.* 2023).

Animals

This study used 20 male rats (*Mus musculus*) aged 8–11 weeks with body weight of 25–30 g (Ilyas *et al.* 2023), which were physically healthy and anatomically normal, obtained from the Animal House at the Faculty of Mathematics and Science, Universitas Sumatera Utara, Indonesia. They were then separated into four groups of T0, T1, T2 and T3. Before conducting the study, all mice were adapted to experimental cages for seven days with ad libitum administration of drinks and food. Health of the mice was maintained until the study began; they did not experience any weight change exceeding 10%, and it was clear that they continued to behave normally. The dosages of test preparations in experimental animals were determined based on the results of previous research, and set at the LD50 value of 315.071 mg/kg body weight. Test doses were also determined, such as Control/0[T0], 100[T1], 200[T2], and 300[T3] mg/kg body weight. Testing was conducted for 56 days with doses administered once per day in the morning orally with an oral needle[2]. Provision of test materials in the form of nano-powder dissolved in CMC 1:1 with the help of vortex mixers. The control group was given a solution of CMC-NA with standard feed. Histopathological observations of the liver and kidneys were conducted on days 0, 14, 28, 42 and 56. Blood counts and histopathological analysis of the heart, brain, and lungs were completed on day 56, after mice were euthanized. This study was approved by the Health Research Ethics Committee of USU Medan (No. 0395/KEPH-FMIPA/2023).

Physiological analysis

Measurements of complete blood counts resulting from the physiological effects of herbs, as well as kidney and liver function, were analyzed using a COBAS 6000.

Histopathology

After fixation with formalin buffer, the liver, kidney, heart, brain, and lung specimens were washed under running water and soaked with serial alcohol for 5 mins at 70, 80, 90% and then with pure alcohol. Soaked with alcohol solution: xylen (ratios 3:1, 2:1, and 1:1) and xylene for 5 min each. Organs were then infiltrated with hard paraffin at a temperature of 56–58°C (2 h) in an incubator. Paraffin blocks formed when removed from the incubator. Paraffin blocks 5–7 µm thick were then cut and placed in warm water (56–58°C), then the paraffin tape object was taken with a glass so that it could stick to the appropriate place and not fold the tissue. Next, tissue staining with hematoxylin and eosin was performed. The tissue was immersed in xylene solution for 15 min and then immersed in series in 100, 90, 80 and 70% ethanol for 5 min at each concentration to cause dehydration. Then, rehydration was performed through washing in running water. Samples were then put in a hematoxylin solution for 5 min before being washed under running water, stained with eosin, and then submerged in 70, 80, 90% and pure ethanol for 10 min each. Finally, samples were put in xylene for 5 sec before being covered with glass. The sample was then ready to examine under a microscope at a magnification of 400x (Ilyas 2018).

Data analysis

Data were collected and analyzed using Microsoft Excel 2021 and SPSS 24. For numerical data that were normally distributed and homogeneous, a variance analysis (ANOVA) was carried out to determine significant differences in blood hematology parameters. Ordinal histopathological data were analyzed with a Kruskal-Wallis test to determine differences between treatment groups. A 95% confidence interval was used for tests with a test significance level of 5%. If the test result was $P < 0.05$, then we proceeded with the Duncan test for ANOVA or the Mann-Whitney U test for Kruskal-Wallis (Ilyas *et al.* 2018).

Table 1: Liver biochemical parameters

G	AST (U/L)					ALT (U/L)				
	0th days	14th day	28th day	42th day	58th day	0th days	14th day	28th day	42th day	58th day
T0	42.80±1.92^{a}	44.00±3.54^{a}	44.80±2.95^{a}	46.60±4.83^{a}	49.20±4.32^{a}	38.00±3.81^{a}	45.20±5.26^{a}	46.00±2.30^{a}	48.00±4.85^{a}	47.40±7.54^{a}
T1	43.60±4.39^{a}	45.40±2.30^{a}	45.80±1.92^{a}	48.60±7.67^{a}	51.40±9.61^{ab}	42.80±2.39^{a}	46.20±4.15^{a}	47.60±2.79^{a}	57.00±8.57^{b}	60.60±1.82^{b}
T2	44.00±2.92^{a}	46.60±1.82^{a}	47.40±3.21^{a}	49.20±5.97^{a}	55.20±2.59^{bc}	46.40±3.58^{a}	47.80±2.70^{a}	51.20±2.17^{a}	59.40±2.19^{b}	63.20±7.66^{b}
T3	45.00±3.39^{a}	46.20±2.39^{a}	49.00±3.32^{a}	50.40±3.65^{b}	59.20±4.92^{c}	50.00±5.83^{a}	51.60±7.73^{a}	53.00±2.55^{a}	60.20±6.10^{b}	65.00±1.58^{b}
G	ALP (U/L)					Total protein (g/L)				
	0th days	14th day	28th day	42th day	58th day	0th days	14th day	28th day	42th day	58th day
T0	45.4±1.82^{a}	45.6±5.54^{a}	44.8±1.00^{a}	44.8±2.24^{a}	48.4±3.16^{a}	45.4±0.89^{a}	45.6±1.95^{a}	44.8±3.70^{a}	44.8±2.95^{a}	48.4±7.02^{a}
T1	46.2±3.27^{ab}	46.6±4.21^{ab}	46.4±1.48^{a}	54.2±3.27^{b}	64.2±3.51^{b}	46.2±1.30^{b}	46.6±5.27^{a}	46.4±2.61^{a}	54.2±9.58^{b}	64.2±2.77^{a}
T2	45.4±2.17^{ab}	47.2±5.26^{ab}	47.6±2.39^{a}	59.2±2.88^{c}	68.2±4.56^{c}	46.4±0.55^{c}	47.2±4.09^{b}	47.6±3.21^{b}	59.2±3.96^{b}	68.2±5.81^{a}
T3	46.6±2.30^{b}	48.8±2.51^{b}	48.6±3.71^{b}	62.8±0.84^{d}	77.2±5.59^{d}	46.6±0.89^{c}	48.8±7.79^{b}	48.6±5.77^{b}	62.8±2.17^{b}	77.2±2.49^{b}
G	Bilirubin Direct (mg/dL)					Albumin (g/dL)				
	0th days	14th day	28th day	42th day	58th day	0th days	14th day	28th day	42th day	58th day
T0	0.32±0.07^{a}	0.34±0.08^{a}	0.35±0.02^{a}	0.36±0.04^{a}	0.41±0.03^{a}	19.20±1.30^{a}	19.80±0.84^{a}	21.80±2.39^{a}	22.20±1.92^{a}	23.00±1.30^{a}
T1	0.34±0.04^{a}	0.36±0.22^{a}	0.36±0.50^{a}	0.42±0.01^{ab}	0.43±0.02^{a}	20.60±1.95^{a}	21.00±1.58^{a}	22.00±2.74^{a}	23.80±1.79^{ab}	24.00±1.92^{a}
T2	0.34±0.01^{a}	0.37±0.01^{a}	0.37±0.04^{a}	0.56±0.15^{bc}	0.57±0.17^{b}	21.40±1.52^{ab}	21.80±2.05^{ab}	22.20±1.10^{a}	24.20±1.00^{ab}	25.00±1.58^{a}
T3	0.35±.0.03^{a}	0.38±0.05^{a}	0.38±0.05^{a}	0.63±0.16^{c}	0.76±0.11^{c}	22.20±2.17^{b}	23.80±1.30^{b}	24.40±1.67^{a}	25.60±1.34^{b}	29.80±1.92^{b}

Note: p^{a,b}<0,05 between groups (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 mg/kg BW)

Results

Hematological analysis

Table 1 shows various blood measurements following the administration of *V. gracilis* nanoherbs. A significant decrease (P<0.05) was observed in all blood variables except red blood cells (erythrocytes), with the reductions continuing after 56 days of treatment. Notably, a significant reduction in MCH and MCHC occurred on day 56 at a dose of 300 mg/kg body weight, while no significant (P>0.05) difference was seen at doses of 100 and 200 mg compared to the control. Leukocyte counts showed a significant decrease at the 300 mg/kg dose, but there was no significant (P>0.05) difference at the lower doses of 100 and 200 mg doses compared to the control.

Liver biochemistry

Fig. 1 presents measurements of liver biochemical parameters, including Aspartate Aminotransferase (AST), Alanine Aminotransferase (ALT), Alkaline Phosphatase (ALP), total protein, bilirubin, and albumin, which reflect liver physiological function under the influence of *V. gracilis* nanoherb exposure. A comparison of the control group (T0) and treatment groups (T1, T2, and T3) revealed significant (P < 0.01) differences on 42 and 56 days. Notably, ALT and direct bilirubin levels showed significant increases on both days 42 and 56, particularly in group T3 on day 56. ALT is known to be released into the bloodstream when liver damage occurs, and its increase is a marker of worsening liver damage. Furthermore, erythrocyte abnormalities were linked to elevated bilirubin levels, with the most significant increase seen in group T4.

Significant increases in AST, ALP, total protein, and serum albumin were observed at higher doses towards the end of the herbal administration, particularly in group T3. Elevated ALP levels were unique to this group, appearing between 14 and 56 days, while other treatment groups showed no significant increases in ALP.

Table 2: Uric Acid Content (mg/dL)

Groups	0th day	14th day	28th day	42nd day	56th day
T0	2.40±0.55^{a}	2.80±0.45^{a}	3.00±0.71^{a}	3.00±0.71^{a}	3.40±0.89^{a}
T1	2.80±0.84^{a}	3.00 ±0.71^{a}	3.20±1.10^{a}	5.00±1.22^{b}	5.40±0.55^{b}
T2	3.00±0.71^{a}	3.00±0.71^{a}	4.40±0.55^{b}	5.80±0.84^{b}	6.20±1.30^{b}
T3	3.20±0.84^{a}	3.40±0.55^{a}	5.60±0.89^{c}	6.20±0.84^{b}	8.40±0.55^{c}

Note: p^{a,b}<0,05 between groups (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 mg/kg BW)

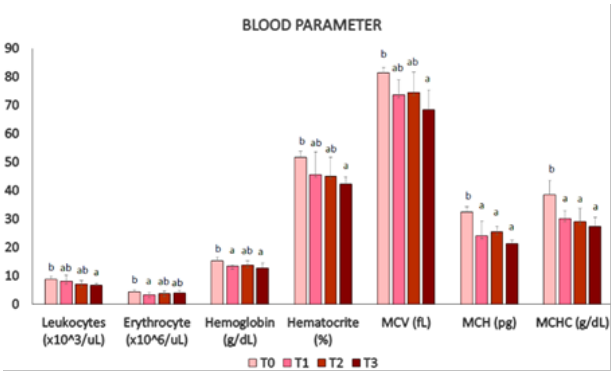

Fig. 1: Results of analysis of rat blood parameters (hematology) after 56 days. Note: pa,b<0.05 between groups (T0-T3)

Biochemical parameters of the kidneys

Table 2 shows the renal biochemical parameters from day 0 to day 56. The mean uric acid level in control mice (T0) ranged between 2.40±0.55 and 3.40±0.89 mg/dL. A significant increase in uric acid levels was observed on days 28, 42 and 56, particularly in the group receiving 300 mg/kg body weight of *V. gracilis* nanoherbs. Similarly, serum creatinine levels, within the accepted range of 2.40–3.40 mg/dL (Table 3), exhibited abnormal increases starting from day 42 in almost all treatment groups. By day 56, these elevated levels suggested a decline in renal function.

The urea/BUN levels, typically 10–30 mmol/L (Table 4), also exceeded the standard limit at the 300 mg/kg body weight dose on days 28, 42 and 56. Uric acid parameters showed abnormal increases beginning on day 28 at the highest dose. At the same time, a significant improvement was seen on day 28 at a dose of 200 mg/kg, although this level remained elevated.

Histology of organs after administration of *V. gracilis* nanoherbs

Liver histopathology: In Table 5, there were significant differences in hepatocyte cells, parenchymal degeneration, and necrosis, with the exception of hydropic degeneration. Higher doses (H-T0 to H-T4) affected the average number of hepatocytes after 56 days of exposure to the nanoherb *V. gracilis* (Fig. 2). In particular, the degeneration of the parenchyma was more pronounced in the H-T3 group. The activity of *V. gracilis* nanoherbs at 100 and 200 mg/kg BW in group H-T1 appeared to greatly increase the number of normal hepatocytes, in reducing other mild symptoms of liver damage such as parenchymal degeneration, as well as in reducing the number of necrotic cells (Table 5). Necrotic degeneration was irreversible. Therefore, high doses (300 mg/kg BW) of the nanoherb *V. gracilis* did not improve the histological characteristics of the mice liver more than the control group. Degeneration can also be the result of cellular aging and disease. The ageing process can take place as a result of the exposure of the cells to free radicals. Injury, reduced blood supply, poisoning by certain substances, and accumulated exposure to high doses of substances can also cause degeneration (Hernandez-Gerez *et al.* 2019) (Table 6).

Pulmonary histopathology: In the histology of the lungs, three criteria were assessed, namely: inflammation, degeneration of the parenchyma, and narrowing of the alveolar lumen. Inflammation was determined to be average (0), mild (1), moderate (2), or severe (3). The degree of parenchymal degeneration was determined to be average (0), 0-30% damage (1), 31-60% damage (2), or >61% damage (3). The degree of narrowing of the alveolar lumen was determined to be 0, average (0), 0–30% narrowed (1), 31-60% narrowed (2), or > 61% narrowed (3). As showen in Fig. 2, in the control group(T0), various conditions of the inflammation level (1), parenchymal damage level (1), and narrowing of the alveolar lumen level (2) were found. L (T1), administration of a dose of 100 mg/kg BW L(T1) reduced the degree of inflammation to level (1), the degree of parenchymal damage to level (1), and the narrowing of the alveolar lumen to level (1). The histological features of the lungs in L(T2) and L(T3) were similar in terms of inflammatory conditions and parenchymal damage, both being at level 2. The difference was found in the narrowing of the alveolar lumen, with the greatest degree of narrowing occurring in the 300 mg/kg body weight group (L-T2).

Renal histopathology: The histopathological assessment of the kidneys is summarized in Fig. 2, with detailed results presented in Table 7. In the control group (K-T1), mild renal abnormalities were observed, including glomerular, endothelial, and tubulointerstitial changes, all classified at level 1. Following exposure to the *V. gracilis* nanoherb at a dose of 100 mg/kg body weight (T1) for 56 days, the rate of

Table 3: Cratinine Content (mg/dL)

Groups	0th day	14th day	28th day	42nd day	56th day
T0	1.40±0.55^{a}	1.60±0.55^{a}	1.80±0.84^{a}	2.00±0.71^{a}	1.60±0.55^{a}
T1	1.60±0.55^{a}	1.80±0.45^{a}	1.80±0.84^{a}	2.00±0.71^{a}	2.00±1.00^{a}
T2	2.00±0.71^{a}	2.00±0.71^{a}	2.20±0.45^{a}	2.20±0.84^{a}	2.20±0.84^{a}
T3	2.20±0.45^{a}	2.40±0.55^{a}	2.60±0.55^{a}	2.60±0.55^{a}	3.40±0.55^{b}

Note: $p^{a,b}$<0,05 between groups (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 mg/kg BW)

Table 4: Blood urea nitrogen (BUN) mmol / L) content

Groups	0th day	14th day	28th day	42nd day	56th day
T0	10.40±1.14^{a}	11.40±1.52^{a}	11.60±1.14^{a}	12.60±1.14^{a}	13.40±1.95^{a}
T1	12.00±1.22^{ab}	12.40±1.14^{ab}	12.80±1.30^{ab}	19.00±3.81^{b}	26.20±5.67^{b}
T2	12.60±1.34^{b}	12.80±1.30^{ab}	13.40±1.14^{ab}	28.80±3.19^{c}	30.60±2.30^{b}
T3	13.00±1.41^{b}	14.00±1.00^{b}	14.40±1.52^{b}	33.20±1.92^{d}	35.40±1.95^{c}

Note: $p^{a,b}$<0,05 between groups (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 mg/kg BW)

Table 5: Liver Histology Degree

Groups	Hepatocytes (400×)	Parenchyma degeneration (%)	Hydropic degeneration (%)	Necrosis (%)
T0	380.00±10.68^{a}	21.80±2.39^{a}	21.20±2.39^{a}	2.74±0.18^{a}
T1	374.60±17.10^{a}	22.60±2.70^{ab}	23.00±1.58^{ab}	3.34±0.69^{ab}
T2	252.60±21.24^{b}	23.80±1.30^{ab}	24.00±4.30^{ab}	5.36±2.88^{bc}
T3	243.00±6.04^{b}	25.80±2.77^{b}	26.60±3.91^{b}	6.74±1.03^{c}

Note: $p^{a,b}$<0,05 between groups (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 mg/kg BW)

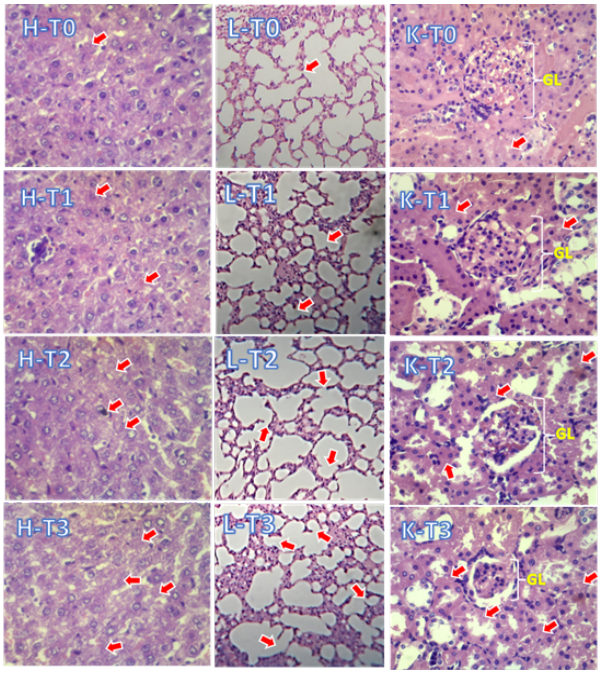

Fig. 2: Histology of liver (H) and lung (L), kidney (K). Description: (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 kg mg/BW). Red arrow = necrosis, green arrow = Purkinje. GL cells: glomerulus, (400×)

renal damage was slightly lower compared to the control group, as illustrated in Fig. 2 (K). However, higher doses (T2 and T3) resulted in significant renal tubular inflammation and an increase in necrotic cell numbers, as depicted in Fig. 2 (K-T2 and K-T3).

Purkinje cell histopathology: Table 8 and Fig. 3 show the number of Purkinje cells after administration of the nano herb *V. gracilis*. The number of Purkinje cells in the treatment group at the end of the observation day 56 decreased significantly when compared to control (T0) at T1 (100 mg/kg BW), T2 (200 mg/kg BW), and T3 (300 mg/kg BW); respectively, their values were 140.80 ± 4.21, 131.80 ± 5.07, and 124.20 ± 3.11 of cells/field of view. This decrease in number was due to the presence of Purkinje cells that died either by apoptosis or necrosis.

Discussion

A significant decrease in hematological parameters, except for red blood cells, suggests that *V. gracilis* nanoherbs, particularly their flavonoid content, might affect the bone marrow where red blood cells are formed. Flavonoids benefit human health, and some studies suggest they improve bone health, including promoting bone marrow growth (Navolokin *et al.* 2017). However, the effects of flavonoids on bone marrow growth are still a subject of ongoing investigation. The decrease in MCH and MCHC at 300 mg/kg dose implies potential interference in hemoglobin formation, possibly due to flavonoids affecting iron absorption (Wang *et al.* 2021).

A significant reduction in leukocyte counts at 300 mg/kg dose could indicate an effect on immune cells. However, the absence of significant changes at lower doses suggests that certain enzyme activities, such as cytochrome c related to mitochondrial functions, were not disrupted, allowing the leukocytes to avoid cell death. These findings align with previous studies, which showed no significant effects of *V. gracilis* at 100 mg/kg in active rats (Wasnis *et al.* 2022). Administering *V. gracilis* nanoherbs induced notable physiological changes in blood parameters, particularly at higher doses, supporting earlier reports of its pro-apoptotic effects through caspase-3 activation and increased interleukin-6 levels.

The results demonstrated that *V. gracilis* nanoherbs significantly increased liver enzyme levels, such as ALT and bilirubin, which are markers of liver function and potential damage. ALT plays a role in energy production from protein molecules, and its elevation is associated with liver damage, as noted in previous studies (Ballotin *et al.* 2021; Saito *et al.* 2022). The increased direct bilirubin in group T3 may indicate erythrocyte abnormalities, likely resulting from impaired bilirubin metabolism.

A significant rise in several liver biochemical parameters, including AST and ALP, toward the end of the treatment suggests that prolonged exposure to *V. gracilis* nanoherbs, particularly at higher doses, can stress liver

Table 6: Degree of damage to the lungs

Groups	Alveolar	Damage to parenchyma (%)	Narrowing of the alveolar lumen
T0	1.20±0.45^{a}	1.40±0.55^{a}	1.40±0.55^{a}
T1	1.20±0.45^{a}	2.00±0.71^{a}	1.40±0.55^{a}
T2	1.60±0.55^{a}	1.80±0.45^{a}	1.60±0.55^{a}
T3	1.40±0.55^{a}	1.80±0.45^{a}	1.80±0.45^{a}

Note: $p^{a,b}$<0,05 between groups (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 mg/kg BW)

Table 7: Degree of kidney damage

Groups	Tubule	Glomerulus	Tubular Endothelium	Tubular interstitial
T0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
T1	1.00±0.00^{b}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{b}
T2	1.80±0.45^{c}	2.00±0.00^{a}	1.00±0.00^{a}	1.80±0.45^{c}
T3	1.00±0.00^{b}	1.00±0.00^{a}	1.00±0.00^{a}	1.00±0.00^{b}

Table 8: Purkinje cell of cerebellum

Groups	Count (x10^{3})
T0	149.20±2.95^{a}
T1	140.80±4.21^{b}
T2	131.80±5.07^{c}
T3	124.20±3.11^{d}

Note: $p^{a,b}$<0,05 between groups (T0: control, T1: 100 mg/kgBW, T2: 200 mg/kgBW, T3: 300 mg/kg BW)

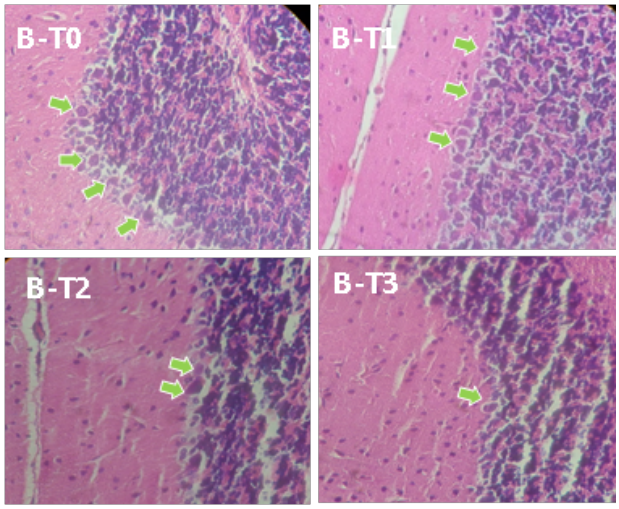

Fig. 3: Histology of Brain (B). Description: (T0: control, T1: 100 mg/kg BW, T2: 200 mg/kg BW, T3: 300 kg mg/BW). Green arrow = Purkinje (400×)

function (Park *et al.* 2022). An elevated ALP in the T3 group indicates possible cholestasis or liver injury, although all parameters remained within normal physiological ranges. Despite these changes, the liver function remained intact, as most values were still within acceptable limits, suggesting that while *V. gracilis* nanoherbs can influence liver biochemistry, they may not cause overt liver toxicity at the recommended dosage and duration. However, the findings support limiting the administration period to one month to prevent potential liver damage, aligning with earlier studies on herb-induced liver injury (HILI) (He *et al.* 2019).

The recommended dosage of 300 mg/kg body weight for one month strikes a balance between therapeutic efficacy and safety, providing liver health benefits without exceeding safe biochemical thresholds.

In this study, *V. gracilis* nanoherb administration on renal function particularly at higher doses, led to significant changes in renal biochemical parameters. The elevation in uric acid levels across all treatment groups indicated possible disruptions in renal excretion, as uric acid is a waste product filtered by the kidneys. Creatinine, a marker of kidney function, began to rise abnormally from day 42 onward, signaling compromised renal function with prolonged exposure to the nanoherbs (Oh *et al.* 2017; Deng *et al.* 2024). By day 56, elevated creatinine levels suggested a further decline in kidney performance.

An increase in urea/BUN levels, especially at higher doses, further reinforces the hypothesis of compromised renal filtration. These elevations indicate reduced kidney efficiency in excreting waste products, which might result from metabolic processes influenced by the nanoherb. Urea, BUN, creatinine, and ammonia levels are closely related to diet quality and metabolic efficiency, suggesting that administering *V. gracilis* may affect these processes (Hernandez-Gerez *et al.* 2019). While the nanoherb showed some therapeutic potential at lower doses (e.g., 200 mg/kg BW), the overall trend suggests that higher doses, such as 300 mg/kgBW, negatively impacted renal function, possibly due to increased metabolic by-products and renal burden. This underscores the need to monitor and limit dosage for prolonged use to avoid potential kidney damage, stressing the need for caution in administering *V. gracilis* nanoherb.

The histopathological examination indicated that *V. gracilis* nanoherbs induced significant changes in liver tissue, particularly at higher doses. A notable degeneration of parenchyma in H-T3 group suggested that while lower doses (100 and 200 mg/kg BW) may exert protective effects by increasing the number of normal hepatocytes, excessive exposure (300 mg/kg BW) leads totissue injury. The increase in normal hepatocytes at lower doses aligns with the therapeutic potential of flavonoids in promoting liver health (Lu and Yip 2023). However, an irreversible necrotic degeneration highlights a critical threshold for dosage, beyond which beneficial effects are overshadowed by cellular damage. This may be attributed to the oxidative stress and damage caused by high concentrations of nanoherb, consistent with findings that link cellular aging and degeneration to oxidative damage (Hernandez-Gerez *et al.* 2019).

Structure of inflammation is related to the fact that the ends of bronchioles, alveolar ducts, and adjacent alveoli are the most important sites for inhaling small particles (Ryu *et al.* 2020). The matrix surrounding the cells consisted of damaged collagen and elastin fibers, as shown by the correlation between the alveoli in the control group. In addition, the alveolar lumen was clearly visible, with damage to the alveolar cells resulting in damage to the microanatomical structure of the lung. Free radicals are detoxified by macrophages, neutrophils, and eosinophils (Patel *et al.* 2021). Therefore, an excessive increase in oxidants can lead to the migration of neutrophils, macrophages, and eosinophils, triggering an inflammatory response and increasing cell death (Shen *et al.* 2023). In this study, there was no significant correlationbetween the administration of *V. gracilis* nanoherbs on changes in lung histology. The duration of herb administration was closely related to the duration of herb administration in chronic toxicity tests, with administration of up to 56 days having a more pronounced pharmacological effect.

The histopathological findings indicated that administering *V. gracilis* nanoherbs at a lower dose of 100 mg/kg body weight resulted in milder renal abnormalities, suggesting a potential protective effect on kidney architecture. This is evidenced by the reduced glomerular and tubulointerstitial damage observed in the T1 group compared to the control. However, higher doses of 200 mg/kg and above led to significant renal tubular inflammation and increased necrotic cell counts, indicating nephrotoxic effects. These results align with existing literature, emphasizing the importance of dose management in herbal administration, as prolonged exposure to higher doses can exacerbate renal damage (Naber and Purohit 2021; Borkar *et al.* 2022). Therefore, limiting the dosage of *V. gracilis* nanoherbs below 200 mg/kg body weight, particularly for extended durations, is crucial to mitigate the risk of renal complications while potentially harnessing their therapeutic benefits.

The decrease in Purkinje cells was due to the occurrence of cellular apoptosis through the pathway of the substance content in the nanoherbs after prolonged administration. Cell death can occur if doses are too high and administration is too long (Villaescusa *et al.* 2023). Apoptosis or necrosis may be the type of cell death that occurred here. A number of distinct morphological and biochemical changes occur during apoptosis, such as cell shrinkage, chromatin condensation, nuclear fragmentation, loss of membrane integrity, and formation of apoptotic bodies containing nuclear material. There is no evidence of inflammation occurring in such cases, although the apoptotic body is eventually engulfed by phagocytes. In biochemical terms, apoptosis is further characterized by protein degradation and activation of caspases. It is regulated by B cell lymphoma family protein 2 (BCL-2), which consists of anti- and pro-apoptotic proteins that must be balanced to promote or inhibit apoptosis (Ding *et al.* 2017; Olopade *et al.* 2021; Stoessel and Majewska 2021; Erekat 2022; Enogieru and Idemudia 2024).

Conclusion

The histology of the kidneys, liver, heart, and lungs improved with the administration of nano-herbal *V. gracilis* in chronic toxicity. It also improved the body's physiological function through complete blood count, kidney, and liver

biochemistry, and blood electrolyte parameters. The dose range that did not cause symptoms of chronic damage was 100–200 mg/kg BW not exceeding one month of administration.

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Author Contributions

Syafruddin Ilyas was involved in the design of the study, interpretation of the data, and drafting of the manuscript. Muswita and Dina Khairani participated in data collection. Dini Prastyo Wati contributed to the preparation of the manuscript. Salmi, Husnarika Febriani were involved in the preparation of this manuscript and in its critical revision.

Conflict of Interests

The authors declare no conflicts of interest.

Data availability

Data presented in this study will be available on a fair request to the corresponding author.

Ethics Approval

All experimental procedures in this study were carried out in accordance with the Institutional Animal Care guidelines of the Animal House of the Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara (USU) and this study was approved by the Health Research Ethics Committee of USU Medan (No. 0395/KEPH-FMIPA/2023).

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