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Sub-chronic toxicity study of green peas protein hydrolysate in rats

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ABSTRACT

Despite green peas protein hydrolysate benefits, very few studies have described the potential toxicity. The acute oral toxicity of green peas protein which hydrolyzed by bromelain at a single dose of 5000 mg/kg BW has been evaluated in Swiss Webster mice and did not produce treatment-related signs of toxicity or mortality in any of the animals tested during the 14-day observation period. The present study aimed to evaluate the sub-chronic toxicity effects of Protein Hydrolysate Green Peas Bromelain (PHGPB) in Sprague Dawley rats by the regulations of the Indonesian Food and Drug Supervisory Agency 2013. In the repeated dose 28-day oral toxicity study, the administration of 100 mg/kg, 200 mg/kg, and 400 mg/kg/day of green peas protein hydrolysate per body weight revealed no significant difference ($P > 0.05$) in body weight change, hematological parameters, relative organ weights, and gross findings compared to the control group. Clinical biochemistry analysis and histopathology examinations of liver and kidney showed slight morphological alteration. The oral sub-chronic toxicity test of PHGPB for 28 days did not induce noticeable signs of toxicity. The no-observed adverse effect level (NOAEL) of PHGPB in the sub-chronic toxicity study was dose of the 200 mg/kg BW. The results of our studies PHGPB indicate a lack of toxicity and support the use of functional foods.

1. Introduction

Despite there are many green peas protein hydrolysate benefits, very few studies have described the potential toxicity. Although peas are a common food ingredient, their consumption as a medicinal plant must use the right dosage because, with the right dosage, the drug can be beneficial and efficacious, but if excessive can potentially be toxic and endanger health. Information that is not supported by scientific facts and adequate studies can make the use of traditional medicines risky to be toxic and dangerous [1]. An important part of assessing potential toxic effects is evaluating the toxicological effects of medicinal plant extracts for use in animals or humans [2].

Toxicity testing evaluation is one way to measure the safety of a drug to be used as a product. A sub-chronic toxicity test is a test used to determine the toxicity of a compound carried out on experimental animals with a minimum of three dose levels, generally within a period of 28 or 90 days [3–5].

Pisum sativum or more commonly known as green or yellow peas is a very beneficial legume because it contains fiber, protein, starch, and various phytochemicals that are good for the human body. Pea protein powders are potentially an excellent choice for additional protein in

patients with kidney disease. Several important studies have recently shown that eating vegetarian protein has a protective effect against worsening staging of CKD compared to animal protein [6]. The study of researchers in Canada shows that peas in their hydrolysate form have many therapeutic effects, for example in the treatment of hypertension and chronic kidney disease [7–9]. The green peas in their natural form do not have effects as good as their hydrolysate form.

The enzymatic hydrolysis process of protein is often used to improve the function and nutrition of protein sources, because it has several advantages, including the production of more efficient peptides without toxic by-products or the destruction of amino acids [10,11]. Hydrolyzed peptides are used as functional foods that have a therapeutic effect to prevent various diseases and damage caused by oxidative stress [12].

Research on the protein hydrolysate of green peas (*Pisum sativum*) which was hydrolyzed using the bromelain enzyme showed effective results in increasing the rats' kidney function parameters induced by Gentamicin or Cisplatin [13,14]. Besides, this hydrolysate is proven to have antioxidant activity, hypolipidemic effects, and has a high content of several essential amino acids so that it is expected to be beneficial for the health of CKD sufferers [13–15]. With this potential, in the future, this protein hydrolysate of green peas bromelain (PHGPB) has the

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potential to be used as a supportive treatment for CKD sufferers in long term, so it is necessary to know the safety aspect of this hydrolysate. In this PHGPB, an acute toxicity test has been carried out by the regulations of the Indonesian Food and Drug Supervisory Agency (BPOM RI) 2013 [3,5], with results PHGPB classified as Practically non-toxic, LD50 of PHGPB is above 5000 mg/kg BW, at very high doses does not show abnormal behavior, no effect on body weight and no deaths occurred at 14 days of observation on female Swiss Webster mice [16].

With the aim of knowing the long-term and repeatable safety aspects of the PHGPB sample material, a sub-chronic toxicity test of PHGPB which was made based on a simple method of producing green peas hydrolysate [13] was carried out for 28 days under the provisions of the 2013 BPOM Republic of Indonesia [3–5].

2. Materials and methods

2.1. Preparation of protein hydrolysate of green peas bromelain (PHGPB)

Green Peas split 20016. USA. Trinidad Benham Corp. Denver, CO 80237 were prepared to be hydrolyzed using previously method (13). Dry seeds (500 g) of green peas were washed, sieved through a 120mesh sieve, and dissolved in 2000 mL of water. Ten percent (w/v) of bromelain was added to each solution and then left for 72 h on a stirrer at room temperature (25–30 °C). The solution was then transferred into a tube and centrifuged at 2500 g at 4 °C for 10 min (Tomy Portable Refrigerated Centrifuge MX-201). The supernatant was filtered using a filter paper. SDS-PAGE was used to separate and determine the molecular weight of the protein hydrolysates, with gel concentration of 15%, Voltage 0 f 90 V and time for 120 min (17). The total protein content in PHGPB calculated using the Bradford method [17].

2.2. Experimental animals

Male and female Sprague Dawley (SD) rats were used for sub-chronic toxicology studies. The rats were obtained from iRATco Veterinary Laboratory Services. The animals were acclimatized to laboratory conditions for 7 days prior to the experiments. The rats were maintained at a room temperature of 22–24 °C, with a 12 h light/dark cycle. During acclimatization, the animals were housed in ventilated cages with a standard pellet diet and tap water ad libitum. The food pellets for the experimental animals were purchased from iRATco Veterinary Laboratory Services, with 18% crude protein. Physical observation signs of toxicity in their skin, fur, eyes, mucus membrane, or behavioral changes, diarrhoea, tremors, salivation, sleep, and coma were observed during the study. All procedures in this study were performed according to the Animal Ethics Committee, Universitas Kristen Maranatha, Decree no 059/KEP/VI/2020.

2.3. Sub-chronic oral toxicity study

The sub-chronic oral toxicity test was performed following the protocol described by the BPOM Republic of Indonesia 2013 guidelines for chemicals testing. In the sub-chronic toxicity test, at least three different dose groups had to be used, one control group and two satellite groups (high dose group and control group) since there were six groups in total. The three-dose groups used: the highest doses that are doses may cause toxic effects but do not cause death or severe toxicity symptoms; moderate doses cause milder toxic symptoms while the lowest doses cause no toxic symptoms at all or No-Observed-Adverse-Effect Level (NOAEL) [5]. In the current study, the lowest dose was the dosage of 100 mg/kg BW/d based on a previous study that dose has shown a therapeutic effect, although not optimal and predicted was not toxic. The moderate dose was chosen based on the most effective dose according to the results of previous study and will be used in the future as a long-term therapy, which is 200 mg/kg BW/d [13]. Sub-chronic administration of this effective dose has not been tested for toxicity. The highest dose

used was 400 mg/kg BW/d which is double the effective dose which may have the potential to cause toxic effects but not cause mortality.

Hundred and twenty rats (60 males and 60 females) were assigned randomly into six groups: two control groups and four treatment groups ($n = 20$; ten males and ten females). The PHGPB was administered orally daily for 28 days at single doses of 100, 200, and 400 mg/kg BW/d, while the control group received only distilled water. After 28 days of treatments first control group, group doses of 100, 200, and 400 mg/kg BW/d of both sexes, a total of 4 groups (males and females) were killed. Two remainder groups (second control group) and highest dose satellite group (400 mg/kg BW/d) were devised as the satellite group namely the control group satellite to observe the reverse sign of any toxicity. The highest dose satellite group was given the only dose of 400 mg/kg/day for the first 28 days, and there was no further treatment for the following 28 days before termination of the study.

All rats were weighed and visual observations for mortality, behavioral pattern (salivation, fur, lethargy, and sleep), changes in physical appearance, injury, pain, and signs of illness were conducted once daily during that period. At the end of the experiment, all animals were anesthetized with 100 mg/kg BW of Ketamine and 10 mg/kg BW of Xylazine. Blood samples were collected via cardiac puncture into plain blood collection tube for biochemical and blood collection with EDTA for hematological analyses, respectively. After cardiac puncture, the rats were euthanized by perfusion methods. The organs were excised, weighed, and examined macroscopically. The relative organ weight was calculated. Two vital organs (liver and kidney) were preserved in fixation medium of 10% solution of buffered formalin for histopathological study. Hematological analyses were performed at DVL animal care Laboratory Services, Tangerang (www.dvl.co.id) using Mindray hematology analyzer. Biochemical analyses were performed using Cobas Roche kit at Purwakarta Laboratory (https://diagnostics.roche.com/global/en/products/systems/cobas_-6000-analyzer-series.html).

2.4. Histopathological study

After sacrificing the rats, parts of the liver and kidney were collected for histological studies. The tissues were washed in normal saline and fixed immediately in 10% formalin for a period of at least 24 h, dehydrated with alcohol, embedded in paraffin, cut into 4–5 μ m thick sections, and stained with haematoxylin-eosin dye for photo-microscopic observation. The microscopic features of the organs of male and female rats were compared with the control group.

2.4.1. Liver histopathological examination

The liver histopathological examination with three parameters, lobular inflammation, steatosis, and ballooning degeneration, will be based on the Brunt scoring system [18].

Assessment of lobular inflammation. Score 0: No inflammatory focus; Score 1: Fewer than two inflammatory foci found; Score 2: 2–4 foci of inflammation found; Score 3: More than four inflammatory foci were found. Assessment of Steatosis. Score 0: The area affected by steatosis is less than 5%; Score 1: The area that has steatosis 5–33%; Score 2: The area that has steatosis is more than 33–66%; Score 3: The area that experienced steatosis was more than 66%. Assessment of ballooning degeneration: Score 0: None; Score 1: Multiple cells with ballooning degeneration; Score 2: Many cells have ballooning degeneration.

Each parameter was examined in nine fields of view and the results are recorded in the scores form of 0–3. The results of the score were taken as the median value.

2.4.2. Kidney histopathological examination

The four parameters examined in the kidneys were histopathological, degeneration of the swollen cloudy tubules, nuclear necrosis, hyaline cast, and fibrosis. Histopathological observations were carried out through a light microscope with an objective magnification of 10 and 40 times, in 5 fields of view continue interpreted in the form of scoring. The

parameters observed were tubular degeneration (cloudy swelling), core necrosis, Hyaline cast, and fibrosis [19].

Assessment of Tubular degeneration. The score of 0 for null; 1 for focal, and 2 for diffuse. Assessment of Nuclear necrosis. The score of 0 for none; 1 for focal and 2 for diffuse. Assessment of Hyaline cast. The score of 0 for null, 1 for < 50%, and 2 for \geq 50%. Assessment of Fibrosis. Score of 0 for null; 1 for focal, and 2 for diffuse.

2.5. Statistical Analysis

All values are expressed as mean \pm STD. Comparisons between groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests using SPSS statistical software. A *P* value of < 0.05 was considered significant. Histopathological results were analysed using Kruskal Wallis test, if significant, continued with Mann-Whitney test.

3. Results

3.1. Preparation of protein hydrolysate of green peas bromelain (PHGPB)

There were 10 bands were identified from SDS PAGE sample (molecular weight between 5.20 and 66.86 kDa) of PHGPB. Among the 10 bands there are 4 thick bands between 8.3 and 22.62 kDa (Fig. 1). The total protein content in PHGPB calculated using the Bradford method [17] was 49.779 mg/mL with pH 4.52.

3.2. Sub-chronic oral toxicity

3.2.1. Clinical signs, necropsy findings, and food and water consumption

Daily oral administration of PHGPB for 28 days did not induce any obvious symptom of toxicity in rats of both sexes, including the highest dose tested at 400 mg/kg body weight. No deaths or obvious clinical signs were found in any group throughout the experimental period. Physical observation of the treated rats throughout the study indicated that none of them showed any signs of toxicity in their skin, fur, eyes, mucus membrane, or behavioral changes, diarrhea, tremors, salivation, sleep, and coma. Normal body weight gains of both sexes were observed during the study period compared to the control group (Fig. 2). After 28 days without treatment, the bodyweight of male control satellite rats was 228.6 \pm 19.05 g, while the average body weight of rats in the high dose group was lighter, namely 181.1 \pm 46.90 g. Meanwhile, for female rats, the bodyweight of female satellite control rats was 132.3 \pm 11.43 g, while the mean bodyweight of the high dose group rats was heavier, namely 145.0 \pm 16.50 g. however, they are not statistically significant.

No abnormal gross findings were observed in the necropsies of any of the rats. The food and water consumptions of the treated rats, which were measured throughout the study, were also not significantly

different compared to the control rats.

3.2.2. Organ and relative organ weights (ROW) of male and female rats

Organ and relative organ weights of 28-day treated rats are shown in Table 1. The relative organ weight of each organ recorded at necropsy in the treatment groups did not show a significant difference (*P* > 0.05) compared to the control (Table 1).

3.2.3. Hematology and clinical biochemistry analysis

The effects of sub-chronic administration of PHGPB on hematological and biochemical parameters are presented in Tables 2 and 3. All of hematology measures: leucocyte (total white blood cell), erythrocyte (red blood cell), hemoglobin, hematocrit, MCV, MCH, MCHC, lymphocyte, monocyte, neutrophil, eosinophil, thrombocyte (platelete), RDW (red cell distribution width), PDW (platelete distribution width) in treated rats were not significantly different from the controls, all results of treatment were in the range of normal value (Table 2).

The results of clinical biochemistry analysis after 28 days of treatment, triglyceride levels of female control rats were significantly different compared to all treatments (low, medium, and high doses). Triglyceride levels of the control rats were higher than the treated rats.

No statistically significant differences in liver function parameters (ALT and AST) were noted. In the highest dose group male rats had no effect on renal function parameters (urea, creatinine, and uric acid) and no relevant changes were found after administration of PHGPB. However, the low and moderate doses in male rats and low doses in female showed significant differences with the control, even though the creatinine levels were still within the normal range, and the urea levels of all rats, both control and treatment groups, showed results above the normal range. In female rats, the highest dose of PHGPB did not affect urea and creatinine parameters, but there was an increase in uric acid levels (Table 3). Other blood biochemical profiles (lipid profile: total cholesterol, LDL, HDL) and glucose, did not differ significantly compared to controls.

3.2.4. Macropathology and histopathology

Macroscopic examination of the vital organs of PHGPB treated animals revealed no abnormalities in the color or texture when compared with the organs of the control group. The light microscopy examinations of the transverse section of the liver and kidney organs of the treated animals and control group rats are shown in Fig. 3. Liver histopathological examination of the control group and PHGPB treated rats showed normal structure and absence of any gross pathological lesion in organs, except the median score of liver histopathology parameters for lobular inflammation of the high dose group, is 3, which is higher compared to the control group which score is 1. The median value of three liver histopathological scores based on 3 parameters in rats treated for 28 days was presented in Table 4. Analysis results of the median score of liver histopathology using Kruskal Wallis test showed *p* > 0.05 which means there is no significance different between groups, except the parameter of lobular inflammation group dose of 400 mg/kg BW/d is statistically different relative to the control group (*p* < 0.05).

In all groups, neither the control group nor the treatment group showed symptoms of steatosis and ballooning degeneration. The abnormalities that arose were only lobular inflammation either in the treatment groups or in the control group. Lobular inflammation indicated that there was early-stage inflammation of the hepatocytes, however, it occurred in the entire group of rats; however, the inflammation was mild to medium, ie less than 2 to more than four foci of inflammation are found (score 1–3). After 28 days of recovery, the high-dose satellite group showed significant improvement (total score 0).

The median value of three kidney histopathological scores based on 4 parameters in rats treated for 28 days was presented in Table 5. Analysis results of the median score of kidney histopathology using the Kruskal Wallis test showed *p* > 0.05 which means there is no significant difference between groups.

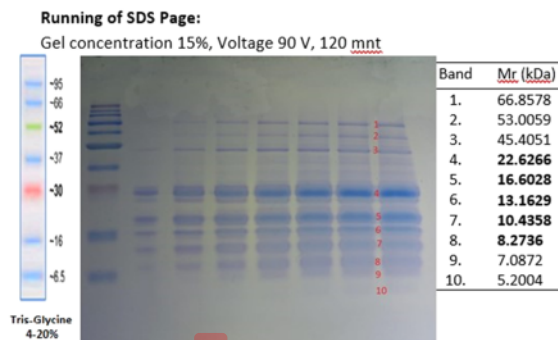


Fig. 1. SDS PAGE of Protein Hydrolysate of Green Peas Bromelain.

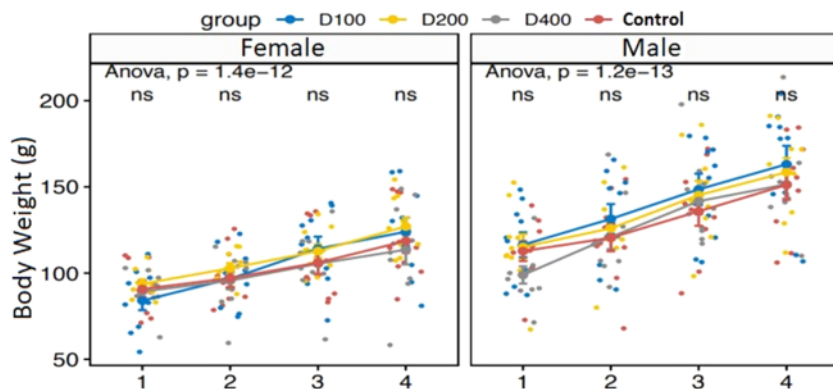


Fig. 2. Body Weight of 28-day Treated Rats.

Table 1
Data organ and relative organ weight of 28-day treated rats.

	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kg BW	Satellite 400
Male (g)						
Liver	6.80 ± 1.04	8.90 ± 0.62	8.11 ± 1.64	7.21 ± 1.21	7.72 ± 1.41	7.20 ± 2.67
Spleen	1.10 ± 0.11	1.10 ± 0.36	1.30 ± 0.50	1.20 ± 0.32	1.20 ± 0.50	1.51 ± 0.45
Heart	0.60 ± 0.16	0.81 ± 0.13	0.60 ± 0.11	0.60 ± 0.14	0.60 ± 0.06	0.82 ± 0.12
Lung	1.70 ± 0.38	1.90 ± 0.15	1.90 ± 0.41	1.50 ± 0.38	1.61 ± 0.29	1.90 ± 0.13
R Kidney	0.80 ± 0.17	1.00 ± 0.18	0.90 ± 0.16	0.80 ± 0.079	0.80 ± 0.09	0.81 ± 0.23
L Kidney	0.70 ± 0.14	0.80 ± 0.18	0.80 ± 0.17	0.70 ± 0.13	0.80 ± 0.11	0.81 ± 0.25
Body W	151.1 ± 27.52	228.6 ± 19.05	163.1 ± 33.92	158.5 ± 25.95	151.5 ± 26.12	181.1 ± 46.90
Row						
	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kgBW	Satellite 400
Liver	4.56 ± 0.61	3.90 ± 0.38	4.99 ± 0.66	4.54 ± 0.54	5.20 ± 1.36	3.90 ± 0.40
Spleen	0.73 ± 0.17	0.50 ± 0.15	0.81 ± 0.21	0.81 ± 0.33	0.78 ± 0.23	0.80 ± 0.19
Heart	0.42 ± 0.10	0.31 ± 0.03	0.39 ± 0.05	0.38 ± 0.09	0.39 ± 0.06	0.40 ± 0.08
Lung	1.17 ± 0.33	0.82 ± 0.08	1.19 ± 0.34	0.95 ± 0.19	1.09 ± 0.17	1.1 ± 0.21
R Kidney	0.50 ± 0.08	0.40 ± 0.10	0.52 ± 0.06	0.53 ± 0.07	0.54 ± 0.07	0.50 ± 0.04
L Kidney	0.48 ± 0.09	0.30 ± 0.06	0.50 ± 0.05	0.46 ± 0.10	0.52 ± 0.04	0.40 ± 0.05
Female (g)						
	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kg BW	Satellite 400
Liver	6.4 ± 1.26	6.7 ± 0.51	6.5 ± 1.35	6.8 ± 1.45	6.1 ± 1.84	5.9 ± 0.89
Spleen	1.2 ± 0.36	1.3 ± 0.27	1.3 ± 0.30	1.1 ± 0.22	1.0 ± 0.37	1.0 ± 0.43
Heart	0.5 ± 0.08	0.5 ± 0.08	0.5 ± 0.11	0.5 ± 0.14	0.4 ± 0.06	0.6 ± 0.10
Lung	1.4 ± 0.29	1.8 ± 0.20	1.7 ± 0.50	1.6 ± 0.42	1.3 ± 0.23	1.4 ± 0.21
R Kidney	0.6 ± 0.14	0.7 ± 0.10	0.7 ± 0.10	0.7 ± 0.11	0.6 ± 0.11	0.5 ± 0.08
L Kidney	0.6 ± 0.12	0.7 ± 0.10	0.6 ± 0.13	0.7 ± 0.16	0.6 ± 0.10	0.6 ± 0.18
Body W	118.6 ± 22.06	132.3 ± 11.43	124.0 ± 26.13	127.0 ± 15.97	133.6 ± 27.10	145.0 ± 16.50
Row						
	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kgBW	Satellite 400
Liver	5.45 ± 0.67	5.1 ± 0.68	5.27 ± 0.42	5.40 ± 1.19	5.29 ± 0.70	4.10 ± 0.63
Spleen	1.05 ± 0.28	1.0 ± 0.25	1.03 ± 0.16	0.90 ± 0.19	0.84 ± 0.23	0.70 ± 0.27
Heart	0.43 ± 0.07	0.4 ± 0.09	0.38 ± 0.11	0.36 ± 0.11	0.39 ± 0.11	0.41 ± 0.06
Lung	1.24 ± 0.27	1.4 ± 0.14	1.33 ± 0.23	1.24 ± 0.33	1.16 ± 0.18	1.00 ± 0.23
R Kidney	0.50 ± 0.11	0.5 ± 0.05	0.54 ± 0.09	0.56 ± 0.08	0.54 ± 0.12	0.40 ± 0.07
L Kidney	0.52 ± 0.07	0.5 ± 0.05	0.51 ± 0.05	0.57 ± 0.14	0.60 ± 0.28	0.40 ± 0.15

Values are expressed as mean ± STD (n = 10 for each group). Relative organ weight was calculated as (organ weight divided with body weight) × 100%. *P values < 0.05 were considered significant using one-way ANOVA followed by Tukey's multiple comparison tests. Asterisks denote significant difference compared to control.

In kidney histopathology analysis, neither the control group nor the treatment group showed symptoms of cloudy swelling, nuclear necrosis, and fibrosis. The only abnormalities that arose in the treatment group were hyaline cast; this condition is the earliest marker of inflammation of the renal tubules in the early stages, both at low, medium, and high doses; however, the hyaline cast that occurred was classified as mild, i.e. less than 50% per 5 visual fields (score 1). After 28 days of recovery, the high-dose satellite group showed significant improvement (total score 0).

4. Discussion

In the preparation stage to produce natural products to be used as a medicine, usually, the first step to evaluating pharmacological activity is the assessment and evaluation of their toxicity. During these assessments and evaluation of the toxic characteristic, usually, the first step

that must be done is determining the LD50 [3,4]. Studies on the acute toxicological effects of green peas protein hydrolysate have been carried out and have shown that this substance is practically non-toxic because the LD 50 is above 5000 mg/kg BW and observation for 2 weeks after the administration of very high doses, all of the experimental animals showed no toxic symptoms at all or death. However, data on repeated administration in chronic toxicology tests are still lacking. Therefore, a study was undertaken to evaluate the sub-chronic toxicity effects of PHGPB in animal models.

The data from the acute toxicity test can be useful to (a) form the basis for classification and labelling; (b) provide preliminary information on the substance's toxic effects; (c) determine the therapeutic dose of the compound under test; (d) determine the dosage of medication in animal studies; (e) determine the LD50 value [20].

If the acute toxicity test shows non-toxic results, the regulation 2013 of Badan Pengawas Obat dan Makanan (BPOM) or National Agency of

Table 2
Hematology analysis of 28-day treated rats and satellite.

Male	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kg BW	SAT 400 mg/kg BW
Parameters						
Leucocyte	10.58 ± 4.07	15.08 ± 7.99	11.23 ± 2.77	11.01 ± 4.74	7.86 ± 3.60	11.84 ± 3.31
Erythrocyte	6.08 ± 1.52	7.44 ± 0.52	6.36 ± 1.03	5.54 ± 1.75	5.08 ± 1.40	6.24 ± 0.45
Hemoglobin	13.21 ± 2.75	14.14 ± 0.34	13.02 ± 1.56	12.15 ± 3.81	10.54 ± 2.48	13.52 ± 1.27
Hematocrit	37.78 ± 8.19	43.44 ± 1.85	39.04 ± 4.29	35.89 ± 11.05	31.94 ± 7.27	39.12 ± 3.33
MCV	62.73 ± 4.19	58.64 ± 2.59	61.56 ± 10.02	65.12 ± 4.86	64.15 ± 5.56	62.76 ± 2.90
MCH	21.36 ± 1.35	19.12 ± 1.31	20.85 ± 3.07	21.97 ± 1.23	21.17 ± 1.90	21.68 ± 1.05
MCHC	34.06 ± 1.04	32.58 ± 1.07	33.36 ± 0.82	33.79 ± 1.29	33.01 ± 1.31	34.52 ± 0.53
Lymphocyte %	69.50 ± 10.78	76.82 ± 5.50	72.47 ± 5.15	73.62 ± 6.61	75.33 ± 5.37	75.30 ± 5.51
Lymphocyte #	7.35 ± 3.21	11.86 ± 7.31	8.19 ± 2.29	9.15 ± 5.21	5.88 ± 2.65	8.96 ± 2.93
Monocyte%	6.34 ± 3.94	4.36 ± 3.79	5.12 ± 1.97	5.03 ± 1.66	5.23 ± 2.56	3.14 ± 1.93
Monocyte#	0.64 ± 0.48	0.50 ± 0.38	0.57 ± 0.22	0.56 ± 0.27	0.47 ± 0.36	0.34 ± 0.21
Neutrophyl	38.77 ± 9.04	17.92 ± 3.89	20.99 ± 3.31	19.04 ± 5.64	17.69 ± 3.84	20.72 ± 4.71
Neutrophyl #	2.44 ± 1.23	2.56 ± 0.95	2.34 ± 0.59	2.05 ± 0.91	1.41 ± 0.71	2.46 ± 0.87
Basophyl %	N/A	N/A	N/A	N/A	N/A	N/A
Basophyl #	N/A	N/A	N/A	N/A	N/A	N/A
Eosinofil %	1.39 ± 0.55	0.90 ± 0.40	1.42 ± 0.78	2.30 ± 2.87	1.75 ± 0.79	0.84 ± 0.54
Eosinofil #	0.14 ± 0.05	0.16 ± 0.15	0.15 ± 0.05	0.15 ± 0.05	0.12 ± 0.04	0.10 ± 0.07
Thrombocyte	532.0 ± 21.89	844.6 ± 83.35	675.1 ± 154.33	627.9 ± 149.65	465.3 ± 258.28	879 ± 56.40
RDW	16.84 ± 3.46	15.16 ± 0.61	17.92 ± 5.03	16.42 ± 4.47	16.69 ± 3.71	13.58 ± 1.59
PDW	15.93 ± 0.44	15.70 ± 0.16	15.63 ± 0.42	15.84 ± 0.52	15.94 ± 0.54	15.54 ± 0.11
Female	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kg BW	SAT 400 mg/kg BW
Parameters						
Leucocyte	9.71 ± 3.01	9.66 ± 3.83	7.97 ± 4.49	5.93 ± 3.54	7.65 ± 5.95	11.84 ± 3.31
Erythrocyte	5.05 ± 1.20	6.68 ± 0.60	5.29 ± 0.75	4.83 ± 1.25	3.75 ± 1.43	6.24 ± 0.45
Hemoglobin	11.78 ± 2.75	12.80 ± 1.00	12.17 ± 1.88	11.11 ± 2.85	8.67 ± 3.53	13.52 ± 1.27
Hematocrit	35.43 ± 7.69	40.90 ± 3.41	35.6 ± 5.23	32.57 ± 8.44	26.05 ± 10.46	39.12 ± 3.33
MCV	70.76 ± 5.86	61.40 ± 4.14	67.53 ± 4.81	67.53 ± 2.64	68.9 ± 9.15	62.76 ± 2.80
MCH	23.42 ± 1.33	19.22 ± 1.23	23.08 ± 1.75	23.08 ± 0.95	22.8 ± 2.86	21.68 ± 1.05
MCHC	33.22 ± 1.34	31.24 ± 0.38	34.15 ± 1.01	34.20 ± 0.85	33.01 ± 1.67	34.52 ± 0.53
Lymphocyte %	75.32 ± 9.96	76.82 ± 5.50	73.49 ± 5.63	72.4 ± 13.98	75.05 ± 8.72	75.3 ± 5.51
Lymphocyte #	7.35 ± 2.68	11.86 ± 7.31	5.87 ± 3.73	4.58 ± 3.12	6.01 ± 2.06	8.96 ± 2.93
Monocyte%	3.87 ± 2.15	6.36 ± 1.15	4.98 ± 2.74	4.85 ± 3.15	4.93 ± 2.04	3.14 ± 1.93
Monocyte#	0.38 ± 0.11	0.62 ± 0.16	0.32 ± 0.18	0.21 ± 0.13	0.32 ± 0.13	0.34 ± 0.21
Neutrophyl	19.32 ± 8.16	20.30 ± 5.87	21.59 ± 6.32	19.76 ± 10.39	17.64 ± 6.24	20.72 ± 4.71
Neutrophyl #	1.86 ± 0.96	2.08 ± 1.08	1.63 ± 0.90	1.38 ± 0.92	1.20 ± 0.75	2.46 ± 0.87
Basophyl %	N/A	N/A	N/A	N/A	N/A	N/A
Basophyl #	N/A	N/A	N/A	N/A	N/A	N/A
Eosinofil %	1.49 ± 0.59	0.72 ± 0.11	2.41 ± 1.16	2.99 ± 1.89	2.38 ± 1.37	0.84 ± 0.17
Eosinofil #	0.14 ± 0.05	0.06 ± 0.01	0.16 ± 0.05	0.13 ± 0.05	0.15 ± 0.05	0.10 ± 0.07
Thrombocyte	708.0 ± 29.61	824.8 ± 55.22	638.6 ± 28.11	386.8 ± 131.30	332.3 ± 25.22	879.0 ± 56.40
RDW	17.10 ± 3.75	14.84 ± 0.91	16.45 ± 3.44	14.60 ± 1.25	19.24 ± 4.63	13.58 ± 1.59
PDW	16.06 ± 0.29	15.68 ± 0.19	15.98 ± 0.67	16.68 ± 1.02	16.36 ± 0.67	15.54 ± 1.11

Values are expressed as mean ± STD (n = 10 for each group). *P values < 0.05 were considered significant using one way ANOVA followed by Tukey's multiple comparison tests. Asterisks denote significant difference compared to control.

Drug and Food Control of Republic of Indonesia stipulates that further evaluation is required to determine sub-chronic toxicity for 28 or 90 days of test materials in experimental animals to complement the comprehensive toxicological data [4]. In this study due to laboratory limitations in the state of the Covid 19 pandemic, we decided to conduct a 28-day study of oral sub-chronic toxicity tests. The sub-chronic toxicity test assesses the adverse effects of repeated or continuous exposure to the test substance in experimental animals, such as rats. In particular, the advantage of sub-chronic evaluation provides information on toxicity target organs and is designed to identify side effects that are difficult to observe [20]. Sub-chronic evaluation can also help to determine the appropriate dosing regimen for long-term therapy. In this study sub-chronic toxicity test of PHGPB was evaluated in rats at a dose of 100–400 mg/kg BW /day for 28 days based on the results of former studies, the effective dose of PHGPB in lowering kidney function parameters is 200 mg/kg BW. Observations must be made on satellite group with the highest dosage for observation of systemic or toxic effects is delayed or maybe even recovery from its toxic effects, at least 28 days after treatment. This group will be treated and placed under the condition identical to the animal from the main experiment. Satellite group in this study PHGPB was given orally at a daily dose of 400 mg/kg BW/day for 28 days, and no further treatment for the next 28 days before the study was terminated.

These tests revealed that administration of PHGPB for 28 days did

not produce clinical signs of toxicity or death in both sexes of all treatment groups. Besides, the treated rats did not show any significant results changes in water or food consumption (data not shown).

In all groups, there was weight gain with age, but there was no difference between the control and treatment groups. Organ-to-body weight ratios or ROW were commonly calculated and were considered more useful when body weights were affected. Change in organ weight has been recognized as a sensitive indicator of chemically induced organ changes. In toxicology studies, comparisons of organ weights between treated and untreated groups of animals have been used to evaluate the toxic effects of the test material [21,22]. However, the benefits of multi-organ weight analysis, organ weight data presentation, and the value of statistical analysis are equally important, because the evaluation of organ weights is a crucial part of the toxicological and risk assessment of drugs, chemicals, biology, and food additives. The approach to the evaluation and interpretation of organ weights shall be carried out with appropriate scientific rigor and, by following regulatory guidelines.

Liver weight is considered useful especially for the pharmaceutical industry and other industries. Important uses of liver weight are in their sensitivity to predict toxicity, which is a prime target in toxicity studies; useful for evaluating the diagnosis of hepatocellular hypertrophy from liver enzyme induction, peroxisome proliferation, or lipidosis; can reflect physiological and metabolic disorders as a whole; it correlates

Table 3
Clinical biochemistry analysis of 28-day treated rats and satellite.

Male	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kg BW	SAT 400 mg/kg BW
Parameter						
CHOL T (mg/dL)	57.40 ± 3.44	57.20 ± 1.92	55.8 ± 4.82	60.20 ± 3.27	62.00 ± 4.64	57.40 ± 4.67
TG (mg/dL)	62.0* ± 14.35	62.00 ± 13.13	46.60 ± 21.91	47.80 ± 9.20	42.40 ± 9.55	40.60 ± 8.71
HDL (mg/dL)	34.20 ± 2.90	34.72 ± 3.02	34.84 ± 0.84	34.60 ± 1.34	32.24 ± 4.04	33.68 ± 3.70
LDL (mg/dL)	13.38 ± 1.80	13.66 ± 2.10	12.20 ± 1.63	15.90 ± 3.59	16.60 ± 4.05	16.76 ± 1.71
AST (U/L)	174.2 ± 32.11	169.6 ± 28.01	205.0 ± 7.90	184.8 ± 60.69	154.2 ± 26.80	134.6 ± 18.38
ALT (U/L)	89.60 ± 12.34	87.60 ± 9.21	87.60 ± 20.32	84.60 ± 10.85	86.20 ± 18.16	73.80 ± 8.58
UREA (mmol/L)	38.56 ± 1.62	38.56 ± 1.78	51.20* ± 2.86	52.80* ± 6.34	38.20 ± 0.84	36.90 ± 0.82
CREAT (μmol/L)	0.22 ± 0.03	0.24 ± 0.04	0.36* ± 0.02	0.38* ± 0.03	0.20 ± 0.02	0.20 ± 0.02
URIC AC (mmol/L)	1.06 ± 0.12	1.04 ± 0.11	1.15 ± 0.09	1.10 ± 0.25	1.03 ± 0.08	0.93 ± 0.06
GLUCOSE (mg/dL)	186.6 ± 16.47	178.2 ± 21.44	169.0 ± 40.34	168.0 ± 43.08	156.0 ± 15.65	148.2 ± 9.04
Female	Control	Control satellite	100 mg/kg BW	200 mg/kg BW	400 mg/kg BW	SAT 400 mg/kg BW
Parameter	Mean	Mean	Mean	Mean	Mean	Mean
CHOL T (mg/dL)	60.40 ± 5.77	59.80 ± 4.15	54.00 ± 8.45	61.20 ± 8.58	60.60 ± 12.64	55.60 ± 9.91
TG (mg/dL)	72.2* ± 18.21	71.40 ± 17.84	36.60 ± 12.38	38.60 ± 3.58	43.00 ± 8.00	40.40 ± 5.03
HDL (mg/dL)	37.42 ± 3.82	36.98 ± 2.93	32.52 ± 1.32	34.84 ± 0.94	33.70 ± 13.12	34.02 ± 11.60
LDL (mg/dL)	11.36 ± 1.88	11.46 ± 1.61	14.86 ± 5.09	18.70 ± 7.30	13.26 ± 2.03	11.94 ± 1.91
AST (U/L)	162.8 ± 37.88	163.4 ± 30.57	161.6 ± 10.88	201.8 ± 10.21	195.2 ± 42.47	168.2 ± 29.15
ALT (U/L)	85.00 ± 20.58	86.40 ± 16.8	90.80 ± 6.02	72.00 ± 10.88	61.60 ± 7.16	55.00 ± 6.24
UREA (mmol/L)	41.40 ± 1.14	39.94 ± 1.1	53.80* ± 8.32	50.20 ± 6.53	48.40 ± 7.33	38.04 ± 3.11
CREAT (μmol/L)	0.19 ± 0.02	0.20 ± 0.02	0.38* ± 0.03	0.51* ± 0.08	0.20 ± 0.03	0.18 ± 0.01
URIC AC (mmol/L)	1.25 ± 0.23	1.19 ± 0.21	1.28 ± 0.38	1.49 ± 0.22	2.44* ± 0.47	2.06* ± 0.38
GLUCOSE (mg/dL)	142.4 ± 17.11	150.2 ± 13.08	174.6 ± 22.49	150.4 ± 26.47	134.0 ± 18.03	127.6 ± 7.30

Values are expressed as mean ± STD (n = 10 for each group). *P values < 0.05 were considered significant using one way ANOVA followed by Tukey's multiple comparison tests. Asterisks denote significant difference compared to control.

Values are expressed as mean ± SEM (n = 6 for each group). *P values < 0.05 were considered as significant using one way ANOVA followed by Tukey's multiple comparison tests. Asterisks denote significant difference compared to control.

* Significant Different from Control.

well with histopathological changes, and the liver organ is considered important because it functions as the main detoxification organ [21].

The benefits of kidney weighing in toxicity studies are to predict toxicity, enzyme induction, physiological disturbances, and acute injury; the kidneys are often become the target organ for toxicity; this often closely associated with its histopathological changes. Some studies state that weight changes are more sensitive than histopathological changes in kidney organs [21]. The results of this study highlighted that the five essential organs, such as the heart, liver, spleen, kidney, and lungs, did not show any clinical signs of toxicity at all and showed no difference compared to controls. Since there were no significant differences in body and organ weight relative to the control group and the treatment group, we conclude that the PHGPB was non-toxic.

The serum hematology and clinical biochemistry analyses were done to evaluate the possible alterations in hepatic and renal functions influenced by the treatment. The hematology results in the treatment groups did not show any significant difference compared to the control. The non-significant effect of the treatment on total red blood cells, mean cell volume, mean corpuscular Hb, and platelets suggests that the hydrolysate does not affect the erythropoiesis, morphology, or osmotic fragility of red blood cells. In addition, no significant changes were observed in leucocytes, lymphocytes, neutrophils, monocytes, and eosinophils in the treatment groups, confirming the normal hematological profile of the PHGPB treated group and further confirming that doses of 100–400 mg/kg BW of PHGPB are non-toxic.

The results of clinical biochemistry analysis after 28 days of treatment, triglyceride levels of the control rats were higher than the treated rats. Triglycerides in the blood form complexes with certain proteins (apoproteins) to form lipoproteins. Triglycerides are fat that is formed from food, formed in the liver, and stored as fat under the skin and in other organs; the levels increase when the intake of calories consumed is higher than what is needed [23]. In rats that are well maintained, and food intake that is ad libitum or not restricted and is not forced to do activities such as in the control group of sub-chronic toxicity test study, excessive calorie intake (seen from the graph of increased body weight) can occur, which can cause hypertriglyceridemia.

The insignificant changes in ALT, and AST in male and female rats at

all treatment doses suggested that sub-chronic administration of PHGPB did not affect the hepatocyte function in rats. Lipid profile results showed low results of total cholesterol, triglyceride, LDL, and HDL which may be associated with antioxidant [23] and hypolipidemic effects of hydrolysates [15,16]. When correlated with histopathological conditions, the three treatment dose groups showed differences in inflammation scores compared to controls, however, only the highest dose (score 3) is statistically different from the control group (p < 0.05). However, the control group showed an inflammation score of 1, indicating that even under normal conditions, the control rats could experience mild inflammation, which was in line with the ALT and triglyceride levels of the male control group which was higher compared to the treatment group. There is an interesting fact that the liver histopathologic scores of the female group at the dose of 200 mg/kg BW showed no inflammation lobular. While the male group dose of 200 mg/kg BW showed fewer score than the group dose of 100 mg/kg BW which indicates there is lower inflammation in the group dose of 200 mg/kg BW/d. Indeed, previous studies have shown that a dose of 200 mg/kg BW is the most effective dose in improving gentamicin-induced rats' lipid profile and kidney function (Urea and Creatinine) [15].

Renal dysfunction can be assessed by utilizing concurrent measurements of urea, creatinine, and uric acid; and levels normally reflect reduced chances of kidney problems [22]. In low and medium doses of male rats, and low doses of female rats showed increased levels of urea and creatinine, although the creatinine levels of all these groups were still within the normal range (0.2–0.6 μmol/L) [24]. What is surprising is the fact that the male and females in the high-dose group showed no significant difference from the control, indicating that there was no deterioration in their kidney function. Likewise with the condition of the satellite group which shows no difference with the control. Increasing the dose of PHGPB indicates increasing kidney function which shows by lowering the effect of urea and creatinine parameters, possibly related to the antioxidant and essential amino acid contained in PHGPB. When the results of the renal function parameters of the low and moderate dose groups that slightly increased were associated with their histopathological conditions, indeed all three treatment dose groups showed low

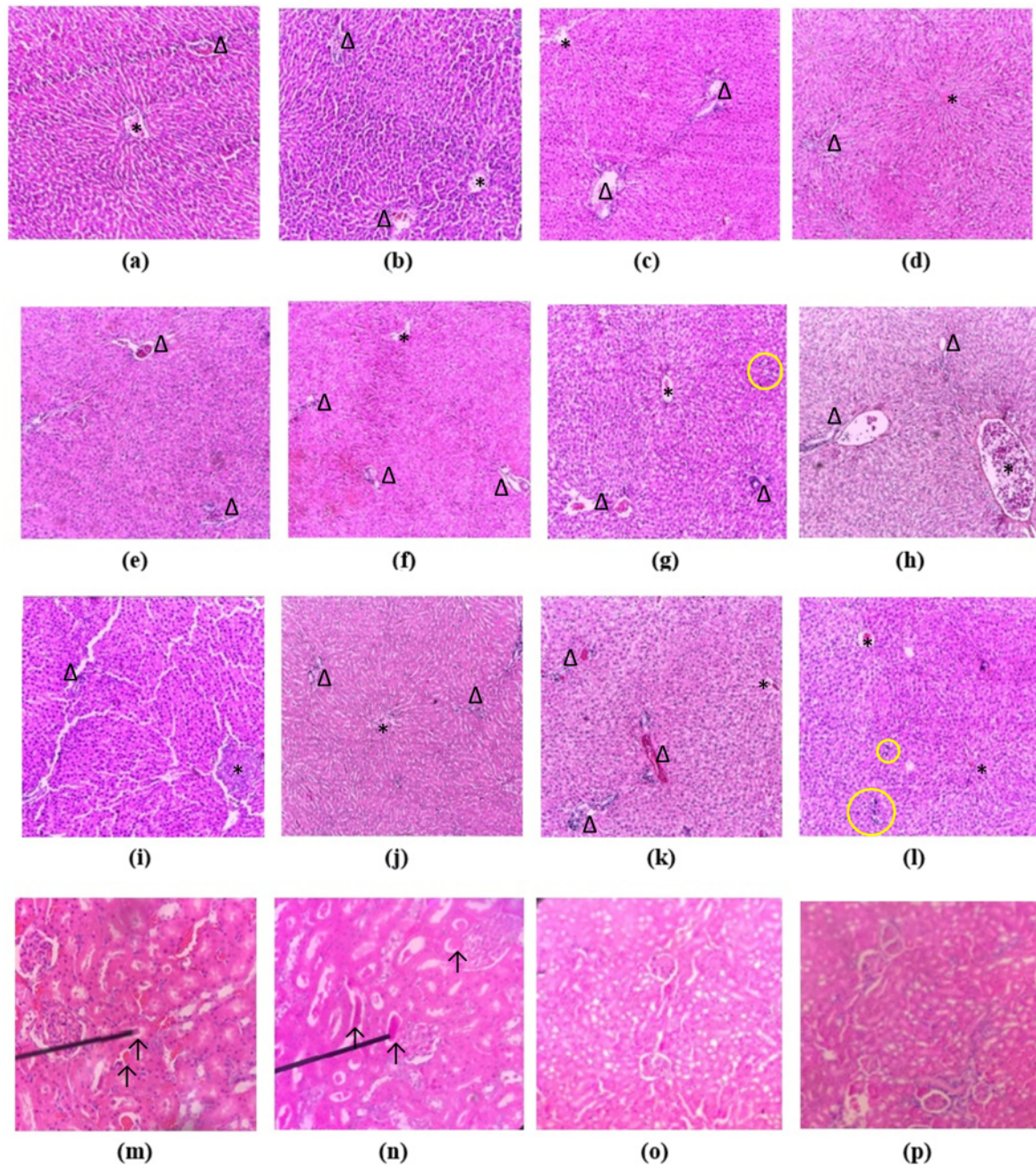


Fig. 3. Effects of various dose of green peas protein hydrolysate on Liver and Kidney rats histomorphologies in sub-chronic oral toxicity study. **Liver Male Rats** (a) 100 mg/kg BW, (b) 200 mg/kg BW, (c) 400 mg/kg BW, (d) Control, (e) Satellite Control, (f) Satellite 400 mg/kg BW. **Liver Female Rats** (g) 100 mg/kg BW, (h) 200 mg/kg BW, (i) 400 mg/kg BW, (j) Control, (k) Satellite Control, (l) Satellite 400 mg/kg BW. (*) central vein. (Δ) portal triad. (yellow circle) lobular inflammation. **Kidney Rats** (m) Hyalin Cast score 1 (10x), were found in Male rats dose of 100, 200 and 400 mg/kgBW and Female rats dose of 200 and 400 mg/kgBW (n) Hyalin Cast score 1 (40x), (o) Male Rat Control, (p) Female Rat Control. (↑) hyalin cast.

hyaline cast scores (score 1) except in the high-dose satellite group. In prerenal Acute Kidney Injury (AKI), the hyaline cast can result from urinary volume depletion and is often not associated with tubular injury/necrosis. Hyaline cast mainly consists of uromodulin produced by the loop of Henle cells and can be seen when there is decreased renal

perfusion and slow urine flow. A hyaline cast can also be seen in the presence of dehydration. Sometimes severe renal hypoperfusion can lead to "patchy" tubular injury, which can co-occur with the prerenal physiology creating the hybrid form of AKI [25].

The increase in uric acid in female rats in the high dose group also

Table 4
Median score of liver histopathology parameters in rats treated for 28 days.

Liver male	Group					
	Control	Satellite	100	200	400	Satellite
Lobular Inflammation	1	0	2	1	3*	0
Steatosis	0	0	0	0	0	0
Ballooning degeneration	0	0	0	0	0	0
Liver female	Group					
	Control	Satellite	100	200	400	Satellite
Lobular Inflammation	1	0	1	0	3*	0
Steatosis	0	0	0	0	0	0
Ballooning degeneration	0	0	0	0	0	0

Kruskal Wallis test showed $p > 0.05$ (NS) except * $p < 0.05$.

Table 5
Median score of kidney histopathology parameters in rats treated for 28 days.

Kidney male	Group					
	Control	Satellite	100	200	400	Satellite
Cloudy Swelling	0	0	0	0	0	0
Nuclear Necrosis	0	0	0	0	0	0
Hyaline Cast	0	0	1	1	1	0
Fibrosis	0	0	0	0	0	0
Kidney female	Group					
	Control	Satellite	100	200	400	Satellite
Cloudy Swelling	0	0	0	0	0	0
Nuclear Necrosis	0	0	0	0	0	0
Hyaline Cast	0	0	0	1	1	0
Fibrosis	0	0	0	0	0	0

Kruskal Wallis test showed $p > 0.05$ (NS).

needs to be a concern, but after 28 days the treatment was stopped the condition became normal. It is necessary to be aware of this increase in uric acid levels, even in male rats, this does not happen. This condition is probably because female rats are much more sensitive than male rats. To ensure safety and toxicity data, it is better if chronic toxicity tests are carried out in the future for a longer time, for example, 90 days or more.

5. Conclusion

Based on our findings, it was concluded that the oral sub-chronic toxicity test of PHGPB for 28 days did not induce noticeable signs of toxicity. The NOAEL of PHGPB in the sub-chronic toxicity study was the dose of 200 mg/kg BW. The results of our studies PHGPB indicate a lack of toxicity and support the use of functional foods.

Based on the current study, if the circumstances allow, further studies are recommended to ensure the safety of PHGPB in more detail through a larger population or longer study time eg 90 days, or chronic exposure with increasing doses up to 1000 mg/kg BW/day.

At the next stage of preparation to produce natural ingredients to be used as drugs, after the completion of the toxicity test, it can be considered to prepare a drug formulation with the aim of absorbing the drug effects for human subjects in clinical trials. One of the formulation methods that can be developed is Nano-chitosan as a carrier for PHGPB so that the active substance can reach the target organ (kidney) precisely.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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