IN VIVO ASSESSMENT OF ACUTE AND SUBCHRONIC TOXICITY OF NANOCHITIN IN EXPERIMENTAL ANIMALS

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This study aimed to evaluate the safety of Nanochitin through oral administration in experimental animals. The acute toxicity was determined in mice at ascending doses and the subchronic toxicity was evaluated in rats with oral doses of 15.6 mg/kg b.w/day and 46.8 mg/kg b.w/day for 30 days. As a result, in the course of the acute toxicity test, Nanochitin at the highest dose of 750 mg/kg did not express acute toxicity in mice. Along with the subchronic toxicity test, Nanochitin had no deleterious effect on hematological parameters, hepato-renal functions, macroscopic and microscopic images of the livers and kidneys of rats. In conclusion, Nanochitin does not appear to produce acute and subchronic toxicities in experimental animals.

Keywords: Nanochitin, acute toxicity, subchronic toxicity, experimental animals.

I. INTRODUCTION

Nature has been a source of medicinal agents from the ancient times and medicinal plants, especially have formed the basis of the wide variety of traditional medicines used in various countries worldwide.[1] The exclusive use of herbal drugs for the management of variety of ailments continues due to easy access, better compatibility and for economic reasons. According to the World Health Organization (WHO), up to 80% of developing country populations uses traditional medicine for their primary health care. However, lack of evidence-based approaches and lack of toxicological profiling of herbal preparations form the biggest concern of medicinal plants use. Thus, the evaluation of their toxicity plays a vital role in recognizing these effects, assisting in their characterization, evaluating their risk to human, and in proposing measures to mitigate the risk particularly in early clinical trials.[2]

Toxicity refers to unwanted effects on biological systems. In order to evaluate biological toxicity, it is very important to choose the correct system, since no effect may otherwise be seen. Toxicity of a substance can be impacted by many factors, such as the route of exposure (skin absorption, ingestion, inhalation, or injection); the time of exposure (a brief, acute, subchronic, or chronic exposure); the number of exposures (a single dose or multiple doses over a period of time); the physical form of the toxin (solid, liquid, or gas); the organ system involved (cardiovascular, nephro-, hemo-, nervous-, or hematopoietic-system); and even the genetic makeup and robustness of the target cells or organisms.[3] Subchronic systemic toxicity is

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Received: 08/04/2025
Accepted: 23/04/2025

defined as adverse effects occurring after the repeated or continuous administration of a test sample for up to 90 days or not exceeding 10% of the animal's lifespan.[4]

Nanochitin was made from shells of white leg shrimp (*Litopenaeus vannamei*). So far, there has been few reports available on the safety of Nanochitin in the world as well as in Vietnam. Therefore, in the present study, we aimed to validate the acute and subchronic toxicity of Nanochitin in experimental animals.

II. MATERIALS AND METHODS

1. Subjects

The preparation of Nanochitin

Nanochitin was formulated in the form of white powder, made from shells of white leg shrimp (*Litopenaeus vannamei*). Nanochitin was a test product of the study "Research on creating nanochitin from shrimp shell chitin for salt reduction application in food processing" conducted by the group of authors Nguyen Thi Cha, Assoc.Prof.Dr. Ho Phu Ha, and Dr. Tien-Thanh Nguyen from 2021 - 2026. The recommended dosage in humans was 2.6 mg/kg b.w of Nanochitin per day.

Experimental animals

Healthy *Wistar* rats (180 - 220g) and *Swiss* mice (18 - 22g) were used in this study. The animals were housed in cages (groups of ten rats or mice/cage) in a room with access to standard certified rodent diet and water ad libitum. They were acclimated to housing for at least 5 days prior to investigation at the Department of Pharmacology, Hanoi Medical University.

2. Methods

Acute toxicity study

Acute toxicity study were carried out according to WHO Guidance.[5]

Before the experiment, mice were fasted overnight. Mice were divided into 10 animals per group and orally administered with Nanochitin at ascending doses that mice could tolerated. Determine the highest dose of Nanochitin at which 0% of exposed animals are lethal and the lowest dose of Nanochitin that under defined conditions is lethal for 100% of exposed animals. The general symptoms of toxicity (vomiting, convulsions, agitation, excretion…) and mortality in each group were recorded within 72 hours of oral administration. All animals found during the study were subjected to gross necropsy. A linear graph was built to calculate the LD_{50} of Nanochitin. Animals that survived after 72 hours were further observed for 7 days after administration of Nanochitin for signs of delayed toxicity.

Subchronic toxicity study

Subchronic toxicity study were carried out according to WHO Guidance.[5]

The study was carried out for 30 days. *Wistar* rats were divided into three groups of ten animals:

- Group 1 (control) was served as the distilled water control. Each rat was applied 1 ml distilled water/100 g/day by oral route of administration.

- Group 2 was applied Nanochitin at 15.6 mg/kg/day (equivalent to human recommended dose, conversion ratio 6).

- Group 3 was applied Nanochitin at 46.8 mg/kg/day (3 times as high as the dose administered to group 2).

Animals were treated daily by oral route of administration once a day in the morning for 30 days and observed once daily to detect signs of toxicity.

The signs and indexes were checked during the study including:

- General condition consists of mortality and clinical signs.

- Body weight changes.

- Hematopoietic function: red blood cells (RBC), hemoglobin (HGB), hematocrit, total white blood cells (WBC), WBC differentials, platelet count (PLT).

- Serum biochemistry: aspartate amino transferase (AST), alanine amino transferase (ALT), total bilirubin, albumin, total cholesterol and creatinine levels.

The parameters were checked before treatment, 15 days after treatment and 30 days after treatment. At the end of experiment, all animals were subjected to a full gross necrospy. 30% rats of each group will be removed livers and kidneys for histopathology examinations.

Statistical analysis

Data were analysed using Microsoft Excel software version 2019. The levels of significance between the experimental groups and the control group were made using student's t-test. Data were shown as mean ± standard deviation. All data were considered significantly at $p < 0.05$.

III. RESULTS

1. Acute toxicity study

Mice were administered orally Nanochitin from the lowest dose to the highest dose (0.25 ml/10 g each time, 3 times within 24 hours). No abnormal sign was observed within 72 hours and an additional 7 days after oral administration. Results were shown at Table 1.

Table 1. Acute toxicity study of Nanochitin

Group	n	Dose (ml/kg)	Dose (g/kg body weight)	The propotion of deaths (%)	Other abnormal signs
Group 1	10	25	250	0	No
Group 2	10	50	500	0	No
Group 3	10	75	750	0	No

2. Subchronic toxicity study

General condition

Animals had normal locomotor activities and good feedings. None of the animals in all treated groups showed any macroscopic or gross pathological changes compared to the control group.

Body weight changes

Table 2. The effect of Nanochitin on body weight changes

Time	Body weight (g)		
	Group 1	Group 2	Group 3
Before treatment	194.00 ± 9.66	194.00 ± 11.74	196.00 ± 21.71
15 days after treatment	197.00 ± 14.18	198.00 ± 12.29	199.00 ± 9.94
30 days after treatment	204.00 ± 17.13	207.00 ± 15.67	206.00 ± 16.47

Table 2 showed that no significant difference in the body weight was observed in groups treated Nanochitin compared to the control group and before treatment ($p > 0.05$).

Effect on hematological examination

Table 3. Effect of Nanochitin on hematopoietic function

Parameters	Group	Before treatment	15 days after treatment	30 days after treatment
Red blood cells count (T/L)	Group 1	8.68 ± 1.10	8.23 ± 1.67	9.19 ± 0.45
	Group 2	8.99 ± 0.35	9.36 ± 1.10	8.49 ± 1.50
	Group 3	8.61 ± 0.63	7.88 ± 1.13	8.56 ± 0.85
Hemoglobin level (g/dL)	Group 1	11.16 ± 1.31	11.00 ± 1.31	11.18 ± 1.23
	Group 2	11.76 ± 1.16	11.98 ± 1.18	11.04 ± 1.48
	Group 3	11.99 ± 1.10	11.44 ± 1.22	12.05 ± 1.15
Hematocrit (%)	Group 1	43.78 ± 5.87	42.13 ± 6.68	45.49 ± 8.64
	Group 2	43.91 ± 1.81	47.28 ± 6.71	41.63 ± 6.57
	Group 3	42.79 ± 3.25	39.20 ± 6.70	45.21 ± 3.73
MCV (fL)	Group 1	51.00 ± 1.94	51.50 ± 1.58	50.90 ± 2.08
	Group 2	49.80 ± 2.62	51.00 ± 1.76	50.20 ± 1.93
	Group 3	49.70 ± 1.16	51.10 ± 2.23	50.70 ± 2.58
Platelet count (G/L)	Group 1	536.20 ± 90.53	501.70 ± 135.33	620.30 ± 112.10
	Group 2	544.30 ± 43.30	491.10 ± 111.41	511.60 ± 123.69
	Group 3	549.40 ± 100.86	441.20 ± 121.28	611.00 ± 95.93

MCV: Mean corpuscular volume

There was no significant difference in red blood cells count, hematocrit, hemoglobin level, MCV and platelet count in groups treated Nanochitin compared to the control group and before treatment ($p > 0.05$) (Table 3).

Table 4. Effects of Nanochitin on total WBC count and WBC differentials

Parameters	Group	Before treatment	15 days after treatment	30 days after treatment
Total WBC count (G/L)	Group 1	8.36 ± 1.47	8.18 ± 1.66	8.78 ± 2.01
	Group 2	7.37 ± 1.67	7.26 ± 1.61	9.30 ± 1.88
	Group 3	8.32 ± 1.81	7.41 ± 1.69	9.38 ± 2.65
Lymphocytes (%)	Group 1	70.37 ± 11.50	69.53 ± 8.40	71.96 ± 3.30
	Group 2	68.89 ± 8.17	71.47 ± 6.59	68.52 ± 4.73
	Group 3	68.26 ± 5.28	70.34 ± 6.91	69.40 ± 8.69

Parameters	Group	Before treatment	15 days after treatment	30 days after treatment
Neutrophils (%)	Group 1	13.27 ± 3.52	14.83 ± 3.74	14.96 ± 3.85
	Group 2	15.50 ± 5.15	14.93 ± 4.29	17.34 ± 4.14
	Group 3	15.74 ± 3.70	14.99 ± 4.07	14.47 ± 2.96

WBC: white blood cells

Table 4 demonstrated that no significant change was observed in total WBC count and WBC differentials in groups treated Nanochitin compared to the control group and before treatment ($p > 0.05$).

Effect on liver parameters

There were no substantial diference in aspartate amino transferase (AST) level, alanine amino transferase (ALT) level, total bilirubin, albumin concentration, and total cholesterol concentration in groups treated Nanochitin compared to the control group and before treatment ($p > 0.05$). The results were shown in table 5.

Table 5. Effects of Nanochitin on liver parameters.

Parameters	Group	Before treatment	15 days after treatment	30 days after treatment
AST level (UI/L)	Group 1	84.70 ± 5.27	81.92 ± 6.33	79.30 ± 7.79
	Group 2	80.30 ± 8.91	74.40 ± 10.89	74.10 ± 8.41
	Group 3	84.30 ± 7.60	78.70 ± 10.70	79.80 ± 6.60
ALT level (UI/L)	Group 1	50.30 ± 6.29	41.50 ± 10.35	42.80 ± 10.12
	Group 2	43.60 ± 8.91	40.40 ± 5.15	47.50 ± 7.41
	Group 3	49.40 ± 7.69	41.30 ± 10.44	49.80 ± 10.76
Total bilirubin (mmol/L)	Group 1	6.83 ± 0.45	7.05 ± 0.78	7.15 ± 0.75
	Group 2	6.81 ± 0.73	7.31 ± 0.81	7.21 ± 0.74
	Group 3	7.10 ± 0.47	7.43 ± 0.55	7.29 ± 0.87
Albumin concentration (g/dL)	Group 1	2.71 ± 0.28	2.89 ± 0.27	2.91 ± 0.22
	Group 2	2.94 ± 0.30	2.78 ± 0.25	2.70 ± 0.34
	Group 3	2.95 ± 0.41	2.66 ± 0.34	2.91 ± 0.28
Total cholesterol concentration (mmol/L)	Group 1	1.12 ± 0.26	1.04 ± 0.26	1.16 ± 0.12
	Group 2	1.06 ± 0.27	1.10 ± 0.20	1.13 ± 0.06
	Group 3	1.06 ± 0.27	1.12 ± 0.22	1.17 ± 0.07

Effect on kidney function

Table 6 illustrated that Nanochitin caused no significant difference in serum creatinine level compared to the control group and before treatment ($p > 0.05$).

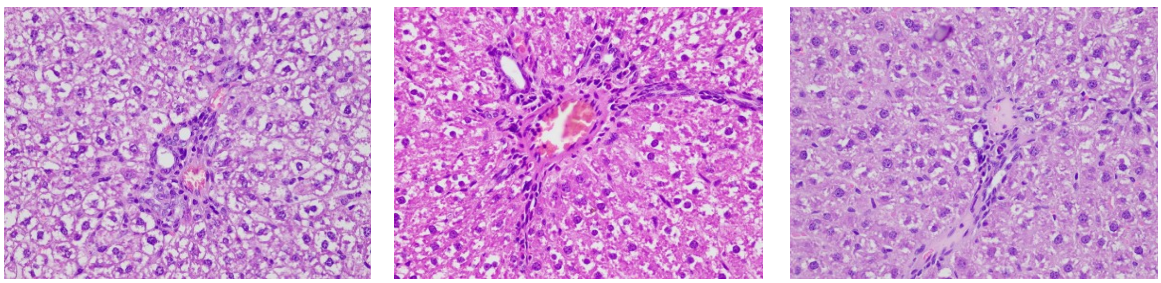
Table 6. Effects of Nanochitin on serum creatinine level

Days	Creatinine level (mg/dl)		
	Group 1	Group 2	Group 3
Before treatment	64.40 ± 4.79	65.00 ± 6.46	62.50 ± 3.14
15 days after treatment	63.30 ± 7.39	69.50 ± 8.63	66.10 ± 6.21
30 days after treatment	65.30 ± 6.17	67.40 ± 7.65	67.90 ± 8.49

Histopathological examination

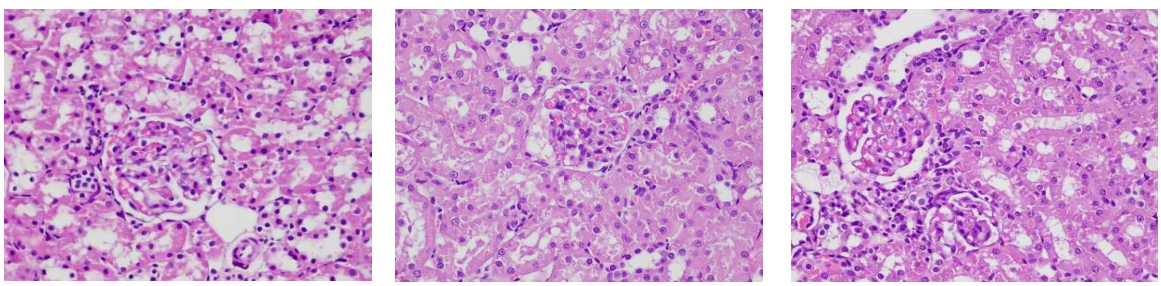
No gross lesion or change in size was observed when all experimental rats were subjected to a full gross necropsy which examined the hearts, livers, lungs, kidneys and abdominal cavities.

There was no significant change in histopathological examination of liver and kidney between mice treated Nanochitin and control group after 30 days of treatment (figure 1 and 2).



Group 1 Group 2 Group 2

Figure 1. Histopathological images of livers (HE × 400)



Group 1 Group 2 Group 3

Figure 2. Histopathological images of kidneys (HE × 400)

IV. DISCUSSION

1. Acute toxicity of Nanochitin

In this experiment, acute oral toxicity test showed that Nanochitin was tolerated up to 750 mg/kg (approximately 24.03 times as high as recommended human dose). Moreover, no sign of toxicity and no mortality was observed for continuous 7 days. As a result, oral LD_{50} of Nanochitin was not determined in mice. As defined by WHO, Nanochitin was the safe product derived herbal medicine.

2. Subchronic toxicity of Nanochitin

Toxicity is the degree to which a substance can harm humans or animals. Toxicity can refer to the effect on a cell (cytotoxicity), an organ (e.g. renal or liver toxicity), or the whole organism. To determine the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animal models to predict toxicity and to provide guidelines for selecting 'safe' therapeutic doses in humans.[6] A subchronic toxicity study provides information on the effects of repeated oral exposure and can indicate the need for further longer term studies.[7] Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity.[8]

The body weight changes serve as a sensitive indication of the general health status of animals.[8] Weights were observed in all animals treated with Nanochitin. It can be stated that Nanochitin did not interfere with the normal metabolism of animals as corroborated by the nonsignificant difference from animals in the distilled water control group.

The hematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in man and animals. Furthermore, such analysis is relevant to risk evaluation as changes in the hematological system have higher predictive value for human toxicity when the data are translated from animal studies.[7] After 15 days and 30 days of the treatment, there were no significantly difference in total red blood cells, hematocrit, hemoglobin level, platelet count, total WBC count, and WBC differentials between Nanochitin treated groups and control group, so it can be concluded that the administration of Nanochitin did not affect the hematological profile and blood formation process.

Analysis of kidney and liver is very important in the toxicity evaluation of drugs and plant extracts as they are both necessary for the survival of an organism. The clinical biochemistry analyses were carried out to evaluate the possible alterations in hepatic and renal functions influenced by the plant products.[9] The liver releases AST, ALT and an elevation in plasma concentration is an indicator of liver damage. As shown at Table 5, there was no subtantial change in the AST and ALT levels between the Nanochitin treated groups and the control group. These results indicated that Nanochitin had no deleterious effect on liver function.

Creatinine level can be used in describing the function of the kidneys.[7] The results presented that no significantly difference between Nanochitin treated groups and the control group ($p > 0.05$), as such Nanochitin did not affect the liver and kidney function.

The histopathological examination revealed the alteration in cell structure when viewed under the light microscope. Further histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of Nanochitin. Our study showed that there was no significant difference in histopathological examination of the liver and kidney between groups treated Nanochitin and the control group.

Overall, the findings of this study indicated that no significant differenceobserved in blood profile, biochemistry parameters and histopathological observations of liver and kidney tissues between Nanochitin treated groups and the control group.

V. CONCLUSION

No signs of toxicity and no mortality was observed in mice treated Nanochitin at dose of 750 mg/kg (approximately 24.03 times as high as recommended human dose). Oral LD_{50} of Nanochitin was not determined in mice.

For continuous 30 days, Nanochitin at doses of 15.6 mg/kg/day and 46.8 mg/kg/day did not produce any toxic signs or evident symptoms of subchronic toxicity.

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