ACUTE AND SUB-CHRONIC ORAL TOXICITY STUDIES OF "TRI 02" POWDER IN EXPERIMENTAL ANIMALS

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This study aims to assess the acute and sub-chronic oral toxicity of "Tri 02" powder in experimental animals. According to World Health Organization Guidance, acute toxicity study was conducted using Swiss mice. LD50 was determined by Litchfield-Wilcoxon method, and sub-chronic toxicity of "Tri 02" at two doses (2.4 g/kg b.w/day and 7.2 g/kg b.w/day) was assessed in Wistar rats for four uninterrupted weeks. Abnormal behavior, toxic symptoms, and death were observed for 7 consecutive days to assess the effects of acute toxicity. The general behavior of the rats was observed daily, and their body weight was recorded weekly. Hematological analysis and biochemical analysis were conducted before treatment, and at 2 and 4 weeks of treatment. Macroscopic examination and histopathological examination of several organs were conducted at the end of the treatment period. The results suggested that "Tri 02" at the highest dose used for mice (187.5 g/kg b.w) did not show acute toxicity and the LD50 was determined. In terms of the sub-chonic toxicity test, after oral administration of "Tri 02" powder, hematological parameters, hepato-renal functions and microscopic images of liver and kidney at both doses were similar to the control group. In conclusion, "Tri 02" powder did not produce the acute and subchronic oral toxicity in experimental animals.

Keywords: "Tri 02" powder, acute toxicity, sub-chronic toxicity, experimental animals.

I. INTRODUCTION

Traditional and complementary medicine has been used for centuries. Herbal medicine is recognized as the most common form of alternative medicine which have been used by the general public and doctors to treat various ailments. In modern medical treatment, plants have been recognized as sources of therapeutic agents, demonstrated by isolation of bioactive compounds and structural elucidation of important compounds in development of drug molecules. Despite the growing popularity and presumed safety of herbal medicines, some components of herbal medicines can be toxic at high doses and may have potentially adverse effect after prolonged use.[1] Consequently, it is important to validate the safety of traditional herbal medicines before their use in clinical settings. Particularly in early clinical trials, the toxic effects of herbs and plants need to be characterized and evaluated, and measures should be developed to mitigate the risks.[2]there is a critical need for reliable toxicity-testing methods to identify, assess and interpret the hazardous properties of any substance. Traditionally, toxicity-testing approaches have been based on studies in experimental animals. However, in the last 20 years, there

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Received: 20/09/2022
Accepted: 15/10/2022

has been increasing concern regarding the sustainability of these methodologies. This has created a real need for the development of new approach methodologies (NAMs Experimental data on the toxicity test of herbal medicine and their extracts need to be obtained to increase confidence in their safety for human use and in the development of pharmaceuticals.

"Tri 02" powder is made from ten medicinal herbs including: *Radix Codonopsis, Pericarpium Citri reticulatae perenne*, *Rhizoma Cimicifugae, Radix Bupleuri chinensis, Radix Astragali membranacei, Radix Angelicae sinensis, Radix et Rhizoma Glycyrrhizae, Rhizoma Atractylodis macrocephalae*, *Semen Nelumbinis nuciferae*, and *Semen Coicis*. The constituents of "Tri 02" powder have been studied extensively.[3-5] However, evaluating toxicity of this combination is crucial to determine the safety of certain dosages of "Tri 02" and to assess its pharmacological applications. The purpose of this study is to investigate the acute and sub-chronic oral toxicity of a "Tri 02" powder on experimental animals.

II. MATERIALS AND METHODS

1. The preparation of "Tri 02" powder

"Tri 02" was formulated in powder form and was made of ten medicinal herbal components. Ingredient for "Tri 02" are *Radix Codonopsis 35g, Pericarpium Citri reticulatae perenne 20g, Radix hedysari 35g, Rhizoma Cimicifugae 20g, Radix Bupleuri chinensis 20g, Radix Angelicae sinensis 20g, Radix et Rhizoma Glycyrrhizae 10g, Rhizoma Atractylodis macrocephalae 20g, Semen Nelumbinis nuciferae 20g, and Semen Coicis 20g*.

The quality control of raw materials and powder is in accordance with the Vietnamese Pharmacopoeia V. The maximum expected dose of "Tri 02" for human is 2 times per day, 10 grams each time. "Tri 02" powder product is manufactured by Traphaco High Tech Joint Stock Company.

2. Experimental animals

Healthy *Wistar* rats of both sexes were purchase from Dan Phuong Experimental Animal Center. Rats were of eight weeks old, and weighed between 180 - 220g.

Healthy *Swiss* mice of both sexes weighing between 18 - 22g were provided by National Institute of Hygiene and Epidemiology.

The animals were housed in cages with ten rats or mice per cage under standard environmental conditions of temperature and humidity. Rats received exposure to 12h of light/dark cycle, and were acclimatized for seven days before the experiment began in the laboratory at the Department of Pharmacology - Hanoi Medical University.

3. Method

Study design of acute toxicity study

Acute toxicity experiment was carried out according to World Health Organization (WHO) Guidance and lethal dose in 50% (LD_{50}) was determined by Litchfield - Wilcoxon method.[6,7]

Before the experiment, *Swiss* mice were fasted on the first night and divided into 4 groups with ten mice per group. Mice were orally administered with "Tri 02" at ascending doses with the same volume to evaluate the minimum lethal dose that kills all mice and the maximum dose with no death. Mice were assessed within 72 hours by monitoring animal conditions after being given "Tri 02". Finally, the researcher took care wof the mice until the 7th day of this experiment.

Sub-chronic oral toxicity study

Sub-chronic toxicity study were carried out according to WHO Guidance.[6]

Wistar rats were randomly divided into three

groups (10 rats per group) and "Tri 02" powder was administered orally in the morning for 4 consecutive weeks.

Groups (n = 10)			Administration
1	Control group		Water at 10 ml/kg b.w/ day
2	"Tri 02" powder	low dose	"Tri 02" at the dose of 2.4 g/kg b.w/day
3		high dose	"Tri 02" at the dose of 7.2 g/kg b.w/day

Observation of the rats were performed daily to note any mortality and signs of toxicity. Rats were weighed biweekly during the study. Blood samples of animals were collected via saphenous vein puncture in tubes for hematological and biochemical analysis. The parameters were checked before treatment and at 2 weeks and 4 weeks after treatment. At the end of the experiment, all animals were subjected to a full gross necropsy. The livers and kidneys of 30% rats of each group were taken for histopathology examinations. The micro-histological examination was carried out at Center for Research and Early Detection of Cancer (CREDCA). Assoc.Prof. Le Dinh Roanh, Director of CREDCA provided the results of pathological image analysis.

4. Statistical analyses

Data were analyzed using Microsoft Excel software version 2010. The results are expressed as means ± standard Deviation (SD). Avant-après test was employed for between and within group comparison and student's t-test was used for paired comparison. A p of < 0.05 was considered statistically significant.

III. RESULTS

1. Acute toxicity study

Table 1. Acute toxicity study of "Tri 02" powder

Groups	n	Dose (ml/kg)	Dose (g/kg b.w)	The propotion of deaths (%)	Other abnormal signs
Group 1	10	30	75	0	No
Group 2	10	45	112.5	0	No
Group 3	10	60	150	0	No
Group 4	10	75	187.5	0	No

Oral administration of "Tri 02" at 187.5 g/kg resulted in no mortality or clinical signs of acute toxicity in mice as observed during a short period of 24h and a prolonged period of 7 days. The mice survived until the end of the observation period and did not show signs of acute toxicity such as piloerection, lacrimation, or changes in locomotion and respiration. (Table 1)

2. Subchronic toxicity study

General condition, body weight changes

Compare to rats in the control group, rats that received daily oral administration of the "Tri 02" powder at doses of 2.4 g/kg and 7.2 g/kg for 4 consecutive weeks did not present in any significant change in the overall behaviors.

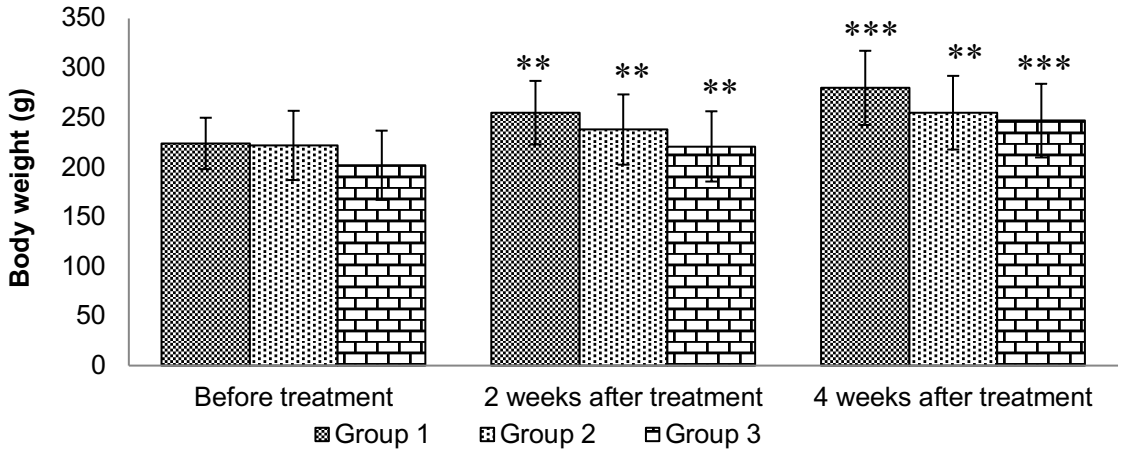

Figure 1. Effect of "Tri 02" powder on the body weight change

**p < 0.05, **p < 0.01, ***p < 0.001 were significant changes compared to before treatment*

Figure 1 showed that after 2 weeks and 4 weeks, no significant differences in body weight were observed between control and treated groups (p > 0.05).

The effect of "Tri 02" powder on hematological system in rats

'Tri 02" at two doses did not produce any significant changes in red blood cell, hematocrit, hemoglobin, WBC, neutrophils, lymphocytes and platelet counts compared to control group (p > 0.05) (Table 2 and Table 3).

Table 2. The effect of "Tri 02" powder on hematopoietic function

Parameters	Group (n = 10)	Before treatment (X ± SD)	After treatment (X ± SD)	
			2 weeks	4 weeks
Red blood cells count (T/L)	Group 1	9.57 ± 0.92	9.88 ± 1.00	9.86 ± 1.08
	Group 2	9.47 ± 1.47	9.10 ± 1.80	9.03 ± 1.38
	Group 3	10.19 ± 0.98	10.43 ± 0.57	9.52 ± 0.93
Hemoglobin level (g/dL)	Group 1	12.64 ± 1.02	12.05 ± 2.02	12.57 ± 1.50
	Group 2	12.70 ± 1.82	12.16 ± 2.18	11.96 ± 1.54
	Group 3	13.61 ± 1.45	12.96 ± 1.43	12.80 ± 1.50
Hematocrit (%)	Group 1	51.64 ± 3.07	50.61 ± 7.54	52.89 ± 8.00
	Group 2	51.43 ± 7.27	49.02 ± 9.06	53.18 ± 4.45
	Group 3	50.11 ± 6.46	48.02 ± 5.21	49.08 ± 5.70
MCV (fl) (Mean Corpscular Volume)	Group 1	50.30 ± 2.45	50.70 ± 3.30	48.30 ± 4.08
	Group 2	51.60 ± 2.99	51.20 ± 3.71	49.80 ± 4.87
	Group 3	52.00 ± 3.89	52.40 ± 1.51	50.60 ± 3.78

Parameters	Group (n = 10)	Before treatment (X ± SD)	After treatment (X ± SD)	
			2 weeks	4 weeks
Platelet count (G/L)	Group 1	671.00 ± 120.90	728.30 ± 109.79	699.00 ± 177.25
	Group 2	755.90 ± 171.28	727.80 ± 167.10	812.00 ± 184.17
	Group 3	784.20 ± 137.10	770.10 ± 138.60	672.44 ± 139.65

Table 3. The effects of "Tri 02" powder on WBC and differential white blood cell count values of rats

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Total WBC count (G/L)	Group 1	10.57 ± 2.89	9.51 ± 1.73	10.74 ± 1.48
	Group 2	10.28 ± 1.35	9.17 ± 1.39	10.17 ± 2.06
	Group 3	10.51 ± 1.62	10.45 ± 1.87	10.64 ± 1.23
Lymphocytes (%)	Group 1	68.45 ± 7.89	71.71 ± 3.59	65.47 ± 2.84
	Group 2	67.17 ± 6.12	68.85 ± 5.67	65.12 ± 7.11
	Group 3	66.81 ± 7.40	66.71 ± 9.44	64.33 ± 5.32
Neutrophils (%)	Group 1	13.27 ± 3.90	12.24 ± 1.88	15.10 ± 1.82
	Group 2	15.09 ± 3.20	14.56 ± 3.37	18.64 ± 4.95
	Group 3	16.90 ± 4.23	14.50 ± 3.42	17.32 ± 4.12

The effect of "Tri 02" powder on biochemical parameters

The biochemical parameters of the "Tri 02" groups and control group are shown in Table 4 and Figure 2. The "Tri 02" groups and the control group had no significant difference before treatment ($p > 0.05$). After treatment, the parameters pertaining to liver and kidney functions remained similar in the "Tri 02" groups and control group.

Table 4. The effect of "Tri 02" powder on serum biochemical parameters in rats

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
AST level (UI/L)	Group 1	92.60 ± 15.47	84.10 ± 13.72	84.30 ± 17.96
	Group 2	102.70 ± 18.63	97.60 ± 16.90	92.50 ± 19.35
	Group 3	104.60 ± 17.63	97.70 ± 17.71	96.00 ± 19.83
ALT level (UI/L)	Group 1	37.90 ± 6.17	32.60 ± 5.83	33.10 ± 4.93
	Group 2	35.20 ± 6.83	32.50 ± 7.79	34.30 ± 8.17
	Group 3	40.40 ± 9.14	36.70 ± 8.97	35.30 ± 6.99

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Total bilirubin (mmol/L)	Group 1	13.39 ± 0.31	13.52 ± 0.37	13.39 ± 0.30
	Group 2	13.67 ± 0.59	13.54 ± 0.3	13.31 ± 0.25
	Group 3	13.43 ± 0.45	13.34 ± 0.37	13.44 ± 0.32
Albumin (g/dL)	Group 1	3.13 ± 0.31	3.22 ± 0.45	3.09 ± 0.40
	Group 2	2.93 ± 0.25	3.16 ± 0.24	3.00 ± 0.18
	Group 3	2.99 ± 0.27	3.21 ± 0.25	2.98 ± 0.27
Total cholesterol (mmol/L)	Group 1	1.27 ± 0.26	1.16 ± 0.2	1.13 ± 0.1
	Group 2	1.22 ± 0.36	1.37 ± 0.32	1.06 ± 0.21
	Group 3	1.30 ± 0.17	1.37 ± 0.31	1.15 ± 0.29

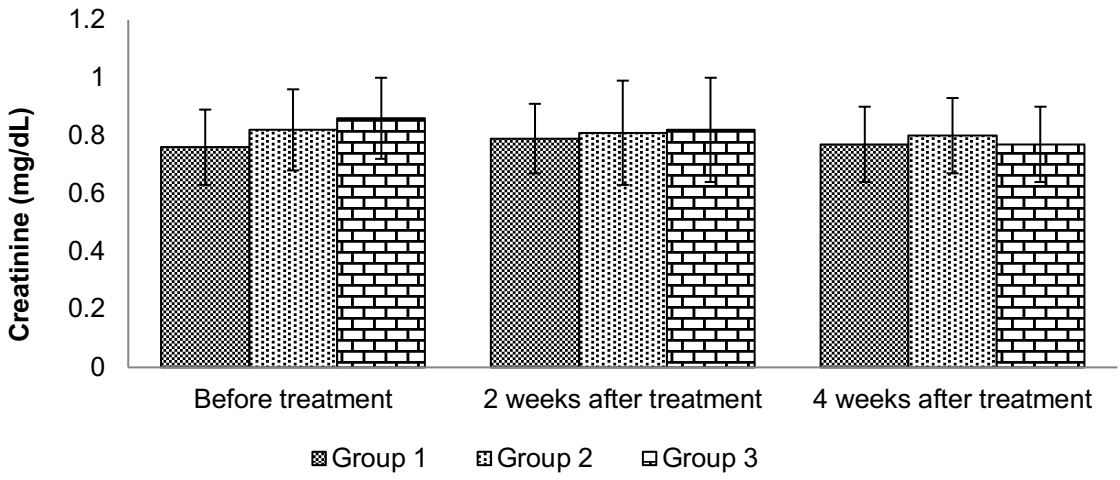

Figure 2. The effects of "Tri 02" powder on serum creatinine level

Histopathological examination

No significant gross lesions or changes were detected in liver and kidney tissues in all groups.

After 4 weeks of treatment, histopathalogical examination of "Tri 02" groups revealed no differences in liver morphology in the central vein, portal vein, hepatocytes, sinusoids, and bile ducts, compared to control group. There were no changes in the structure of kidneys with respect to glomeruli, distal and proximal tubules.

IV. DISCUSSION

Acute exposure refers to the first 24h time period in which animals are exposed to chemicals. Using the information obtained from this exposure, one may estimate the lethal dose (LD_{50}) or find out the extent to which the dosage can be safe. The data can also provide insight into the pharmacological effects of different traditional medicines of natural source. Sub-chronic toxicity and chronic toxicity studies can provide information on the potential toxicity due to repeated exposure and accumulation.[8] A

sub-chronic toxicity study provides information on target organ toxicity with longer time using herbal medicine. The exposure period for sub-chronic and chronic toxicity is usually 1-3 months and 3 months, respectively.

The results of the acute oral toxicity test showed that "Tri 02" powder was tolerated up to 187.5 g/kg b.w (approximately 39.06 times as high as recommended human dose). Moreover, there was no dead mice after 72 hours and after seven consecutive days, and no abnormal signs were observed in all animals. Therefore, this study was unable to identify LD_{50} of "Tri 02" powder in mice. According to Dong-Gu Kim (2021), after single oral dose administration of *Glycyrrhiza radix*, no significant toxicological changes or mortality was observed up to 5000 mg/kg in Sprague-Dawley rats.[9] Besides, species of the *Codonopsis* have long been used as traditional medicines are reported to have almost no toxicity. As defined by WHO, "Tri 02" powder may be considered relatively safe on acute exposure.

Oral gavage is the most convenient method for testing the toxicity of plant extracts.[10] In the 4-week repeated oral dose toxicity study, no treatment-related adverse effects were observed involving mortality, clinical signs, and general behavior. There was no significant change in body weight in the treatment groups compared to the control group during the 4-week repeated oral dose toxicity test.[5]

The hematopoietic system serves as an important target for the toxic chemicals. Analysis of risk as changes in the hematological system have higher predictive value for human toxicity when the data are translated from animal studies.[11]1991a,b In this study, after 2 weeks and 4 weeks of "Tri 02" powder treatment, there were no significantly difference in total red blood cell count, hematocrit, hemoglobin level, platelet count, total WBC count, and WBC differentials between the "Tri 02" groups and control group. These results suggested that the "Tri 02" powder might have no effect on the hematological system. Serum biochemical analysis is an important tool to assess the effects of herbal medicine. The common biomarkers for liver or kidney's damage and dysfunction include alanine amino transaminase (ALT), aspartate amino transaminase (AST), serum albumin, total cholesterol, total bilirubin, and creatinine levels. The results of this study showed no significant changes in the liver and kidney serum biomarkers between "Tri 02" powder groups and control groups, suggesting that there was no sign of impaired renal function and liver function. Addtionally, histopathological examination revealed no changes in liver and kidney morphology in rats that received doses of 2.4 and 7.2 g/kg b.w/day. This suggests that the "Tri 02" power is appropriate to be prescribed at these doses. According to previous research, *Astragalus membranaceus has* low toxicity and side effect.[5] The pharmacological mechanisms of these *Codonopsis* species related to biological activity and clinical application remain largely unexplained and the toxicity of *Codonopsis* has not been reported in the scientific literature.[9] In a oral dose toxicity study, no adverse effects, no mortality, and no toxicological changes in water consumption, hematology and target organs were observed in dosage of up to 5000 mg/kg/day *Glycyrrhiza radix* extract.[9]

Overall, the findings of this study showed no significant differences were observed in blood parameters, biochemistry parameters and histopathological observations of liver and kidney tissues between the "Tri 02" treated groups and the control group.

V. CONCLUSION

This study showed that “Tri 02” did not have any acute toxicity in experimental animals at dose of 187.5 g/kg b.w (approximately 39.06 times higher than the recommended human dose). Oral LD_{50} of “Tri 02” powder was not determined in *Swiss* mice.

For continuous 4 weeks, “Tri 02” powder at doses 2.4 g/kg b.w/day and 7.2 g/kg b.w/day did not result in any toxic signs or symptoms of sub-chronic oral toxicities in *Wistar* rats.

ACKNOWLEDGMENTS

The authors would like to thank the Traphaco High Tech Joint Stock Company, Tan Quang Ward, Van Lam District, Hung Yen Province, Vietnam for their helps and supports for the study.

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