EVALUATION OF ACUTE AND SUBCHRONIC TOXICITY OF "TRAN CHAU NGUU HOANG HOAN" IN EXPERIMENTAL ANIMALS

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"Tran chau nguu hoang hoan" was prepared from 12 herbal ingredients. So far, the safety of this product, has not been reported yet. Thus, this study aimed to evaluate the acute and subchronic toxicity of "Tran chau nguu hoang hoan" through oral administration in experimental animals. The acute toxicity was determined by the method of Litchfield Wilcoxon in mice at the doses of 2.42 g/kg b.w/day to 6.04 g/kg b.w/day. The subchronic toxicity was evaluated followed the Guideline of WHO and OECD in rats with oral doses of 58.0 mg/kg b.w/day and 174.0 mg/kg b.w/day for 12 consecutive weeks. As a result, in the course of the acute toxicity test, the mice showed no abnormal sign or death. In terms of the subchonic toxicity test, hematological indexes, hepato-renal functions and microscopic images of liver and kidney were unchanged. In conclusion, "Tran chau nguu hoang hoan" does not appear to produce acute and subchronic toxicities in mice and rats.

Keywords: "Tran chau nguu hoang hoan", acute toxicity, subchronic toxicity, experimental animals.

I. INTRODUCTION

Nature has been a source of medicinal agents from the ancient times and medicinal plants, especially, are the basis of a 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 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, in order to characterize, evaluate their risk for 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 effects 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

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organisms.[3] Subchronic systemic toxicity is 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]

"Tran chau nguu hoang hoan" was derived from ancient remedy in Mongolia pharmacopoeia in 13th century. This product aimed to support the improvement of blood circulation and the reduction of the risk of embolic stroke. "Tran chau nguu hoang hoan" was prepared from 12 natural materials.Studies about toxicities of some components in "Tran chau nguu hoang hoan" were conducted in several reports. *Ophiocordyceps sinensis* did not pose toxicological concern in rats in 28-days subacute toxicity study and 90-day subchronic toxicity study.[5,6] The acute toxicity study and the repeated dose for 28-day oral toxicity study of *Aquilaria crassna* indicated that *Aquilaria crassna* was might be a safe material.[7] However, so far, there has been no reports available on the toxicity of the combination of these components (as "Tran chau nguu hoang hoan") in the world as well as in Vietnam. Therefore, in present study, we aimed to validate the acute and subchronic toxicity of "Tran chau nguu hoang hoan" in experimental animals.

II. METHODS

1. The preparation of "Tran chau nguu hoang hoan"

"Tran chau nguu hoang hoan" was prepared in form of pills. Each pills contained 12 herbal ingredients including *Aquilaria crassna* Pierre ex Lecomte, *Calculus Bovis artificialis*, *Ophiocordyceps sinensis*, *Concretin silicea Bambusa*, *Avicula martensii*, *Cornu bubali*, *Radix Achyranthis bidentatae, Herba Dendrobii, Radix et Rhizoma Salviae mitiorrhzae*, *Carthamus tinctorus*, *Pterocarpus indicus* and *Radix et Rhioma Glycyrrhiea*.

"Tran chau nguu hoang hoan" was produced by Asean Functional Foods Co., LTD and distributed by Viet Pharmaceutical Jsc.

2. Chemicals and laboratory machines

Kits for testing enzymes and metabolites in blood: ALT (alanin aminotransferase), AST (aspartat aminotransferase), total bilirubin, albumin, total cholesterol, creatinine kits from Hospitex Diagnostics (Italy) và DIALAB GmbH (Austria) were used for Screen Master machine of Hospitex Diagnostics (Italy). Blood-testing solutions ABX Minidil LMG of ABX Diagnostics were used for Vet abcTM Animal Blood Counter. Chemicals for tests and histopathological examination.

3. Experimental animals

Healthy *Swiss* mice (20-22 g) and *Wistar* rats (150-200 g) 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 1 week prior to investigation at the Department of Pharmacology, Hanoi Medical University.

4. Acute toxicity study

Acute toxicity study were carried out according to WHO Guidance and Organization for Economic Co-operation and Development guidelines (OECD guidelines).[8,9]

Group of mice (10 per group) were fasted for 12h and orally administered with "Tran chau nguu hoang hoan" at ascending doses that mice could be tolerated. The general symptoms of toxicity and mortality in each group, within 24 hours, were recorded. The median lethal dose (LD50) was estimated by Litchfield Wilcoxon method.[10] Animals that survived after 24 hours were further observed for 7 days for signs of

delayed toxicity.

5. Subchronic toxicity study

Subchronic toxicity study were carried out according to WHO Guidance and OECD guidelines.[8,9]

The study was carried out in a consecutive 12-week period. *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/100g/day by oral route of administration;
- Group 2 was applied "Tran chau nguu hoang hoan" at the dose of 58.0 mg/kg/day (equivalent to human recommended dose, conversion ratio 6);
- Group 3 was applied "Tran chau nguu hoang hoan" at the dose of 174.0 mg/kg/day (3 times as high as the dose at group 2).

Animals were treated daily by oral route of administration of distilled water and "Tran chau nguu hoang hoan" with the volume 10 mL/kg b.w once a day in the morning for 12 consecutive weeks and observed once daily to detect signs of toxicity. Pills were dissolved with distilled water (the solvent of "Tran chau nguu hoang hoan") before giving orally for rats.

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, 4 weeks, 8 weeks and 12 weeks post treatment. At the end of experiment, all animals were subjected to a full gross necrospy. 30% rats of each group will be removed liver and kidney 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 performed pathological image analysis.

6. Statistical analysis

Data were analysed using Microsoft Excel software version 2010. The levels of significance between the experimental groups and the control group were made using student's t-test and Avant-après test. Data was shown as mean ± standard deviation. All data were considered significantly at $p < 0.05$.

*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared with the control group.

$^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared with the time point "before treatment".

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, animals treated "Tran chau nguu hoang hoan" showed no mortality, up to highest dose level (6.04 g/kg body weight) within 24 h and for consecutive 7 days. Also, animals 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

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

Table 1. Acute toxicity study of "Tran chau nguu hoang hoan"

Group	n	Dose (ml/kg)	Dose (g/kg body weight)	The propotion of deaths (%)	Other abnormal signs
Group 1	10	30	2.42	0	No
Group 2	10	45	3.62	0	No
Group 3	10	60	4.83	0	No
Group 4	10	75	6.04	0	No

Table 2. The effect of "Tran chau nguu hoang hoan" on body weight changes

Time	Body weight (g)		
	Group 1	Group 2	Group 3
Before treatment	184.00 ± 24.59	189.00 ± 30.71	175.00 ± 25.93
4 weeks after treatment	225.00 ± 47.67 $^{\Delta}$	208.00 ± 39.94 $^{\Delta\Delta}$	214.00 ± 38.64 $^{\Delta\Delta\Delta}$
8 weeks after treatment	245.00 ± 52.76 $^{\Delta\Delta\Delta}$	219.00 ± 36.65 $^{\Delta\Delta\Delta}$	212.00 ± 36.15 $^{\Delta\Delta}$
12 weeks after treatment	278.00 ± 38.24 $^{\Delta\Delta\Delta}$	244.00 ± 44.77 $^{\Delta\Delta\Delta}$	244.00 ± 34.71 $^{\Delta\Delta\Delta}$

$^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared with the time point "before treatment"

Body weight changes

Table 2 showed that after 4 weeks, 8 weeks and 12 weeks of treatment, body weight in all rats increased substantially as compared with the time point "before treatment". No significant differences were observed between groups treated "Tran chau nguu hoang hoan" and control group (group 1) ($p > 0.05$).

Effect on hematological examination

Table 3. Effect of "Tran chau nguu hoang hoan" on hematopoietic function

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Red blood cells count (T/L)	Group 1	9.75 ± 0.71	9.84 ± 0.83	9.87 ± 0.95	9.60 ± 0.87
	Group 2	9.21 ± 0.72	8.97 ± 1.08	9.20 ± 0.37	8.97 ± 1.22
	Group 3	9.20 ± 1.08	9.03 ± 1.03	9.91 ± 1.16	9.49 ± 0.85
Hemoglobin level (g/dL)	Group 1	13.50 ± 0.85	13.31 ± 1.37	12.74 ± 1.06	12.69 ± 1.45
	Group 2	13.85 ± 0.62	12.70 ± 1.45	12.56 ± 1.61	12.49 ± 1.68
	Group 3	13.73 ± 0.73	12.96 ± 1.14	13.43 ± 1.45	12.92 ± 0.94

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Hematocrit (%)	Group 1	51.86 ± 2.26	49.50 ± 3.19	49.76 ± 4.17	48.77 ± 4.33
	Group 2	49.02 ± 3.67	46.17 ± 3.98	46.93 ± 3.76	45.13 ± 3.92
	Group 3	49.86 ± 7.24	46.10 ± 4.79	50.70 ± 6.07	48.71 ± 4.59
MCV (fL)	Group 1	53.60 ± 1.17	52.40 ± 2.76	51.40 ± 3.57	51.60 ± 4.36
	Group 2	52.43 ± 1.87	51.20 ± 2.10	50.90 ± 2.77	51.60 ± 1.65
	Group 3	52.88 ± 2.36	51.70 ± 1.83$^{\Delta}$	51.70 ± 2.06	51.30 ± 1.64
Platelet count (G/L)	Group 1	567.20 ± 109.16	584.90 ± 96.38	654.90 ± 102.76	628.10 ± 110.43
	Group 2	682.70 ± 75.87	654.00 ± 70.17	662.10 ± 92.28	685.50 ± 88.18
	Group 3	562.50 ± 88.78	595.70 ± 98.87	586.10 ± 119.31	615.50 ± 94.53

MCV: Mean corpuscular volume

$^{\Delta}p < 0.05$ compared with the time point "Before treatment"

There was no significant difference in red blood cells count, hematocrit, hemoglobin level, MCV and platelet count between groups treated "Tran chau nguu hoang hoan" and group 1 ($p > 0.05$) (Table 3).

Table 4. Effects of "Tran chau nguu hoang hoan" on total WBC count and WBC differentials

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Total WBC count (G/L)	Group 1	9.43 ± 2.37	9.85 ± 2.22	9.24 ± 2.30	9.81 ± 1.10
	Group 2	8.69 ± 2.06	10.04 ± 2.47	8.52 ± 2.57	9.79 ± 3.13
	Group 3	9.26 ± 2.48	9.04 ± 1.27	7.93 ± 1.80	9.20 ± 2.47
Lymphocytes (%)	Group 1	76.89 ± 6.63	76.34 ± 8.84	73.44 ± 5.39	74.09 ± 6.68
	Group 2	75.96 ± 4.89	73.35 ± 6.36	70.25 ± 4.83	72.90 ± 3.40
	Group 3	74.15 ± 1.28	74.95 ± 4.93	74.04 ± 4.73	77.94 ± 7.69
Neutrophils (%)	Group 1	8.11 ± 1.85	8.56 ± 2.90	9.30 ± 3.00	9.59 ± 3.09
	Group 2	9.13 ± 1.84	10.74 ± 2.18	11.79 ± 3.13	9.60 ± 3.18
	Group 3	7.15 ± 1.99	8.76 ± 2.78	8.58 ± 1.73	7.57 ± 2.36

WBC: white blood cells

Table 4 demonstrated that at all time points, there was no significant difference in total WBC count, lymphocytes and neutrophils at groups treated "Tran chau nguu hoang hoan" as compared with group 1 and the time point "before treatment" ($p > 0.05$).

Effect on liver parameters

There were no significant diferences in aspartate amino transferase (AST) level and alanine amino transferase (ALT) level, total bilirubin, albumin concentration and total cholesterol concentration between groups treated "Tran chau nguu hoang hoan" and group 1 ($p > 0.05$). The results were shown in table 5.

Table 5. **Effects of "Tran chau nguu hoang hoan" on liver parameters.**

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
AST level (UI/L)	Group 1	105.80 ± 29.17	87.40 ± 22.16	90.20 ± 19.15	81.50 ± 23.02
	Group 2	103.30 ± 11.75	96.30 ± 22.75	82.90 ± 25.04	88.70 ± 14.89
	Group 3	96.20 ± 14.82	82.50 ± 14.08	82.50 ± 13.73	95.10 ± 26.11
ALT level (UI/L)	Group 1	49.30 ± 12.37	49.60 ± 18.73	49.70 ± 14.90	39.40 ± 12.19
	Group 2	43.30 ± 4.30	45.50 ± 12.54$^{\Delta}$	40.00 ± 5.72	40.10 ± 9.37
	Group 3	38.30 ± 7.20	39.30 ± 5.81	42.40 ± 5.64	45.70 ± 9.68
Total bilirubin (mmol/L)	Group 1	13.34 ± 0.54	13.42 ± 0.40	13.40 ± 0.41	13.40 ± 0.54
	Group 2	13.59 ± 0.31	13.53 ± 0.34	13.31 ± 0.38	13.39 ± 0.31
	Group 3	13.52 ± 0.39	13.44 ± 0.39	13.22 ± 0.84	13.54 ± 0.48
Albumin concentration (g/dL)	Group 1	3.59 ± 0.24	3.43 ± 0.32	3.39 ± 0.30	3.33 ± 0.29
	Group 2	3.57 ± 0.26	3.27 ± 0.41	3.24 ± 0.45	3.28 ± 0.28
	Group 3	3.45 ± 0.26	3.40 ± 0.20	3.18 ± 0.28	3.34 ± 0.36
Total cholesterol concentration (mmol/L)	Group 1	1.81 ± 0.22	1.65 ± 0.23	1.64 ± 0.38	1.86 ± 0.39
	Group 2	1.87 ± 0.21	1.69 ± 0.24	1.63 ± 0.35	1.63 ± 0.30
	Group 3	1.97 ± 0.19	1.82 ± 0.25	1.76 ± 0.30	2.02 ± 0.28

$^{\Delta}p < 0.05$ *compared with the time point "Before treatment"*

Effect on kidney function

Table 6 illustrated that "Tran chau nguu hoang hoan" caused no significant differences in serum creatinine level between groups treated "Tran chau nguu hoang hoan" and group 1 ($p > 0.05$).

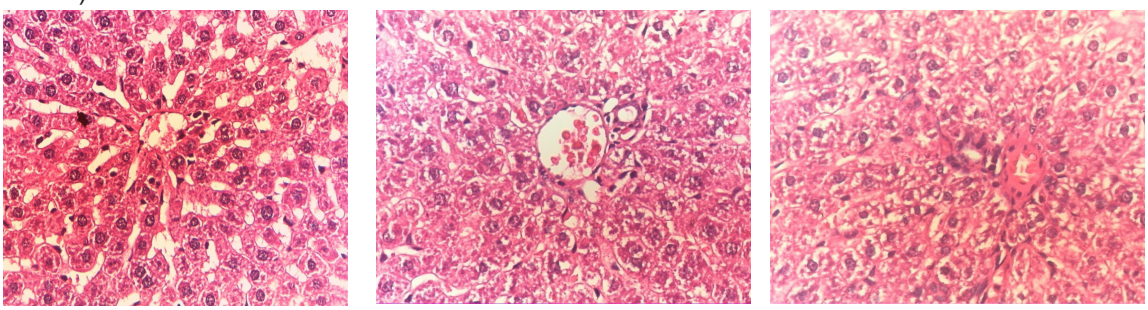
Table 6. Effects of "Tran chau nguu hoang hoan" on serum creatinine level

Days	Creatinine level (mg/dl)		
	Group 1	Group 2	Group 3
Before treatment	0.81 ± 0.22	0.81 ± 0.14	0.80 ± 0.15
4 weeks after treatment	0.88 ± 0.18	0.80 ± 0.14	0.88 ± 0.15
8 weeks after treatment	0.72 ± 0.11	0.81 ± 0.15	0.85 ± 0.19
12 weeks after treatment	0.74 ± 0.13	0.84 ± 0.11	0.86 ± 0.17

Histopathological examination

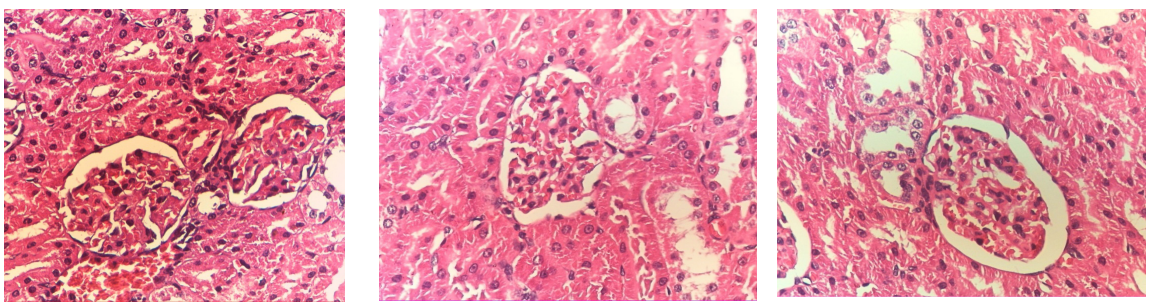
No gross lesions or changes in size was observed when subjected all experimental rats to a full gross necropsy which examined of the hearts, livers, lungs, kidneys and abdominal cavities.

There was no significant difference in histopathological examination of liver and kidney between groups treated "Tran chau nguu hoang hoan" and control group after 12 weeks of treatment (figure 1 and 2).



Group 1 **Group 2** **Group 3**

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



Group 1 **Group 2** **Group 3**

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

IV. DISCUSSION

1. Acute toxicity of "Tran chau nguu hoang hoan"

In this experiment, acute oral toxicity test showed that "Tran chau nguu hoang hoan" was tolerated up to 6.04 g/kg (approximately 52.08 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 LD50 of "Tran chau nguu hoang hoan" was not determined in mice. As defined by WHO, "Tran chau nguu hoang hoan" was the safe product derived herbal medicine.

2. Subchronic toxicity of "Tran chau nguu hoang hoan"

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. A subchronic toxicity study

provides information on the effects of repeated oral exposure and can indicate the need for further longer term studies.[8,11] 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.[12]

The body weight changes serve as a sensitive indication of the general health status of animals.[12] Weights were observed in all animals treated with "Tran chau nguu hoang hoan". It can be stated that *"Tran chau nguu hoang hoan"* 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.[8,11] After 4 weeks, 8 weeks and 12 weeks 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 groups treated "Tran chau nguu hoang hoan" with control group, so it can be concluded that the administration of "Tran chau nguu hoang hoan" 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.[13] The liver releases AST, ALT and an elevation in plasma concentration is an indicator of liver damage.[8] There was no subtantial change in AST level and ALT level between the group treated "Tran chau nguu hoang hoan" and the control group. These results indicated that "Tran chau nguu hoang hoan" had no deleterious effect on liver function.

Creatinine level can be used in describing the function of the kidneys.[11] No significantly differences were observed in blood biochemical parameters between control group and groups treated "Tran chau nguu hoang hoan" at various dose levels ($p > 0.05$). Thus, "Tran chau nguu hoang hoan" 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 "Tran chau nguu hoang hoan". Our study showed that there was no significant difference in histopathological examination of the liver and kidney between groups treated "Tran chau nguu hoang hoan" and the control group.

Overall, the findings of this study indicated that no significant differences were observed in blood profile, biochemistry parameters and histopathological observations of liver and kidney tissues between groups treated "Tran chau nguu hoang hoan" and the control group.

"Tran chau nguu hoang hoan" was derived from ancient remedy in Mongolia pharmacopoeia in 13th century. It contained 12 ingredients including *Aquilaria crassna* Pierre ex Lecomte, *Calculus Bovis artificialis*, *Ophiocordyceps sinensis*, *Concretin silicea Bambusa*, *Avicula martensii*, *Cornu bubali*, *Radix Achyranthis*

bidentatae, Herba Dendrobii, Radix et Rhizoma Salviae mitiorrhzae, *Carthamus tinctorus*, *Pterocarpus indicus* and *Radix et Rhioma Glycyrrhiea*. Historically, this remedy have been used in folklore for improving blood circulation and supporting the recovery of consciousness in coma patients.

Our study was consistent with the results from previous reports about the toxicity of components in "Tran chau nguu hoang hoan". Treatment of *Swiss* mice with essential oil of *A.crassna* did not produce treatment-related mortality at the limit test dose (2000 mg/kg) and besides, throughout the 14 days observation period, no significant change had been discovered in the behavior among the tested animals. In the repeated dose for 28-day oral toxicity study, the administration of 100 mg/kg and 500 mg/kg of essential oil of *A.crassna* per body weight revealed insignificant difference in body weight change, hematological and biochemical parameters, relative organ weights, gross findings or histopathology compared to the control group.[5] Following the study of Fung SY (2017), oral administration of cultivated fruiting body of *O. sinensis* for 28 days, at dosage up to 1000 mg/kg did not pose toxicological concern in rats.[7]

V. CONCLUSION

No sign of toxicity and no mortality was observed in mice treated "Tran chau nguu hoang hoan" at dose of 6.04 g/kg (approximately 52.08 times as high as recommended human dose). Oral LD50 of "Tran chau nguu hoang hoan" was not determined in mice.

"Tran chau nguu hoang hoan" at doses of 58.0 mg/kg/day and 174.0 mg/kg/day administered orally during continuous 12 weeks did not produce any toxic signs or evident symptoms of subchronic toxicity in rats.

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