THE STUDY OF ACUTE AND SUBCHRONIC TOXICITIES OF DA DAI TRANG HVD CAPSULES IN EXPERIMENTAL ANIMALS

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The purpose of this research is to evaluate the acute and subchronic toxicities of DA DAI TRANG HVD capsules through oral administration in experimental animals. The acute toxicity was determined by the method of Litchfield Wilcoxon in Swiss mice. The subchronic toxicity was evaluated by the recommendation of WHO and OECD in Wistar rats with oral doses of 1.44 g/kg/day (equal to recommended human dose) and 4.32 g/kg/day (3 times as high as recommended human dose) in 4 consecutive weeks. As a result, DA DAI TRANG HVD capsules at the highest dose used for mice (99.9 g materials/kg) did not express acute toxicity in mice. In term of the subchonic toxicity test, DA DAI TRANG HVD had no deleterious effect on hematological parameters, hepato-renal functions, macroscopic and microscopic images of livers and kidneys of rats. In conclusion, DA DAI TRANG HVD capsules did not produce the acute and subchronic toxicities in Swiss mice and Wistar rats.

Keywords: DA DAI TRANG HVD capsules, acute toxicity, subchronic toxicity, experimental animals.

I. INTRODUCTION

Nature has been a source of medicinal agents from the ancient times and medicinal plants, form as the basis of a wide variety of traditional medicines used in many 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 use traditional medicine for their primary health care. However, the lack of evidence-based approaches and lack of toxicological profiling of herbal preparations are the biggest concern of medicinal plant use. Thus, the evaluation of herbal toxicity plays a vital role in recognizing, characterizing, and gauging their risk for human, leading to formulate measures to mitigate the risk, particularly in early clinical trials.[2]

Toxicity refers to unwanted effects on biological systems. 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); 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 defined as adverse effects occurring after the repeated or

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continuous administration of a test sample for up to 12 weeks or not exceeding 10% of the animal's lifespan.[4,5]

DA DAI TRANG HVD capsules are prepared from natural materials including *Codonopsis pilosula (Franch)* Nannf, *Nelumbo nucifera* Gaertn., *Lactuca indica* L., *Curcuma longa* L., *Zingiber offcinale* Rosc., *Saussurea lappa* Clarke and *Atractylodes macrocephala* Koidz. Historically, these natural products have been used since ancient times and in folklore for the treatment of many diseases and illnesses. So far, there have been no reports available on the safety of a combination product from these components. Therefore, we aimed to investigate the acute and subchronic toxicities of DA DAI TRANG HVD capsules in animals.

II. METHODS

1. The preparation of DA DAI TRANG HVD capsules

DA DAI TRANG HVD was manufactured by Hai Duong Pharmaceutical Medical Materials Joint Stock Company. Hung Vuong Duong Pharmacy Joint Stock Company was responsible for the product quality and distribution. It was formulated in capsule form and each capsule contained 0.5g *Codonopsis pilosula (Franch)* Nannf, 0.5g *Nelumbo nucifera* Gaertn., 0.15g *Lactuca indica* L., 0.1g *Curcuma longa* L., 0.1g *Zingiber offcinale* Rosc., 75mg *Saussurea lappa* Clarke and 75 mg *Atractylodes macrocephala* Koidz.

The expected dose in human: for children from 6 to 12 years old, 2 times per day, 2 capsules each time and for children over the age of 12 and adults, 2 times per day, 3 – 4 capsules each time.

2. Experimental animals

Wistar rats (150 - 200g) and *Swiss* mice (18 - 22g) were provided by The Center of Experimental Animals, Dan Phuong, Ha Noi. 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.

3. Acute toxicity study

Acute toxicity study were carried out according to WHO Guidance and Organization for Economic Co-operation and Development guidelines (OECD guidelines).[6,7]

Group of mice (10 per group) were fasted for 12h – 16h and orally administered with DA DAI TRANG HVD at ascending doses that mice could be tolerated. The general symptoms of toxicity and the mortality in each group were observed within 72 hours. The median lethal dose (LD50) was detected by Litchfield Wilcoxon method.[8] Animals that survived after 72 hours were further observed for 7 days for signs of delayed toxicity (ref.).

4. Subchronic toxicity study

Subchronic toxicity study were carried out according to WHO Guidance and OECD guidelines.[6,7]

The study was carried out in a course of continuous 4 weeks. *Wistar* rats were divided into three groups of ten animals:

- Group 1 (control group) was given an administration of distilled water;

- Group 2 was administered orally DA DAI TRANG HVD at the dose of 1.44 g/kg/day (equivalent to human recommended dose, conversion ratio 6);

- Group 3 was administered orally DA DAI TRANG HVD at the dose of 4.32 g/kg/day (3 times as high as the dose at group 2).

Animals were given the oral administration of distilled water and DA DAI TRANG HVD with

the volume 10 mL/kg b.w daily for consecutive 4 weeks and observed once daily to detect clinical signs and time points for laboratory tests. The capsules were dissolved with distilled water (the solvent of DA DAI TRANG HVD) before giving orally for rats.

The signs and parameters were checked during the study including general condition, including the 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 test: aspartate amino transferase (AST), alanine amino transferase (ALT), total bilirubin, albumin, total cholesterol and creatinine levels.

The parameters were checked at the time points such as: before treatment and 2 weeks, 4 weeks after treatment. At the end of the experiment, all animals were subjected to a full gross necrospy. The livers and kidneys of 30% rats of each group will be 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 gave results of pathological image analysis.

5. 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 were shown as mean ± standard deviation. All data were considered significantly at $p < 0.05$.

Notes: *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ as compared with group 1 (control group). $^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ as compared with the time point "Before treatment".

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, DA DAI TRANG HVD capsules treated animals showed no mortality at highest dose level (99.9 g materials/kg body weight) within 24h and for additional 7 days. Also, animals did not show signs of acute toxicity such as piloerection, lacrimation or changes in locomotion and respiration (Table 1).

Table 1. Acute toxicity study of DA DAI TRANG HVD capsules

Group	n	Dose (ml/kg)	Dose (g materials/kg body weight)	The propotion of deaths (%)	Other abnormal signs
Group 1	10	45	59.9	0	No
Group 2	10	60	79.9	0	No
Group 3	10	75	99.9	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 when compared to the control group.

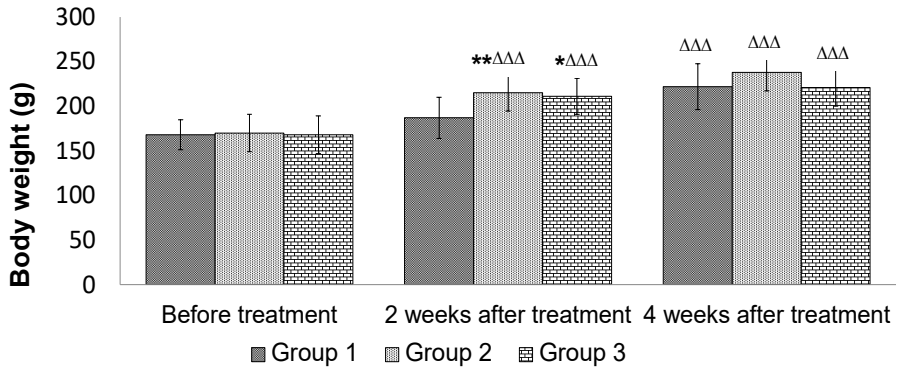


Figure 1. The effect of DA DAI TRANG HVD capsules on body weight changes

**p < 0.05, **p < 0.01 as compared with group 1 (control group)*

ΔΔΔp < 0.001 as compared with the time point "Before treatment"

Body weight changes

Figure 1 showed that at group 1 and group 2, body weight of rats increased dramatically with p < 0.001 after 2 weeks and 4 weeks of treatment as compared with the time point "Before treatment". After 2 weeks of treatment, there was a substantial development in body weight of rats at group 1 and group 2 as compared with the control group (p < 0.01 and p < 0.05 respectively).

The effect of DA DAI TRANG HVD capsules on hematological system

There were no significant differences in red blood cells count, hematocrit, hemoglobin level, platelet count, total WBC count and WBC between DA DAI TRANG HVD capsules treated groups and control group (p > 0.05) (Table 2 and Table 3).

Table 2. The effect of DA DAI TRANG HVD capsules on hematopoietic function

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Red blood cells count (T/L)	Group 1	10.41 ± 1.16	9.00 ± 2.25	10.21 ± 1.22
	Group 2	9.38 ± 1.19	9.24 ± 0.98	10.26 ± 0.81
	Group 3	9.62 ± 1.48	9.66 ± 1.06	10.68 ± 0.85
Hemoglobin level (g/dL)	Group 1	14.09 ± 1.65	12.08 ± 3.29	13.03 ± 1.77
	Group 2	13.44 ± 1.80	12.90 ± 2.43	13.20 ± 1.92
	Group 3	14.13 ± 2.35	15.53 ± 6.26	13.70 ± 1.28
Hematocrit (%)	Group 1	56.17 ± 5.98	51.36 ± 6.17	52.82 ± 7.45
	Group 2	50.69 ± 6.65	46.97 ± 6.15	51.82 ± 7.76
	Group 3	53.61 ± 8.96	49.31 ± 6.67	55.07 ± 5.76
Platelet count (G/L)	Group 1	627.30 ± 106.94	494.90 ± 185.47	638.50 ± 112.32
	Group 2	617.10 ± 141.34	628.60 ± 90.02	566.22 ± 72.99
	Group 3	554.40 ± 117.30	658.10 ± 163.57	585.00 ± 177.75

Table 3. The effects of DA DAI TRANG HVD capsules on WBC

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Total WBC count (G/L)	Group 1	10.07 ± 2.03	8.09 ± 2.47	11.45 ± 2.17
	Group 2	8.56 ± 2.34	6.49 ± 2.19	9.73 ± 1.69
	Group 3	11.29 ± 2.17	9.14 ± 2.51	10.22 ± 3.80
Lymphocytes (%)	Group 1	6.9 ± 2.0	5.4 ± 1.7	7.0 ± 2.2
	Group 2	6.0 ± 1.8	4.6 ± 1.6	7.0 ± 1.7
	Group 3	8.4 ± 1.6	6.9 ± 2.1	7.0 ± 2.6
Neutrophils (%)	Group 1	1.3 ± 0.3	1.0 ± 0.4	2.1 ± 1.3
	Group 2	1.2 ± 0.7	1.0 ± 0.4	1.5 ± 0.4
	Group 3	1.3 ± 0.5	1.2 ± 0.4	1.5 ± 0.6

The effect of DA DAI TRANG HVD capsules on liver functions

Table 4. The effect DA of DAI TRANG HVD capsules on liver functions

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
AST level (UI/L)	Group 1	105.10 ± 32.67	82.30 ± 20.41	85.40 ± 18.28
	Group 2	113.20 ± 51.40	87.90 ± 20.75	80.10 ± 12.88
	Group 3	114.20 ± 33.30	91.60 ± 21.62	104.30 ± 23.26
ALT level (UI/L)	Group 1	31.00 ± 8.97	35.90 ± 7.85	30.60 ± 5.19
	Group 2	32.30 ± 6.27	32.20 ± 3.55	32.30 ± 7.36
	Group 3	39.70 ± 18.47	37.20 ±7.79	41.30 ± 10.84*
Total bilirubin (mmol/L)	Group 1	13.34 ± 0.68	13.41 ± 0.46	13.50 ± 0.41
	Group 2	13.48 ± 0.37	13.55 ± 0.57	13.62 ± 0.57
	Group 3	13.49 ± 0.40	13.29 ± 0.48	13.56 ± 0.37
Albumin concentration (g/dL)	Group 1	3.10 ± 0.48	3.08 ± 0.25	3.19 ± 0.40
	Group 2	3.18 ± 0.37	3.24 ± 0.18	3.31 ± 0.28
	Group 3	3.27 ± 0.45	3.02 ± 0.30	3.25 ± 0.28
Total cholesterol concentration (mmol/L)	Group 1	1.46 ± 0.35	1.26 ± 0.22	1.36 ± 0.21
	Group 2	1.64 ± 0.34	1.37 ± 0.30	1.52 ± 0.30
	Group 3	1.71 ± 0.38	1.37 ± 0.47	1.53 ± 0.21

**p < 0.05 as compared with group 1 (control group)*

There were no significant differences in aspartate amino transferase (AST), total bilirubin, albumin

concentration and total cholesterol concentration between DA DAI TRANG HVD capsules treated groups and the control group (p > 0.05). The results are shown in Table 4.

Besides, in terms of alanine amino transferase (ALT) level, at group 2 (DA DAI TRANG HVD at the dose of 1.44 g/kg/day), after 2 weeks and 4 weeks of treatment, there was no statistical difference in ALT level as compared with the control group and before treatment (p > 0.05). At group 3 (DA DAI TRANG HVD at the dose of 4.32 g/kg/day), after 2 weeks of treatment, there was no significant difference in ALT level as compared with the control group and before treatment (p > 0.05). After 4 weeks of treatment, ALT level increased substantially as compared with control group (p < 0.05), however, no significant difference was observed as compared with the time point "Before treatment" and ALT level at group 2 after 4 weeks of treatment was still at normal range in rat.[9]

The effect of DA DAI TRANG HVD capsules on kidney functions

Figure 2 demonstrated that after 2 week and 4 weeks of treatment, DA DAI TRANG HVD capsules caused no significant differences in serum creatinine level between the control group and 2 treated groups (p > 0.05).

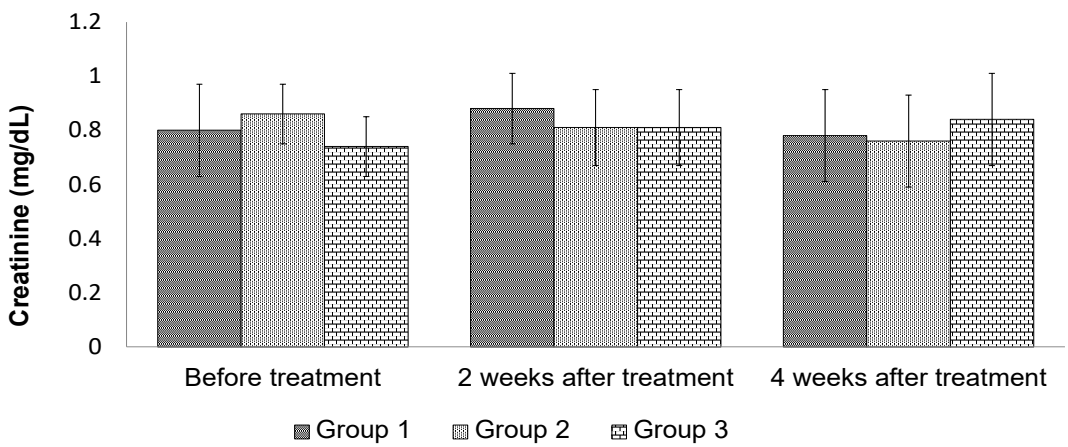
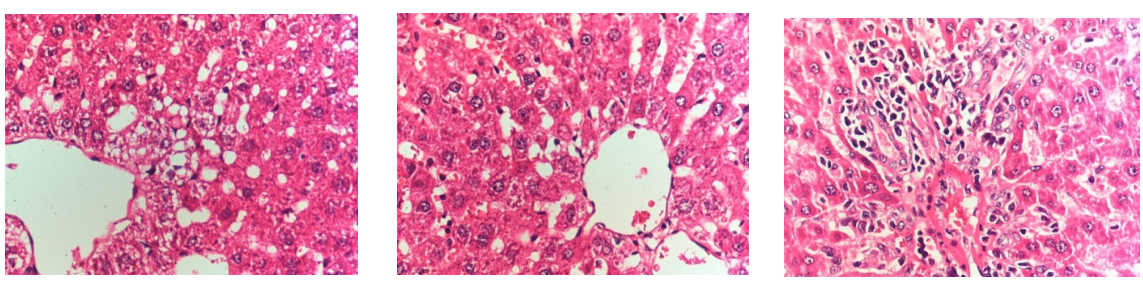


Figure 2. The effects of DA DAI TRANG HVD capsules on serum creatinine level

Histopathological examination

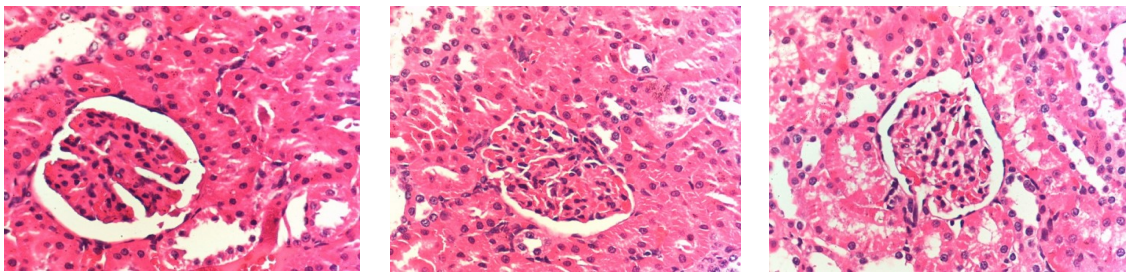
No gross lesions or changes in size were observed when subjected all experimental rats to a full gross necropsy which examined of the hearts, livers, lungs, kidneys and abdominal cavities.

There were no significant differences in histopathological examinations of livers and kidneys between DA DAI TRANG HVD capsules treated rats and control group after 4 weeks of treatment (Figure 3 and 4).



Group 1 Group 2 Group 3

Figure 3. Histopathological morphology of liver (HE × 400)



Group 1 Group 2 Group 3

Figure 4. Histopathological morphology of kidney (HE × 400)

IV. DISCUSSION

1. Acute toxicity of DA DAI TRANG HVD capsules

In this experiment, acute oral toxicity test showed that DA DAI TRANG HVD was tolerated up to 99.9 g materials/kg (approximately 34.68 times as high as recommended human dose). Moreover, no signs of toxicity and no mortality were observed for a continuous 7 days. As a result, oral LD50 of DA DAI TRANG HVD capsules was not determined in mice. As defined by WHO, DA DAI TRANG HVD was a safe herbal medicine.

2. Subchronic toxicity of DA DAI TRANG HVD capsules

Toxicity is the degree of 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.[8] To determine the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animal models to detect 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 long term studies.[6,10] 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.[11]

The changes in body weight are the most basic index to reflect toxicity to organs and systems and also reflect the combined effects of xenobiotics on the body.[11] For all experimental animals, general signs should be observed daily and body weight should be measured periodically.[10] It can be stated that DA *DAI TRANG HVD capsules* did not interfere with the normal metabolism of animals as corroborated by the non-significant difference from animals using the distilled water as the control group.

The blood circulatory system performs important functions, for example, delivering oxygen to all body tissues, maintaining vascular integrity, providing necessary immune factors for host defense reaction, and so on. The hematopoietic system is one of the most sensitive targets of toxic compounds and is an important parameter of physiological and pathological status in human and animals.[6,10] 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. After 2 weeks and 4 weeks of treatment, there were no significantly difference in total red blood cells, hematocrit, hemoglobin

level, platelet count, total WBC count and WBC differentials between the DA DAI TRANG HVD treated groups with control group, so it can be concluded that the DA DAI TRANG HVD capsules have no effect on the hematological system.

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.[12] The changes of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents is a sensitive index to reflect the degree of liver cell damage. When the chronic liver injury happened, AST and ALT would be released from the injury of the liver cells, resulting in an increase in the serum.[8] Creatinine levels can be used in describing the function of the kidneys.[10] There are no significant changes in AST in both male and female rats at all doses. The ALT level at group 3 (DA DAI TRANG HVD at the dose of 4.32 g/kg/day), however, increased significantly after 4 weeks of treatment as compared with control group ($p < 0.05$), but no significant difference was observed as compared with the time point "Before treatment" and ALT level at group 3 was still at normal range in rat.[9] The blood biochemistry level of control and DA DAI TRANG HVD in treated rats at various doses are presented no significantly differences between DA DAI TRANG HVD treated groups and control group ($p > 0.05$). These evidents show that DA DAI TRANG HVD capsules did not affect the liver and kidney functions.

In various organs, the liver and kidney are strong for the drug's affinity and conducive to the elimination of the drug, but also have a certain role in the accumulation. The histopathological examination revealed the alteration in cell structure under the light microscope.[12] Further histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of the DA DAI TRANG HVD capsules. Our study showed that there were no significant differences in histopathological examinations of the livers and kidneys between the DA DAI TRANG HVD treated groups and the control group.

Overall, the findings of this study indicated that no significant differences were observed in blood parameters, biochemistry parameters and histopathological observations of liver and kidney tissues between the DA DAI TRANG HVD treated groups and the control group.

Our study was consistent with the result from the previous report about toxicity of the component in DA DAI TRANG HVD capsules. According to Kunanusorn P (2011), the extract of *Nelumbo nucifera stamens* at a dose of 5000 mg/kg produced no treatment-related signs of toxicity or mortality in any of the animals tested during 14 days of the study. In a 90-day repeated dose oral toxicity test of *Nelumbo nucifera* extract at the doses of 50 mg/kg and 100 mg/kg showed no significant toxicity on body weight, hematological parameters, biochemical parameters, gross and histopathological examinations of rats.[13]

V. CONCLUSION

No signs of toxicity and no mortality was observed in DA DAI TRANG HVD treated mice at dose of 99.9 g/kg (approximately 34.68 times as high as recommended human dose). Oral LD50 of DA DAI TRANG HVD capsules was not determined in *Swiss* mice.

For continuous 4 weeks, DA DAI TRANG HVD capsules at doses 1.44 g/kg/day and 4.32 g/kg/day did not make any toxic signs or symptoms of subchronic toxicities.

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