EVALUATION OF ACUTE AND SUBCHRONIC TOXICITIES OF TD0019 HARD CAPSULES IN EXPERIMENTAL ANIMALS

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TD0019 hard capsules were prepared from the traditional remedy called "duhuojisheng decoction" and integrated dry extract from the bark of Eucalyptus exserta F.v Muell with fermented Glycine Max (L) Merr. Extract. These components have a potential analgesic, acute and chronic anti - inflammatory effects on experimental animals. However, the safety of this product has not been reported . Objects: To evaluate the acute and subchronic toxicities of TD0019 through oral administration in experimental animals. Methods: The acute toxicity was determined by the method of Litchfield Wilcoxon in Swiss mice at two doses 22.84 g/kg and 57.1 g/kg. The subchronic toxicity was evaluated by the recommendation of WHO and OECD in rats with oral doses of 0.822 g/kg/day (equal to recommended human dose) and 2.466 g/kg/day (3 times as high as recommended human dose) in 90 consecutive days. Results: In the course of the acute toxicity test, the mice showed no abnormal signs or death. Along with the subchonic toxicity test, hematological parameters, hepato - renal functions and microscopic images of the liver were unchanged. Compared with the control group, microscopic morphology of kidneys in the TD0019 treated groups were slightly injured in a safe range. In conclusion, TD0019 product does not affect the acute and subchronic toxicities in Swiss mice and Wistar rats.

Key words: TD0019, 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 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 [2]. TD0019 hard capsules are prepared from natural materials including Rehmannia glutinosa (Gaertn.) Libosch, dry extract from the bark of Eucalyptus exserta F.v Muell, fermented Glycine Max (L) Merr. extract, Paeonia lactiflora pall, Angelica sinensis (Oliv) Deils, Poria cocos Wolf, Eucommia ulmoides Oliv, Codonopsis pilosula (Franch) Nannf, Angelica laxiflora Diels, Ligusticum wallichii Franch, Achyranthes bidentata Blume, Loranthus parasiticus (L.) Merr, Gentiana macrophylla Pallas, Cinnamomum cassia Presl, Glycyrrhiza uralensis Fish, Ledebouriella seseloides Wolff, Asarum sieboldii and Prunus persica L. Batsch. Up to now, there is no report available on the toxicity of these mixed components, therefore we aimed to investigate the acute and subchronic toxicities of TD0019 hard capsules in animals.

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II. METHODS

1. The preparation of TD0019 hard capsules

TD0019 hard capsules were provided by Sao

Thai Duong Joint Stock Company. Each capsule contained 107.2 mg Rehmannia glutinosa (Gaertn.) Libosch, 250 mg dry extract from the bark of Eucalyptus exserta F.v Muell, 167 mg fermented Glycine Max (L) Merr. Extract, 71.4 mg Paeonia lactiflora pall, 71.4 mg Angelica sinensis (Oliv) Deils, 71.4 mg Poria cocos Wolf, 71.4 mg Eucommia ulmoides Oliv., 71.4 mg Codonopsis pilosula (Franch) Nannf, 35.9 mg Angelica laxiflora Diels, 17.9 mg Ligusticum wallichii Franch, 35.9 mg Achyranthes bidentata Blume, 35.9 mg Loranthus parasiticus (L.) Merr, 17.9 mg Gentiana macrophylla Pallas, 18 mg Cinnamomum cassia Presl, 18 mg Glycyrrhiza uralensis Fish, 35.9 mg Ledebouriella seseloides Wolff, 9.0 mg Asarum sieboldii Miq and 35.9 mg Prunus persica L. Batsch.

The expected dose in human: 6 hard capsules per day (equivalent to 6.85 g materials per day).

2. Experimental animals

Wistar rats (150 - 200 g) and Swiss mice (20 - 22 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.

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) [3; 4].

Group of mice (10 per group) were fasted for 12h and orally administered with TD0019 at ascending doses that mice could be tolerated. The general symptoms of toxicity and the mortality in each group were observed within 24 hours. The median lethal dose (LD50) was detected by Litchfield Wilcoxon method [5]. Animals that survived after 24 hours were further observed for 7 days for signs of delayed toxicity.

4. Subchronic toxicity study

Subchronic toxicity study were carried out according to WHO Guidance and OECD guidelines [3; 4].

The study was carried out in a course of continuous 90 days. Wistar rats were divided into three groups of ten animals:

- Group 1 (control group) was served with distilled water. Each rat was applied 1 ml distilled water/100g/day by oral route of administration;
- Group 2 was applied TD0019 at the dose of 0.822 g/kg/day as the low - dose group;
- Group 3 was applied TD0019 at the dose of 2.466 g/kg/day as the high - dose group.

Animals were treated daily by oral route of administration once a day in the morning for 90 consecutive days and observed once daily to detect clinical signs and time points for laboratory tests. The capsules were dissolved with distilled water (the solvent of TD0019) depending on the doses of TD0019 before giving orally.

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: 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 30, 60, 90 days 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.

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 $p < 0.05$.

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, TD0019 treated animals showed no mortality at highest dose level (57.1 g/kg body weight) within 24 h 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. Acute toxicity study of TD0019

Group	n	Dose (ml/kg)	Dose (g/kg body weight)	The propotion of deaths (%)	Other abnormal signs
Group 1	10	30	22.8	0	No
Group 2	10	45	34.26	0	No
Group 3	10	60	45.68	0	No
Group 4	10	75	57.1	0	No

2. Subchronic toxicity study

2.1. 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.

2.2. Body weight changes

Table 2. The effect of TD0019 on body weight changes

Time	Body weight (g)			p (t - test student)
	Group 1	Group 2	Group 3	
Before treatment	234.00 ± 47.19	239.00 ± 14.30	238.00 ± 29.46	> 0.05
30 days after treatment	244.00 ± 29.98	248,50 ± 22.61	251.50 ± 28.09	> 0.05
p (before - after)	> 0.05	> 0.05	> 0.05	
60 days after treatment	236.00 ± 33.73	231.00 ± 25.14	238.00 ± 37.36	> 0.05
p (before - after)	> 0.05	> 0.05	> 0.05	

Time	Body weight (g)			p (t - test student)
	Group 1	Group 2	Group 3	
90 days after treatment	232.00 ± 18.74	230.00 ± 18.26	236.00 ± 45.75	> 0.05
p (before - after)	> 0.05	> 0.05	> 0.05	

Table 2 showed that no significant differences were observed between TD0019 treated groups and control group (p > 0.05).

2.3. The effect of TD0019 on hematological system

Table 3. The effect of TD0019 on hematopoietic function

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
Red blood cells count (T/L)	Group 1	8.13 ± 1.27	8.06 ± 0.83	8.14 ± 0.67	8.11 ± 0.89
	Group 2	8.26 ± 0.64	8.45 ± 1.01	8.02 ± 0.98	8.48 ± 0.42
	Group 3	8.29 ± 0.77	8.13 ± 0.55	8.39 ± 0.53	8.66 ± 0.66
	p	> 0.05	> 0.05	> 0.05	> 0.05
Hemoglobin level (g/dL)	Group 1	11.32 ± 1.16	11.52 ± 0.91	11.24 ± 0.33	11.82 ± 1.14
	Group 2	11.57 ± 0.81	12.23 ± 1.17	11.14 ± 0.80	11.20 ± 0.86
	Group 3	11.30 ± 1.10	11.70 ± 0.90	11.34 ± 0.77	11.55 ± 0.90
	p	> 0.05	> 0.05	> 0.05	> 0.05
Hematocrit (%)	Group 1	39.71 ± 2.54	38.21 ± 2.76	37.90 ± 2.41	39.89 ± 2.90
	Group 2	40.12 ± 1.90	40.46 ± 4.25	37.72 ± 3.43	38.63 ± 4.92
	Group 3	37.41 ± 3.45	38.79 ± 3.19	37.32 ± 2.72	38.60 ± 2.78
	p	> 0.05	> 0.05	> 0.05	> 0.05
Platelet count (G/L)	Group 1	596.90 ± 62.83	616.50 ± 103.71	552.80 ± 63.16	566.50 ± 79.54
	Group 2	604.70 ± 103.09	629.20± 126.71	530.10 ± 82.21	598.70 ± 76.77
	Group 3	621.60 ± 101.05	637.00 ± 92.57	581.10 ± 98.82	593.30 ± 74.15
	p	> 0.05	> 0.05	> 0.05	> 0.05

Table 4. The effects of TD0019 on WBC

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
Total WBC count (G/L)	Group 1	8.07 ± 1.87	8.60 ± 1.94	10.18 ± 2.02	9.31 ± 1.88
	Group 2	7.74 ± 0.75	7.88 ± 1.45	9.12 ± 1.81	8.12 ± 1.24
	Group 3	7.74 ± 0.54	7.36 ± 1.64	9.47 ± 2.37	8.84 ± 2.26
	p	> 0.05	> 0.05	> 0.05	> 0.05
Lymphocytes (%)	Group 1	89.10 ± 4.95	86.30 ± 5.03	89.70 ± 2.67	86.90 ± 5.93
	Group 2	87.10 ± 4.41	83.20 ± 6.68	88.00 ± 6.20	85.00 ± 5.66
	Group 3	87.40 ± 7.81	84.40 ± 7.79	90.90 ± 5.76	82.70 ± 5.44
	p	> 0.05	> 0.05	> 0.05	> 0.05
Neutrophils (%)	Group 1	10.90 ± 4.95	13.70 ± 5.03	10.30 ± 2.67	13.10 ± 5.93
	Group 2	12.90 ± 4.41	16.80 ± 6.68	12.00 ± 6.20	15.00 ± 5.66
	Group 3	12.60 ± 7.81	15.60 ± 7.79	9.10 ± 5.76	17.30 ± 5.44
	p	> 0,05	> 0.05	> 0.05	> 0.05

There were no significant difference in red blood cells count, hematocrit, hemoglobin level, platelet count, total WBC count and WBC between TD0019 treated groups and control group (p > 0.05) (Table 3 and Table 4).

2.4. The effect of TD0019 on liver functions

There were no significant difference in aspartate amino transferase (AST), alanine amino transferase (ALT) level, total bilirubin, albumin concentration and total cholesterol concentration between TD0019 treated groups and the control group (p > 0.05). The results are shown in Table 5.

Table 5. The effect of TD0019 on liver functions

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
AST level (UI/L)	Group 1	95.13 ± 19.08	82.89 ± 15.58	82.50 ± 16.37	89.50 ± 3.31
	Group 2	94.05 ± 20.24	94.59 ± 12.17	85.70 ± 17.70	86.60 ± 17.04
	Group 3	92.61 ±17.75	93.87 ± 6.26	74.20 ± 16.92	87.40 ± 12.59
	p	> 0.05	> 0.05	> 0.05	> 0.05

Parameters	Group	Before treatment	After treatment		
			30 days	60 days	90 days
ALT level (UI/L)	Group 1	61.90 ± 18.13	55.10 ± 10.86	58.70 ± 7.36	65.60 ± 12.27
	Group 2	69.70 ± 18.26	63.40 ± 8.86	63.80 ± 9.95	62.80 ± 13.64
	Group 3	62.70 ± 14.45	63.10 ± 8.77	53.90 ± 10.92	56.80 ± 9.80
	p	> 0.05	> 0.05	> 0.05	> 0.05
Total bilirubin (mmol/L)	Group 1	13.60 ± 0.69	13.37 ± 0.62	13.17 ± 0.35	13.46 ± 0.49
	Group 2	13.46 ± 0.63	13.32 ± 0.61	13.52 ± 0.72	13.52 ± 0.39
	Group 3	13.34 ± 0.67	13.49 ± 0.46	13.37 ± 0.57	13.52 ± 0.61
	p	> 0.05	> 0.05	> 0.05	> 0.05
Albumin concentration (g/dL)	Group 1	2.93 ± 0.28	2.84 ± 0.47	2.73 ± 0.34	2.63 ± 0.31
	Group 2	2.92 ± 0.54	2.65 ± 0.30	2.77 ± 0.41	2.63 ± 0.43
	Group 3	2.84 ± 0.44	2.69 ± 0.33	2.58 ± 0.50	2.48 ± 0.20
	p	> 0.05	> 0.05	> 0.05	> 0.05
Total cholesterol concentration (mmol/L)	Group 1	1.41 ± 0.35	1.25 ± 0.20	1.36 ± 0.23	1.25 ± 0.26
	Group 2	1.37 ± 0.25	1.20 ± 0.11	1.38 ± 0.15	1.30 ± 0.20
	Group 3	1.48 ± 0.36	1.23 ± 0.20	1.31 ± 0.22	1.27 ± 0.19
	p	> 0.05	> 0.05	> 0.05	> 0.05

2.5. The effect of TD0019 on kidney functions

After treatment, TD0019 caused no significant difference in serum creatinine level between the control group and 2 treated groups (p > 0.05).

Table 6. The effects of TD0019 on serum creatinine level

Days	Creatinine (mg/dl)			p (t - test Student)
	Group 1	Group 2	Group 3	
Before treatment	1.03 ± 0.08	1.07 ± 0.08	1.04 ± 0.07	> 0.05
After 30 days	1.06 ± 0.08	1.06 ± 0.08	1.06 ± 0.10	> 0.05
p (before – after)	> 0,05	> 0.05	> 0.05	
After 60 days	1.04 ± 0.10	1.02 ± 0.08	1.04 ± 0.10	> 0.05

Days	Creatinine (mg/dl)			p (t - test Student)
	Group 1	Group 2	Group 3	
p (before - after)	> 0.05	> 0,05	> 0.05	
After 90 days	1.07 ± 0.05	1.07 ± 0.11	1.04 ± 0.08	> 0.05
p (before – after)	> 0.05	> 0.05	> 0.05	

2.6. Histopathological examination

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

There were no significant difference in histopathological examinations of livers and kidneys between TD0019 treated mice and control group after 90 days of treatment (Figure 1 and 2).

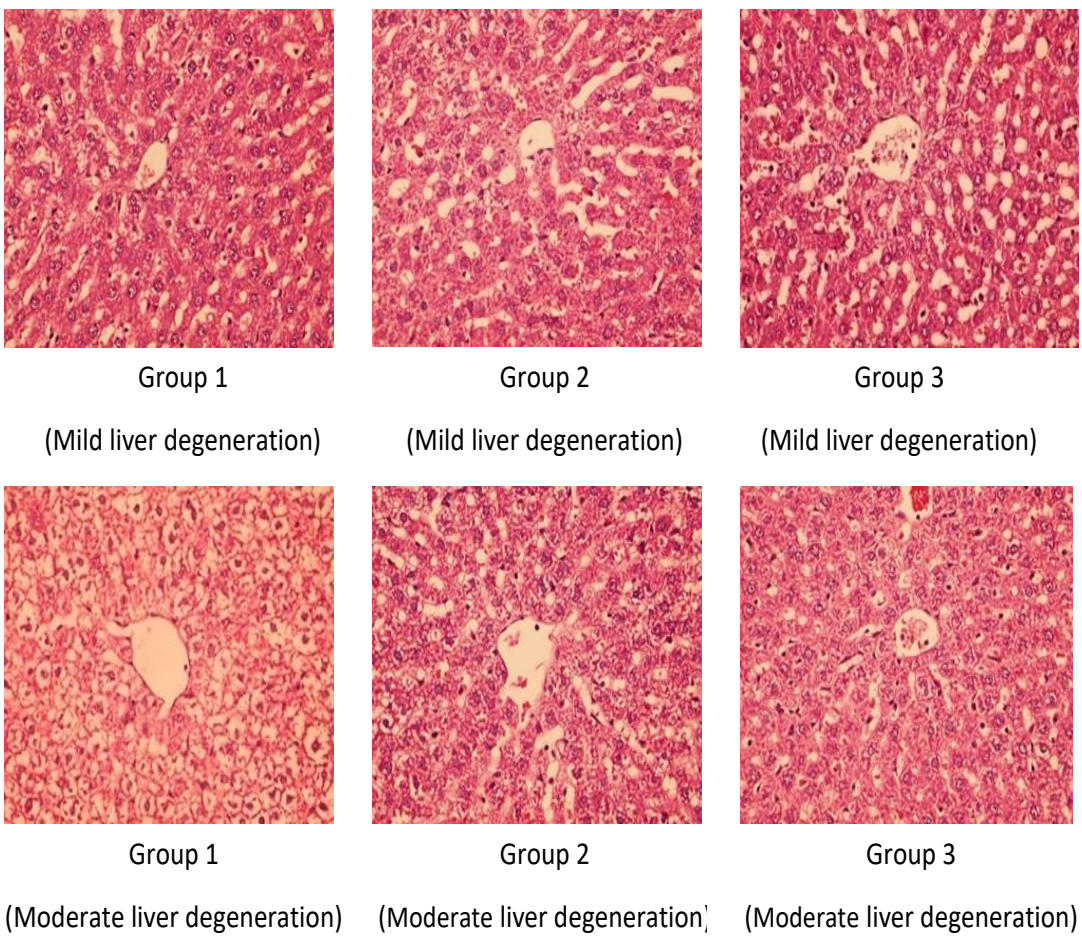
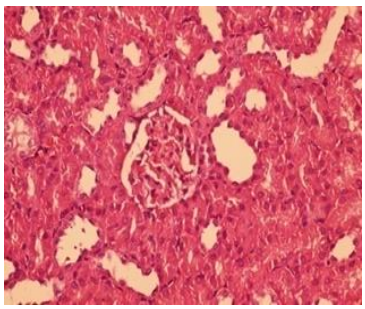
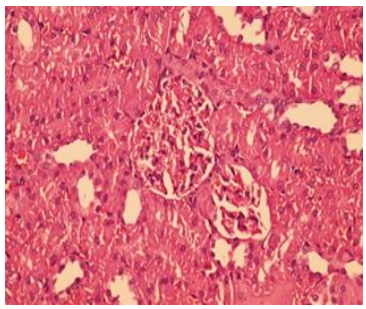


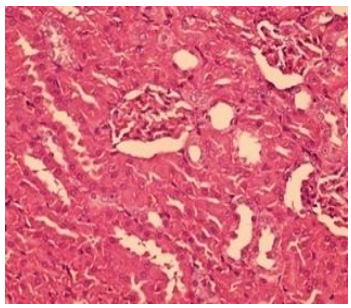
Figure 1. Histopathological morphology of liver (HE × 400)



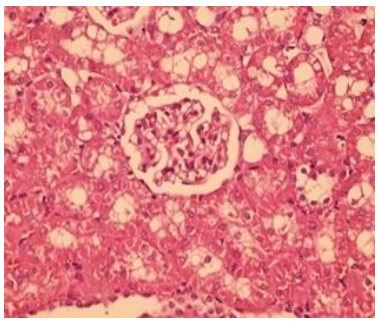
Group 1
(Normal kidney)



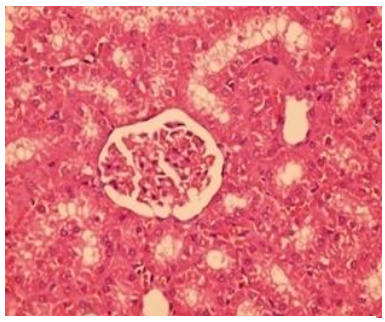
Group 2
(Normal kidney)
2mg/kg/day



Group 3
(Normal kidney)
6mg/kg/day



Group 2
(Mild kidney degeneration)
2mg/kg/day



Group 3
(Mild kidney degeneration)
6mg/kg/day

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

IV. DISCUSSION

Acute toxicity of TD0019 hard capsules

In this experiment, acute oral toxicity test showed that TD0019 was tolerated up to 57.1 g/kg (approximately 34 times as high as recommended human dose). Moreover, no sign of toxicity and no mortality were observed for a continuous 7 days. As a result, oral LD50 of TD0019 hard capsules was not determined in mice. As defined by WHO, TD0019 was a safe herbal medicine.

Subchronic toxicity of TD0019 hard capsules

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 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 longer term studies [3; 6]. 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 [7].

The body weight changes serve as a sensitive indication of the general health status of animals [7]. Weights were observed in all animals treated with TD0019 hard capsules. It can be stated that TD0019 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 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. 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 [3; 6]. After 30 days, 60 days and 90 days of treatment, there were no significant difference in total red blood cells, hematocrit, hemoglobin level, platelet count, total WBC count and WBC differentials between theTD0019 treated groups and the control group, so it can be concluded that the TD0019 hard 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 conducted to evaluate the possible alterations in hepatic and renal functions influenced by the plant products [8]. The liver releases aspartate amino transferase (AST), alanine amino transferase (ALT) and their concentrations in plasma are indicators of liver damage [3]. There is no significant change in ALT and AST in both male and female rats at all doses, which indicate that TD0019 had no deleterious effect on liver function. Creatinine level can be used in describing the function of the kidneys [6]. The blood biochemistry level of control and TD0019 in treated rats at various doses presented no significantly difference between TD0019 treated groups and control group ($p > 0.05$). These evidents show that TD0019 hard capsules did not affect the liver and kidney functions.

The histopathological examination revealed the alteration in cell structure under the light microscope. Further histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of the TD0019 hard capsules. Our study showed that there were no significant difference in histopathological examinations of the livers and kidneys between the TD0019 treated groups and the control group.

Overall, the findings of this study indicated that no observed significant difference in blood parameters, biochemistry parameters and histopathological observations of liver and kidney tissues between the TD0019 treated groups and the control group.

Our study was consistent with the results from previous reports about toxicity of some components in TD0019 hard capsules. There were no dead mice in acute toxicity experiment and no significant difference between Eucommia ulmoides seed and bark in bone marrow micro nuclear test. In chronic toxicity experiments, E. ulmoides affected the utilization rate of food, the routine blood test and liver function, and the organ coefficient of the liver, spleen, testis and ovary, but there were no obvious abnormality in histologic examination [9]. Body weight differences were not significant between group treated with Cinnamon Cassia extract and control group [10].

V. CONCLUSION

No signs of toxicity and no mortality was observed in TD0019 treated mice at dose of 57.1g/kg (approximately 34 times as high as recommended human dose). Oral LD50 of TD0019 hard capsules was not determined in Swiss mice.

For 90 continuous days, TD0019 hard capsules at doses 0.822g/kg/day and 2.466g/kg/day did not produce any toxic signs or symptoms of subchronic toxicities.

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