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Acute and Semi-chronic Toxicity of *Panax stipuleanatus* H. T. Tsai et K. M. Feng Saponin Enriched Extracts in Animal Model

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Abstract: *Panax stipuleanatus* H. Tsai et K.M. Feng exerted multiple effects on vascular bed by inducing the smooth muscle relaxation via releasing NO production as well as inhibited the platelet aggregation. Despite the obvious beneficial effects, toxicity of *Panax stipuleanatus* H. Tsai et K.M. Feng extracts needed to be further elucidated. The toxicity effects of the extracts were elucidated by acute toxicity tests in mice, semi-chronic toxicity test in rabbits by hematology, biochemistry and pathohistology. The results of acute toxicity showed that lethal dose of 50% *Panax stipuleanatus* H. Tsai et K.M. Feng extracts was 23.74 (20.59 – 26.75) g/kg. Using doses of 0.01g/kg/24h (dose 1) and 0.03g/kg/24h (dose 2) by oral administration up to 4 weeks for semi-chronic toxicity test, there was no significant difference in hematology, kidney and liver biochemistry function, coagulation and organ morphology in experimental rabbits at all the time points. The conclusion was *Panax stipuleanatus* H. Tsai et K.M. Feng with biological doses did not cause semi-chronic toxicity to experimental animals in this study.

Keywords: Panax stipuleanatus, acute toxicity, semi-chronic toxicity, mice, rabbits

INTRODUCTION

Panax stipuleanatus (H.T. Tsai et K. M. Feng) containing high amount of saponin is known to exert multiple effects on vascular bed, for example inducing eNOS phosphorylation and NO release for smooth muscle relaxation (Thom, 2018a) or platelet aggregation (Thom, 2018b). *Panax stipuleanatus* has been reported for anti-thrombosis owing to its potentially functional compound. However, the current anti-thrombosis drugs face side effects leading to bleeding, organ injury and blood content modulation (Morowski, 2013). Recently, Heptadeca-8-En-4,6-Diyne-3,10-Diol, a new polyacetylene compound, isolated from *Panax stipuleanatus* was considered as a toxic substance for potentialcancer treatment (Tuyen, 2018; Okoli, 2019). Another compound of *Panax stipuleanatus* known as oleanane-triterpenoid caused toxicity to HepG2, a liver cancer cell line, through NF-κB inhibition pathway by inhibition of iNOS and COX-2 expression (Liang, 2013). Our previous studies observed the effect of saponin isolated from *panax* on anti-thrombosis activity, therefore this study aims to estimate the acute and semi-chronic toxicity of saponin-enriched extracts of *Panax stipuleanatus.* The results can be investigated for achieving anti-thrombosis activity for future clinical applications.

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MATERIAL AND METHODS

Plant collection: Panax stipuleanatus H.T. Tsai et K.M. Feng was collected at Hoang Su Phi, Ha Giang province and characterized by Dr. Pham Thanh Huyen at National Institute of Medicinal Materials. Saponin-enriched extracts were obtained from our previous study (Thom, 2018a).

Experimental Animal growth: For acute toxicity study 40 female healthy Swiss mice provided by Military Medical University were used. Animals were grown in the standardized animal room at School of Medicine and Pharmacy, Vietnam National Univeristy Hanoi with the temperature about 24±2°C, humidity at 50±5%, artificial light with 12 hours light to 12 hours dark circle (7am:7pm). The standardized food and clean water were supplied unlimited prior to dosing. Every 5 mice were housed in a cage for at least 3 days prior experiment to allow acclimatisation to the laboratory conditions. Before dosing, mice were fasted for 3-4 hours, but were hydrated enough. The fasted body weight of each mouse was recorded. The dose was calculated according to the body weight. For semi-chronic toxicity study 30 adult rabbits (both female and male) with average weight of 2.0 ± 0.2 kg supplied by Military Medical University were used. These experimental rabbits were raised at least one week before dosing for acclimatisation to the laboratory conditions. The general state of these animals was checked and recorded every day.

Acute toxicity experiments: Addressing the adverse effects of a substance by single or multiple exposures in a short period of time is considered as acute toxicity and in a long-term period of time is considered as chronic toxicity. For acute toxicity, mice were randomly devided into 5 groups, 8 mice each. The oral administration of plant extracts was applied with various concentrations of 15, 20, 25, 30, 35 g/kg. After the extract administration, the activities of mice were observed and recorded during first 4, 24, 48 and 72 hours. The death of mice for each time point was recorded and the lethal dose 50% (LD50%) was calculated.

Semi-chronic toxicity experiments: Total rabbits were randomly divided into 3 groups with 10 rabbits each. Three groups were made including control, dose 1 and dose 2 groups. Animals were administrated with 1% NaCl in control group; with 0.01g/kg/24 hours of *Panax stipuleanatus* saponin rich extract in dose 1 group; with 0.03g/kg/24 hours of *Panax stipuleanatus* saponin rich extract in dose 2 group for 28 days. Experimental animal blood samples were collected at T0 (starting point), T2 (after 2 weeks) and T4 (after 4 weeks) for hematology, biochemistry and coagulation. The histology samples (liver, kidney and spleen) were only obtained at the 4th week on the last day of experiment.

Statistical analysis: Data sets were entered, edited and analysed using SPSS 22.0 software (IBM, USA). Appropriatestatistical analysis was applied with $P < 0.05$, considered as significant difference.

Ethic approval: This study was adhered to the ethic guideline on Ministry of Health and approved by the ethic committee (IRB-VN01016) of School of Medicine and Pharmacy.

RESULTS

Acute toxicity of Panax stipuleanatus: Acute toxicity of *Panax stipuleanatus* was accessed by oral administration of various plant extract doses to mice and followed up for 24, 48 and 72 hours after injection. The number of dead mice were evaluated shown in Table 1.

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Table 1. Number of death mice induced by *Panax stipuleanatus* in acute toxicity assays

Animal batch	Dose of Panax stipuleanatus/ animal weight	Number of mice	Number of death mice			Total dead mice
			24 hours	48 hours	72 hours	
1	15 g/kg	8	0	0	0	0
2	20 g/kg	8	2	1	0	3
3	25 g/kg	8	3	1	0	4
4	30 g/kg	8	4	2	1	7
5	35 g/kg	8	4	4	0	8

From these data, LD_{50} of *Panax stipuleanatus* was calculated using the formula:

$$LD_{50} = 23.74\ (20.59 – 26.75)\ g/kg, \text{ with } p = 0.05$$

Semi-chronic toxicity of Panax stipuleanatus: The semi-chronic toxicity was addressed by oral administration of saponin-enriched extracts of *Panax stipuleanatus* to the experimental rabbits with two doses of 0.01 g/kg/24 hours (dose 1) and 0.03 g/kg/24 hours (dose 2). Body weight data, blood and tissue samples were collected and analyzed for hematology, biochemistry, anticoagulation and histology at time point before, after 2 weeks and after 4 weeks of *Panax stipuleanatus* extract administration.

Effect of saponin-enriched extracts of Panax stipuleanatus on experimental animal body weight: There was no change of rabbit body weight between experimental groups and between different time points (before, 2 weeks and 4 weeks) of oral administration of saponin enriched extracts. The detail results are presented in Table 2.

Table 2. Effect of saponin enriched extracts of *Panax stipuleanatus* on rabbit body weight

Groups	n	Time point of *Panax stipuleanatus* administration			p_{ANOVA}
		T0	T2	T4	
Control	10	1.92 ± 0.08	1.94 ± 0.09	1.96 ± 0.07	**> 0.05**
Dose 1	10	1.88 ± 0.08	1.90 ± 0.07	1.92 ± 0.08	**> 0.05**
Dose 2	10	1.99 ± 0.07	2.00 ± 0.06	2.03 ± 0.06	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	

Note: T0: Before; T2: 2 weeks; T4: 4 weeks after saponin extract administration

Effect of saponin-enriched extracts of Panax stipuleanatus on experimental animal plasma hematological parameters: After administration of saponin-enriched extracts of *Panax stipuleanatus* before, after 2-week and 4-week time points, main hematological parameters of experimental animals were presented in Table 3. Most of hematological parameters were in normal limit. The number of red blood cells, hemoglobin, hematocrit, red blood cell volume, number of white blood cells and platelet number was not affected by plant extracts in comparison to control group. There was also no significant difference of these parameters between time point before plant extract administration and the time points of after 2 weeks and after 4 weeks, respectively. Only platelet count after 4 weeks tent to decrease versus the first time point, however it was not a statistically significant difference in this study.

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Table 3. Effect of saponin-enriched extracts of *Panax stipuleanatus* on rabbit hematological parameters

		Time point of *Panax stipuleanatus* administration			
Groups	**n**	**T0**	**T2**	**T4**	**p_{ANOVA}**
Number of red blood cells (x10^{12} g/L)					
Control	10	4.91 ± 0.99	5.03 ± 0.22	4.74 ± 0.83	**> 0.05**
Dose 1	10	5.02 ± 0.59	4.93 ± 0.33	5.04 ± 0.47	**> 0.05**
Dose 2	10	5.17 ± 0.26	5.00 ± 0.52	5.14 ± 0.40	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Hemoglobin (g/L)					
Control	10	112.50 ± 7.07	110.30 ± 4.99	109.50 ± 7.18	**> 0.05**
Dose 1	10	107.60 ± 10.34	107.30 ± 6.93	107.40 ± 7.96	**> 0.05**
Dose 2	10	110.10 ± 6.69	109.40 ± 9.91	112.30 ± 9.18	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Hematocrit (%)					
Control	10	33.00 ± 5.89	32.93 ± 1.66	30.79 ± 4.55	**> 0.05**
Dose 1	10	32.74 ± 3.95	32.31 ± 2.38	32.95 ± 2.82	**> 0.05**
Dose 2	10	33.40 ± 2.29	32.68 ± 3.73	33.80 ± 2.85	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Red blood cell volume (fl)					
Control	10	66.10 ± 3.00	65.20 ± 1.93	65.40 ± 4.33	**> 0.05**
Dose 1	10	65.30 ± 3.86	65.40 ± 2.59	65.40 ± 3.80	**> 0.05**
Dose 2	10	64.60 ± 2.27	65.30 ± 2.67	65.70 ± 2.87	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Number of white blood cells (g/L)					
Control	10	9.48 ± 2.27	9.37 ± 2.20	9.56 ± 3.11	**> 0.05**
Dose 1	10	9.23 ± 2.34	10.17 ± 3.51	9.10 ± 2.20	**> 0.05**
Dose 2	10	10.08 ± 2.44	9.44 ± 2.35	9.44 ± 2.01	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	**> 0.05**
Number of Platelet (g/L)					
Control	10	597.10 ± 224.39	402.60 ± 111.62	427.50 ± 163.29	**> 0.05**
Dose 1	10	566.70 ± 236.13	516.50 ± 203.08	425.20 ± 241.62	**> 0.05**
Dose 2	10	536.10 ± 174.43	412.20 ± 121.90	407.60 ± 131.59	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	

Note: Data was presented as mean ± SD; T0: Before; T2: 2 weeks; T4: 4 weeks after saponin extract administration

Effect of saponin-enriched extracts of Panax stipuleanatus on experimental animal plasma biochemical parameters: Liver function was examined by measuring the plasma concentration of

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AST, ALT, total albumin and total cholesterol (table 4). Similar results as hematological parameters were obtained and, there was no effect of *Panax stipuleanatus* extracts on liver function biochemical parameters in different experiment groups at all time points of examination. Interestingly, the total plasma cholesterol level seemed to decrease in plant extract treatment groups in comparison with control group, however, this decrease was not statistically significant.

Table 4. Effect of saponin-enriched extracts of *Panax stipuleanatus* on rabbit plasma biochemical parameters for liver function

Group	**n**	**Time point of *Panax stipuleanatus* administration**			**p_{ANOVA}**
		T0	**T2**	**T4**	
AST (U/L)					
Control	10	57.40 ± 26.00	52.30 ± 29.63	61.30 ± 23.02	**> 0.05**
Dose 1	10	54.50 ± 27.81	50.60 ± 22.55	55.60 ± 30.41	**> 0.05**
Dose 2	10	56.30 ± 27.83	52.30 ± 21.45	49.50 ± 22.84	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
ALT (U/L)					
Control	10	69.20 ± 17.65	70.60 ± 14.73	71.20 ± 44.08	**> 0.05**
Dose 1	10	64.70 ± 19.44	71.50 ± 28.40	72.60 ± 39.23	**> 0.05**
Dose 2	10	67.20 ± 17.43	68.50 ± 17.80	71.50 ± 26.10	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Total Albumin (g/L)					
Control	10	34.60 ± 2.41	34.90 ± 2.28	36.40 ± 2.91	**> 0.05**
Dose 1	10	34.90 ± 3.07	33.60 ± 2.63	35.20 ± 3.16	**> 0.05**
Dose 2	10	34.50 ± 2.92	34.30 ± 0.67	35.50 ± 2.76	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Total Cholesterol (mmol/L)					
Control	10	2.72 ± 1.28	2.83 ± 1.17	2.79 ± 1.63	**> 0.05**
Dose 1	10	1.76 ± 0.67	1.67 ± 0.91	1.90 ± 0.88	**> 0.05**
Dose 2	10	1.67 ± 1.09	1.69 ± 0.56	1.64 ± 0.68	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	

Note: Data was presented as mean ± SD; T0: Before; T2: 2 weeks; T4: 4 weeks after saponin extract administration

Kidney function was tested by urea and creatinine levels and the results are presented in Table 5. Clearly, *Panax stipuleanatus* saponin-enriched extracts did not induce kidney injury and function at all concentrations and time points.

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Table 5. Effect of saponin-enriched extracts of *Panax stipuleanatus* on rabbit plasma biochemical parameters for kidney function

Group	n	Time point of *Panax stipuleanatus* administration			p_{ANOVA}
		T0	T2	T4	
Ure (mmol/L)					
Control	10	6.25 ± 1.63	6.32 ± 2.41	4.92 ± 0.60	**> 0.05**
Dose 1	10	6.67 ± 1.41	7.13 ± 2.13	5.82 ± 2.41	**> 0.05**
Dose 2	10	6.08 ± 2.53	5.72 ± 1.71	5.17 ± 2.49	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	
Creatinin (mmol/L)					
Control	10	87.00 ± 18.27	77.60 ± 13.23	79.80 ± 4.69	**> 0.05**
Dose 1	10	87.30 ± 19.89	80.20 ± 10.10	85.90 ± 7.59	**> 0.05**
Dose 2	10	86.40 ± 27.27	81.10 ± 26.65	83.30 ± 26.63	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	**> 0.05**	

Note: Data was presented as mean ± SD; T0: Before; T2: 2 weeks; T4: 4 weeks after saponin extract administration

Effect of saponin-enriched extracts of Panax stipuleanatus on experimental animal organ patho-histology: Beside the blood tests for organ function, tissue samples were collected to see whether *Panax stipuleanatus* saponin-enriched extracts induced liver, kidney or spleen injury.A patho-histology analysis was performed with animal organ tissues. The results in Figure 1 showed that rabbits administrated with 0.01g/kg/24 hours (dose 1) and 0.03g/kg/24 hours (dose 2) continuously for 28 days did not cause liver, kidney or spleen damage or injury.

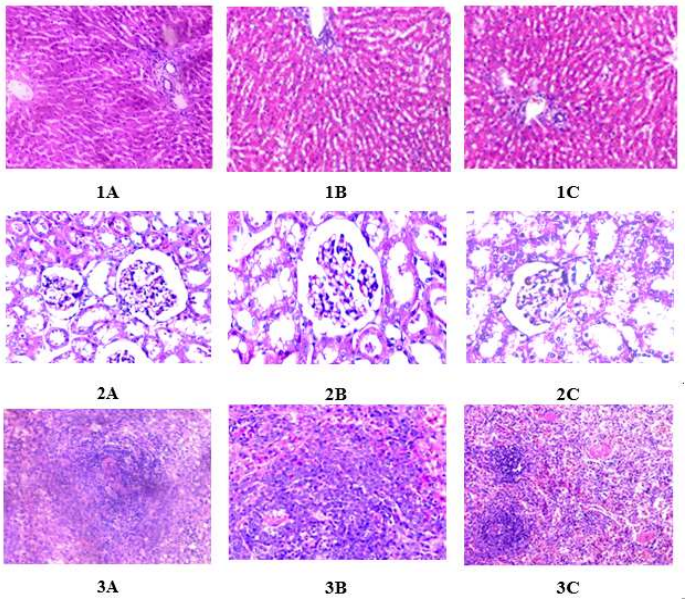

Figure 1. HE stainning for liver (1), kidney (2), spleen (3) tissues with magnification of 200x; A: control group; B: Dose 1 group; C: Dose 2 group

As seen in Figure 1, there was no difference between liver tissue structures of dosing groups (1B - 1C) and the control group's (1A): liver lobes still ensured structural integrity including central veins (Terminal hepatic venule) in the middle of capillary lobes,

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hepatic sinusoids and liver cells, the periportal space with the hepatic artery, the portal vein and the bile duct also presented clearly the normal structure. In particularly, our research's results did not record inflammatory signs such as congestion, rupture of tissue and cells, hemorrhage and bile, edema in liver tissues of drug groups. The effects of the drug on kidney tissue were depicted in picture 2B-2C. It had clear that the glomerular structure characterized by capillary beams and Bowmann capsules, renal tubules with complete epithelium like the control group (2A). Evidents of damageability such as edema, necrosis or congestion were not observed in drug group's kidney tissue. In the description of the spleen in drug mice groups (3B-3C), the splenic tissue structure were clearly shown with lymphoid follicles centered around arterioles, non-edematous, congenital venous sinuses. The red pulp were obvious composed of two elements: venous sinuses (VS) and the splenic cords (of Billroth), the tissue that lies between the sinuses.

Effect of saponin-enriched extracts of Panax stipuleanatus on experimental animal plasma anticoagulant parameters: Similar to our previous study on anticoagulation of human blood *in vitro*, the saponin-enriched extracts were not affected on experimental animal blood coagulant activity after 4 weeks as presented in Table 6.

Table 6. Effect of saponin-enriched extracts of *Panax stipuleanatus* on rabbit plasma anticoagulant parameters

Group	n	PT (s)	APTT (s)	p_{ANOVA}
Control	10	13.68 ± 2.83	23.15± 3.27	**> 0.05**
Dose 1	10	13.15 ± 1.81	22.36 ± 4.09	**> 0.05**
Dose 2	10	11.89 ± 2.88	22.49 ± 3.78	**> 0.05**
p_{ANOVA}		**> 0.05**	**> 0.05**	

Note: Data was presented as mean ± SD

DISCUSSION

Panax stipuleanatus from China contained high content of saponin with oleane and dammarane structures such as stipuleanoside R1 and R2 from root or ginsenoside Rb1, Rc, Rb3 and Rd (Qiao, 2018). Chemical compound characterization of Vietnamese *Panax stipuleanatus* root, as per our previous data showed high amount of saponin with two major structures of stipuleanoside R2 and araloside A methyl ester (Thom, 2018b). Huong et al. (2009) studied pharmacological activities of Vietnamese *Panax stipuleanatus* and found that the extract from root of this medicinal plant improved immune response, anti-stress and anti-oxidative stress.

This study showed the acute toxicity in mice with LD50% of 8.8 g/kg; anti-stress archived with concentration range of 44, 88 and 176 mg/kg in experimental mice. In our study, the LD50% of saponin extracted from *Panax stipuleanatus* was 23.74 g/kg in mice that was nearly three times higher than study of total root extract in Huong's study in 2009. Liang et al. (2013) isolated 15 compounds from root of *Panax stipuleanatus* including saponin 1, saponin 2, polyyn 16, polyyen 17 that caused toxicity effect to HL-60 hemato-cancer cell line and colon cancer cell line HCT-116 with IC50 values from 0.13 to 41.45 μM by inducing apoptosis process. The question arised whether saponin isolated from root of *Panax stipuleanatus* in our study induced the semi-chronic toxicity in the animal? Multiple semi-chronic toxicity tests performed in experimental rabbits using the equivalent concentrations for *in vitro* bioactivity testing showed that there was no toxic effect on hematological parameter as well as biochemical kidney and liver function or blood coagulation (Thom, 2018ab). In this study, we confirmed the safety concentrations for oral administration of saponin isolated from Vietnamese *Panax stipuleanatus* by various acute and semi-chronic toxicity assays.

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CONCLUSION

Identifying the lethal dose of 50% (LD50%) in mice of *Panax stipuleanatus* saponin extract was 23.74 g/kg. Mice showed fatal signs of acute toxicity. In general, saponin extract of *Panax stipuleanatus* with 3 time higher of expected treatment dose, continuously supplying for 28 days did not induce the semi-chronic toxicity in experimental rabbits.

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DECLARATION OF CONFLICT OF INTEREST

No conflict of interest associated with this work.

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