TOXICITY EVALUATION OF ACUTE AND SUB-CHRONIC ORAL TOXICITY OF HAMO NK HARD CAPSULE IN EXPERIMENTAL ANIMALS.

Pham Quoc Binh[1], Pham Thi Van Anh[2], Dang Thi Thu Hien[2], Nguyen Trong Thong[1] and Pham Thuy Phuong[1,✉]

[1]Vietnam University of Traditional Medicine,

[2]Hanoi Medical University Hospital

[3]Phenikaa University

Hamo NK hard capsule is composed of dry extracts formulation of herbal medicine which had shown good properties for effective treatment of dyslipidemia. However, there are no scientific report of its toxicological properties which would guarantee its safe usage as a potent treatment of dyslipidemia. Therefore, the present study was to investigate acute and sub-chronic toxicity of Hamo NK on mice and rats through oral administration. Acute toxicity study was performed in Swiss mice at tolerable ascending doses in mice and followed up to 7 days. Subsequently, the sub-chronic administration of Hamo NK was studied on Wistar rats. The animals were orally exposed to 0.25g/kg and 0.75 g/kg b.w/day of Hamo NK for 12 consecutive weeks. Physical observations and body weight were made during the study period. At the end of the experiment, blood samples were collected for hematology and clinical chemistry evaluations. Gross pathology and histopathology of livers and kidneys were assessed. No mortality or any major signs of morbidity was recorded for acute toxicity up to the the tolerated dose of 17.85g/kg. In sub-chronic toxicity, no major alteration was observed in the evaluated parameters at two doses of 0.25 g/kg per day and 0.75 g/kg per day. The histopathologic analysis of the livers (control and HAMO NK low dose) and kidneys indicated architecture showed no difference among groups. However, histopathological alterations were seen in the liver of HAMO NK at high dose, so a subchronic study for other doses consumed should be further carried out to assess the effects of HAMO NK on histological of liver. Collectively, these data demonstrate that Hamo NK hard capsule has a high margin of safety.

Keywords: Hamo NK, acute toxicity, sub-chronic toxicity, experimental animals.

I. INTRODUCTION

Traditional medicine is an important part of health care system which has a long history of use in disease prevention and treatment.[1] Dyslipidemia is one of the common metabolic disorders which is an increasing global health problem especially in developing countries.[2] In Vietnam, traditional medicine has been used to treat dyslipidemia and traditional knowledge of herbal medicine are still being explored and researched.[3] Hamo NK hard capsule is prepared from dry extract herbal plants including *Pericarpium Citri reticulatae perenne, Rhizoma Smilax ferox, Radix Achyranthis bidentatae, Rhizoma Imperataecylindricae, Semen Cassiae torae, Flos Styphnolobii japonici imaturi, Folium Nelumbinis nuciferae, Spica Prunellae, Rhizoma Typhonium trilobatum.* According to folk experiment and documents on traditional medicine, the researchers found that each of these herbal medicines had shown ameliorating effect on dyslipidemia.[4,5,6,7] Despite studies of each of these herbal medicines had already started broadly many years ago, the safety of their combination in Hamo NK has not been

Corresponding author: Pham Thuy Phuong
Vietnam University of Traditional Medicine
Email: thuyphuongdhctvn@gmail.com
Recieved day: 04/09/2020
Accepted day: 10/112020

evaluated. Thus, the toxicity assessment of this hard capsule was carried out to provide their safety and guidance for subsequent experiment. The objective of this study is to evaluate the acute and sub-chronic toxicity of Hamo NK in experimental animals for oral administration.

II. SUBJECTS AND METHODS

1. Plant materials and preparation of extract

Ingredients: dry extracts of each hard capsule: *Extractum Pericarpium Citri reticulatae perenne siccus* (25 mg), *Extractum Rhizoma Smilasis ferox siccus* (52 mg), *Extractum Radix Achyranthis bidentatae siccus* (112 mg), *Extractum Rhizoma Imperataecylindricae siccus* (188mg), *Extractum Semen Cassiae torae siccus* (64 mg), *Extractum Flos Styphnolobii japonici imaturi siccus* (22mg), *Extractum Folium Nelumbinis nuciferae siccus* (1mg), *Extractum Spica Prunellae siccus* (23mg), *Extractum Rhizoma Typhonium trilobatum siccus* (38 mg).

The materials were in compliance with the standards of Vietnamese Pharmacopoeia V and standard basis ISO/IEC17025 VILLAS 486170 No.10/2019. Grinding and filtering the materials up to a prescribed degree of fragmentation according to the microbiological safety and Pharmacopoeia V requirements. The dry extract (extractum siccum) materials were weighed based on the well ratio of the remedy. Dry extracts, along with pharmaceutical excipients are mixed to form granulate then filled automatically in hard capsules. Hamo NK hard capsule was prepared in Tuetinh Institute of Traditional Pharmaco – Medicine. The expected dose in clinical is 4 hard capsules per day (equivalent to 2.1 g dry extract per day)

2. Animals

The healthy Swiss mice of both sex, weighed 18 – 22 g was provided by National Institute of Hygiene and Epidemiology. The healthy *Wistar* rats of both genders from of 8 to 10 weeks old weighing from 140 – 180 g were obtained from The Center of Experimental Animals, Danphuong, Hanoi. All animal studies were acclimated to housing condition of the laboratory of the Department of Pharmacology, Hanoi Medical University for 7 days before and during the study period; they were provided with free access to standard diet and tap water *ad libitum* (housed at (25 ± 2oC) temperature and (80% ± 10 %) humidity under a 12hrs light/12 hrs dark cycle.

3. Acute toxicity experiment

Acute toxicity study was performed based on Litchfield – Wilcoxon method and the World Health Oganization Guidance. [8,9] Before conducting the experiment, the animals were randomly divided into groups with ten mice per group and kept in their cages. All mice were fasted overnight prior to oral administration of Hamo NK hard capsule at ascending doses to determine the highest non-lethal dose to the lowest lethal dose. Animals were fed orally 3-time a day, each time at 0.25 mL/10g; general conditions and the mortality of the animals were observed continuously within the first 72 hours as well as for the next 7 days. Dead mice were operated to observe gross physical examination of vital organ. The lethal dose in 50% (LD50) was determined in accordance with the Litchfield-Wilcoxon method.

4. Sub-chronic toxicity experiment

This study was carried out in compliance with the World Health Organization guidance and the OECD guideline No.4078,10. Thirty rats were randomly distributed into three groups (I, II and III) ten rats each group. Group I served as the control group and received distilled water. Groups II and III were orally administered with

Hamo NK at 0.25 g/kg (low dose- equivalent to clinical dose) and 0.75 mg/kg.(high dose - 3 times-equivalent to clinical dose) per day, respectively, for 90 successive days using oral gavage.

Body weight of rats in each group was assessed. Visual observations for behavioral pattern, feed and water consumption, general morphological changes were made daily for the entire period. Blood samples of animals were collected for hematological analysis (total red blood cells, hematocrit, hemoglobin concentration, total white blood cells and platelet) and biochemical analysis (alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, total cholesterol and creatinine). At the end of experiment, all animals were subjected to a full gross necropsy and three rats in each group were sacrified by cervical dislocation, parts of the livers and the kidneys were dissected for histopathological examination.

5. Statistical analysis

Results were presented as mean ± Standard Deviation (SD). The values were analysed statistically using Microsoft Excel software version 2013 followed by Student't-test and Avant-après test. Differences between groups were considered to be statistically significant at p-values less than 0.05 ($p < 0.05$).

III. RESULTS

1. Acute toxicity study

Oral administraton of Hamo NK hard capsules did not reveal any toxicity signs and symptoms up to highest dose of 17.85 g/kg within the first 72h of treatment and for the next 7 days. Besides, animals did not show no significant acute toxicity signs such as piloerection, muscle twinge and lethargy. As the result, the LD50 of the hard capsule could be greater than 17.85 g/kg b.w.

Table 1. Acute toxicity of Hamo NK hard capsule

Group	n	Dosage (ml/kg b.w)	Dosage (g/kg b.w)	Mortarity rates (%)	Other abnormal signs
Group 1	10	30	7.14	0	No
Group 2	10	45	10.71	0	No
Group 3	10	60	14.28	0	No
Group 4	10	75	17.85	0	No

2. Sub-chronic toxicity study

Effect on body Weight, Food and Water consumption

The sub-chronic oral administration of Hamo NK (0.25 g/kg and 0.75 g/kg) did not produce change in behavior, skin, fur colors, mucous membrane, motor activities and no diarrhea, mortality during the experimental period.

The body weight of rats in all of treatment groups showed gradual increased in their body weight. However, there is no statistically significant weight difference between the treated and the control group ($p > 0.05$) (Table1). During the experimental periods, there is no change in feeding and water consumption of all groups by observation.

Table 2. Effect of Hamo NK on body weight of rats during sub-chronic toxicity study.

	Weeks	Control ($\bar{X} \pm SD$)	Hamo NK (g/kg B.W, $\bar{X} \pm SD$)	
			0.25 g/kg	*0,75 g/kg*
Body weight (g)	Before treatment	145.00 ± 11.79	157.30 ± 13.81	157.00 ± 31.99
	Week 4	*161.00 ± 25.14*	*163.00 ± 24.06*	*168.00 ± 34.25*
	Week 8	*168.00 ± 41.31*	*172.00 ± 30.84*	*174.00 ± 39.78*
	Week 12	*178.00 ± 42.90*	*178.00 ± 32.93*	*169.50 ± 33.87*

Effect of Hamo NK on hematological parameters in rats

In the sub-chronic toxicity study, the hematological indexes including RBC, HGB, HCT, MCV, neutrophils, lymphocytes and WBC were not statistical changed between the Hamo NK – treated groups and the control group (Table 3, Table 4).

Table 3. Hematological values of rats in the Hamo NK- treated and control group for 12 consecutive weeks.

Parameters	Groups (n = 10)	Before treatment ($\bar{X} \pm SD$)	After treatment ($\bar{X} \pm SD$)		
			Week 4	Week 8	Week 12
RBC (T/l)	Control	8.24 ± 0.76	8.55 ± 0.97	9.04 ± 0.96	8.87 ± 0.93
	Group I	8.55 ± 1.24	8.63 ± 1.04	8.99 ± 1.45	9.12 ± 0.93
	Group II	8.03 ± 0.79	8.72 ± 1.14	8.72 ± 1.14	8.49 ± 1.44
HGB (g/dL)	Control	11.79 ± 0.84	11.37 ± 1.10	11.76 ± 1.16	12.48 ± 1.08
	Group I	11.29 ± 1.25	11.03 ± 0.94	12.92 ± 1.95	13.07 ± 0.92
	Group II	11.05 ± 1.36	11.68 ± 1.25	12.56 ± 2.06	12.00 ± 1.56
HCT (%)	Control	42.13 ± 3.12	43.33 ± 4.99	45.36 ± 5.12	44.42 ± 4.61
	Group I	41.90 ± 4.32	44.19 ± 4.21	44.78 ± 6.61	44.96 ± 4.86
	Group II	41.38 ± 4.36	44.24 ± 6.75	44.91 ± 5.75	42.83 ± 5.31
MCV (fL)	Control	50.80 ± 2.35	50.79 ± 1.55	50.20 ± 1.40	49.30 ± 2.26
	Group I	51.10 ± 3.35	50.10 ± 2.56	50.00 ± 2.05	49.30 ± 2.06
	Group II	51.40 ± 2.76	50.70 ± 2.75	52.30 ± 4.14	51.00 ± 4.50
PLT (g/L)	Control	570.20 ± 99.76	523.40 ± 85.92	510.10 ± 114.78	597.20 ± 113.38
	Group I	527.20 ± 85.94	558.90 ± 84.37	540.00 ± 131.37	560.00 ± 116.30
	Group II	618.50 ± 81.00	600.00 ± 82.69	595.30 ± 90.88	656.50 ± 100.42

Table 4. Differential white blood cell count values of rats in the sub-chronic toxicity study

Weeks	Groups (n = 10)	Differential white blood cell ($\overline{X} \pm SD$)		
		WBC (T/l)	Neu (%)	Lym (%)
Before treatment	Control	9.80 ± 2.38	71.77 ± 9.64	9.99 ± 2.76
	Group I	9.48 ± 2.77	71.75 ± 10.96	10.23 ± 3.71
	Group II	9.09 ± 1.80	74.77 ± 7.29	9.00 ± 3.11
Week 4	Control	9.59 ± 2.57	71.53 ± 7.37	9.91 ± 2.53
	Group I	11.57 ± 1.85	70.74 ± 13.64	10.61 ± 6.60
	Group II	11.18 ± 2.50	74.40 ± 7.18	8.35 ± 2.96
Week 8	Control	10.12 ± 2.00	71.03 ± 9.45	10.18 ± 3.63
	Group I	10.03 ± 2.49	67.89 ± 7.97	11.66 ± 4.34
	Group II	11.78 ± 2.82	69.39 ± 6.88	11.25 ± 4.59
Week 12	Control	10.47 ± 2.97	70.15 ± 5.93	10.44 ± 1.20
	Group I	10.67 ± 2.87	71.50 ± 8.40	10.26 ± 3.97
	Group II	11.04 ± 1.86	70.00 ± 5.55	11.21 ± 2.39

Effect on serum biochemical parameters

Total cholesterol, total bilirubin, creatinine, aspartate aminotransferase (AST), alanine amino-transferase (ALT) results in the 12-week experimental period are shown in Table 5. There were no significant difference on the concentration of serum markers of liver and kidney functions compared with the control group, except ALT level with its respective control. In particular, statistical findings occurred in the high-dose group. The level of ALT increase statistically compared with the control group after 8 weeks of treatment ($p < 0.001$), but no statistical change was observed between groups for 12 weeks.

Table 5. Effect of orally administration of Hamo NK on serum biochemical

Parameters	Groups (n = 10)	Before treatment	After treatment		
			Week 4	Week 8	Week 12
Albumin (g/dL)	Control	3.35 ± 0.27	3.47 ± 0.28	3.14 ± 0.32	3.34 ± 0.20
	Group I	3.31 ± 0.27	3.33 ± 0.24	3.38 ± 0.29	3.11 ± 0.41
	Group II	3.31 ± 0.17	3.36 ± 0.30	3.09 ± 0.21	3.16 ± 0.26
Total cholesterol (mmol/L)	Control	1.66 ± 0.26	1.78 ± 0.33	1.52 ± 0.42	1.60 ± 0.28
	Group I	1.93 ± 0.46	1.90 ± 0.35	1.62 ± 0.35	1.84 ± 0.26
	Group II	1.71 ± 0.43	1.57 ± 0.33	1.69 ± 0.35	1.62 ± 0.41
Total bilirubin (mmol/L)	Control	13.47 ± 0.43	13.40 ± 0.37	13.43 ± 0.35	13.50 ± 0.51
	Group I	13.34 ± 0.24	13.51 ± 0.48	13.41 ± 0.55	13.46 ± 0.51
	Group II	13.38 ± 0.32	13.54 ± 0.35	13.49 ± 0.36	13.29 ± 0.56

Parameters	Groups (n = 10)	Before treatment	After treatment		
			Week 4	Week 8	Week 12
Creatinine (mg/dL)	Control	0.81 ± 0.14	0.77 ± 0.13	0.80 ± 0.15	0.75 ± 0.12
	Group I	0.80 ± 0.12	0.78 ± 0.14	0.76 ± 0.14	0.76 ± 0.16
	Group II	0.79 ± 0.14	0.71 ± 0.12	0.85 ± 0.11	0.78 ± 0.15
AST (IU/L)	Control	72.50 ± 18.96	85.30 ± 22.67	89.80 ± 25.29	90.60 ± 25.28
	Group I	75.60 ± 22.09	81.10 ± 13.83	92.00 ± 24.68	86.70 ± 21.38
	Group II	71.20 ± 20.32	85.50 ± 13.13	96.90 ± 14.94	91.60 ± 19.28
ALT (IU/L)	Control	36.40 ± 7.59	43.40 ± 11.70	42.10 ± 8.69	44.50 ± 11.50
	Group I	35.50 ± 5.40	41.80 ± 8.90	43.20 ± 10.17	42.70 ± 11.64
	Group II	35.50 ± 5.40	47.50 ± 14.70	72.40 ± 8.72***	42.70 ± 7.80

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

Histopathological changes

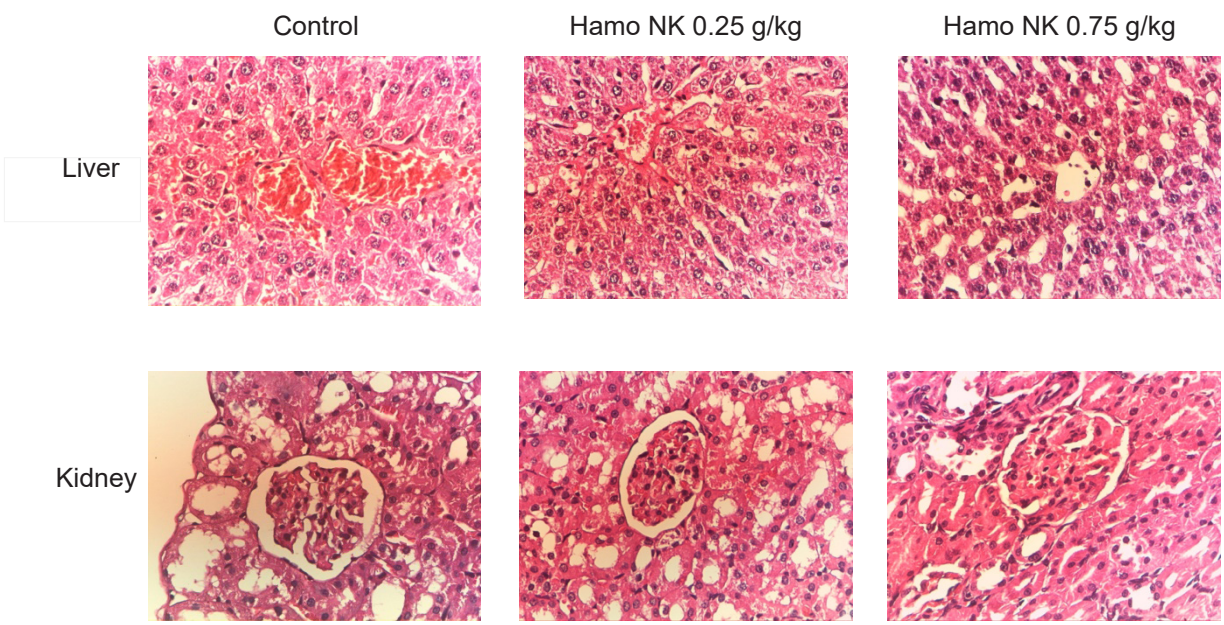

Figure 1. Histopathological images of livers and kidneys from rats treated with Hamo NK for 12 weeks (Selected microphotographs HE staining magnification 400X)

Gross anatomical examination of the vital organs (heart, lung, liver, speen and kidney) in all experiment rats did not reveal any gross pathological lesions.

Histopathological studies of the livers and kidneys sections of rats treated with Hamo NK showed no significant microscopic changes compares with the controls at the end of the treatment period.

IV. DISCUSSION

Toxicity is defined as any harmful effect of chemical or a drug on a target organism. Acute and sub-chronic toxicities have been defined by various experts. The Organization for Economic Co-operation and Development panel of experts (OECD Guidelines) defines acute toxicity as "the adverse effects occurring within a short time of administration of a single dose of a substance or multiple dose given within 24 hours". [11,12] For acute toxicity study, Hamo NK was given to mice at maximum tolerated dose of 17.85 g/kg (approximately 36 times as high as recommended human dose). No mortality was observed in the treated groups during the study period. Therefore, the LD_{50} of Hamo NK was not determined in mice and may be considered relatively safe on acute exposure. Furthermore, Do Linh Quyen's study of the Folium nelumbinis nuciferae (225g gram dry herb medicine) and Pericarpium Citri reticulatae perenne (150 gram herb medicine) did not determined The LD 50 of HVT at a dose of 600g/kg. The maximum dose of Pericarpium Citri reticulatae perenne in Hamo NK used in this study (approximately 0.85g/kg for acute toxicity) and Folium nelumbinis nuciferae (0.034g) are much lower than Do Linh Quyen's study.[13]

Sub-chronice toxicity as the adverse effects occurring as a result of the repeated daily dosing of chemical to experimental animals.[10] During the 12-week oral administration of Hamo NK, no death in rats was observed. The results of clinic symptoms showed no change in behavior, drinking and eating habits. Besides, body weight change is an important index for assessment of toxicity. In this present study, there was a gradual increase in weight gain of control and both treated groups.

Assessment of hematological parameters can be used to determine the extent of harmful effect of compound including herbal medicine on blood. The data obtained from the present study, almost all hematological parameters, including HBC, WBC, Lym, Neu, PLT, HCT, HGB were not statistically changed among treated and control groups. It may suggest that Hamo NK hard capsule did not have toxic effects at these dose regimens in rats.

In toxicological evaluation, biochemical parameters have significant roles as a marker liver and renal funtions tests reveal hepatic and renal toxicity as target organs due to involvement in elimination of xenobiotics. Clinical chemistry indexs are good indicators in determining toxicity. The serum levels of liver-derived enzymes are usually quantified, ALT (alanine amino transferase) and AST (aspartat amino transferase) may increase during hepatocyte injury[14]. Additionally, the liver also plays a role in the metabolism of total bilirubin, albumin and total cholesterol. Thus, the levels of liver injury and liver functions could be assessed by these indicators changes. As shown, the concentration of ALT, AST and hepatic function profiles did not alter significantly in treated rats compared to the control group. However. this was not associated with the histopathological changes of the liver in the Hamo NK at the high dose (0.75g/kg). Based on the histopathological images of the livers, the Hamo NK induces liver cell damage depending on the dosage. On the contrary, previous studies indicated that *Radix Achyranthis bidentatae* and *Folium nelumbinis nuciferae* both induced decreasing activities of serum AST, ALT, ameliorating histopathological liver changes through the inhibition of oxidative and an inhibit the hepatocyte apoptosis.[15,16]

The kidneys are the main organ for excretion. In the histopathological study of the kidney, rats treated with both doses (0.25 and

0.75 g/kg) of the dry extract revealed no histopathological changes observed in kidney, The sections of the kidneys of treated rats showed normal general structure of the kidney and normal appearance of glomeruli and tubules. The result was further supported by the values of biochemical parameter of the blood which is main indicator of kidney damage. The mean values of serum creatinine were within the reference range for rats; as such, the results of the study showed that Hamo NK hard capsule did not affect the kidney function.

V. CONCLUSION

The acute toxicity study of the Hamo NK hard capsules did not produce adverse effects and no motarlity in mice at the maximum tolerated dose of 17.85g/kg. Therefore, the oral LD50 of Hamo NK hard capsule was not determined in mice.

The sub-e toxicity study of Hamo NK hard capsule did not adversely affect the general conditions, hematological and biochemical parameters of tested doses. There was no sign of toxicity observed in the kidneys and livers of treated rats. However, there were injuries in liver was seen in all doses of Hamo NK 0.75g/kg group, which reached significance in the Hamo NK high dose treated rats

Acknowledgments

The authors would like to thank the Professor Le Dinh Roanh, Director of the Center for Research and Early Detection of Cancer, for interpreting the histopathology of liver and kidney of experimental rats.

REFERENCES

1. WHO. WHO Traditional Medicine Strategy 2014-2023. Published online 2013.

2. Fuentes R, Uusitalo T, Puska P, Tuomilehto J, Nissinen A. Blood cholesterol level and prevalence of hypercholesterolaemia in developing countries: a review of population-based studies carried out from 1979 to 2002. *Eur J Cardiovasc Prev Rehabil.* 2010;10(6):411–419.

3. Woerdenbag HJ, Nguyen TM, Vu DV, et al. Vietnamese traditional medicine from a pharmacist's perspective. *Expert Rev Clin Pharmacol.* 2012;5(4):459-477. doi:10.1586/ecp.12.34

4. Zhou T, Luo D, Li X, Luo Y. Hypoglycemic and hypolipidemic effects of flavonoids from lotus (Nelumbo nuficera Gaertn) leaf in diabetic mice. J*ournal of Medicinal Plant Researc*h. 2009;3(4):290–293.

5. Ming Guo,1Yue Liu, Zhu-Ye Gao, Da-zhuo Sh. Chinese Herbal Medicine on Dyslipidemia: Progress and Perpective. *Evidence-Based Complementary and Alternative Medicine.* 2014;2014.

6. Shahriar DM. PHYTOCHEMICAL AND PHARMACOLOGICAL INVESTIGATION OF THE CRUDE EXTRACT OF TYPHONIUM TRILOBATUM (L.) SCHOTT. Accessed October 11, 2020. https://www.academia.edu/11750999/PHYTOCHEMICAL_AND_PHARMACOLOGICAL_INVESTIGATION_OF_THE_CRUDE_EXTRACT_OF_TYPHONIUM_TRILOBATUM_L_SCHOTT

7. Jj Y, J S, Mq Y, Zh L, Gy L. [Correlation between lipid-lowering efficacy and components of Pericarpium Citri Reticulatae]. *Zhongguo Zhong Yao Za Zhi Zhongguo Zhongyao Zazhi China J Chin Mater Medica.* 2019;44(15):3335-3342. doi:10.19540/j.cnki.cjcmm.20190523.304

8. WHO. Working group on the safety and efficacy of herbal medicine", Report of regional office for the western pacific of the World Health Organization. Published online 2000.

9. Litchfield J.T, Wilcoxon F. A simplified method of evaluating dose – effect experiments. *JPharmacolExpTherap.* 96(2):99-113.

10. Organisation for Economic Co-operation and Development. Guidelines for the testing of chemicals repeated dose oral toxicity study in rodents, Environmental Health and Safety Monograph Series on Testing and Assesment No 407. Published online 2004.

11. Organisation for Economic Co-operation and Development. Guideline for testing of chemicals. Published online 2001.

12. IUPAC. *Compendium of Chemical Terminology*. 2nd ed.; 1997.

13. Do Linh Quyen. A study on the toxicity and effectiveness of HVT liquid drug on dyslioiedemia pattern. Published online 2019.

14. Ta Thanh Van. *Clinical Biochemistry.* Medical Publshing House, Hanoi; 2013.

15. Chandrasekaran Chinampudur Velusami, Amit Agarwal, Vijayalakshmi Mookambeswaran. Effect of Nelumbo nucifera Petal Extracts on Lipase, Adipogenesis, Adipolysis, and Central Receptors of Obesity.*Evidenced based complementaryand alternative medicine.* 2013;7.

16. Jinyang Lin,corresponding author, Zhuoying Zhang and Ying Shan. Effect of Achyranthes Bidentata Polysaccharides on the Expression of BCL-2 and Bax in Hepatic Tissues after Exhaustive Exercise in Rats. *Afr J Tradit Complement Altern Med*. 2010;7(4):307-314.