EVALUATION OF ACUTE AND SUBCHRONIC TOXICITY OF "HUYET PHU TRUC U HOAN" IN EXPERIMENTAL ANIMALS

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This research was carried out to evaluate the acute and sub-chronic toxicity of "Huyet phu truc u hoan" (HPTUH) in experimental animals. The acute toxicity was defined on Swiss mice by the Litchfield – Wilcoxon's method and the sub-chronic toxicity on Wistar rat's hematopoietic function, liver, and kidney functions according to the World Health Organization Guidance. As a result, with doses from 10g/kg to 50g/kg, LD_{50} of HPTUH was not determined. With the doses from 2.38g/kg to 7.14g/kg per day on Wistar rat for 8 consecutive weeks, haematological parameters, ALT, AST, urea and creatinine levels were unaffected. In addition, the HPTUH did not produce any toxic effect in the animals's liver and kidney in the treated animals compared to the control.

Keywords: "Huyet phu truc u hoan", acute toxicity, sub-chronic toxicity, experimental animals.

I. INTRODUCTION

According to the World Stroke Organization, stroke is the second-leading cause of death and the third-leading cause of death and disability combined after cardiovascular disease and cancer (as expressed by disability-adjusted life-years lost - DALYs) in the world.[1] The frequency of stroke increases with age and tends to increase and aging is the most robust non-modifiable risk factor for incident stroke, which doubles every 10 years after age 55 years.[2] In recent years, advances in diagnostic methods, emergency resuscitation, neurosurgery, promoting preventive measures, combining modern medicine and traditional medicine usage, drug free lifestyle have helped doctors achieve many advancement in diagnosis, emergency treatment and recovery treatment. The World Health Organization has proposed a range of primary health care interventions for behavioral and lifestyle risk factors, including management of hypertension, cessation of cigarette smoking, and an unhealthy diet, to prevent and manage non-communicable diseases such as stroke.[3] Besides, the effective treatment for acute ischemic stroke is reperfusion therapy like Tissue plasminogen activator (tPA). However, the narrow treatment window and the risk of complications limit its clinical application.[4] In recent years, several clinical trials and basic research published on herbs medicine have demonstrated their therapeutic effects in preventing and treating stroke. In addition, discovering traditional medicine remedies that have therapeutic effects, limiting the side effects of modern medicine, and overcoming neurological complications caused by stroke are important and necessary. "Huyet phu truc u hoan" (HPTUH) -produced by the Military Institute of Traditional Medicine - -derived from the remedy "Huyet phu truc u thang". This is

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an ancient remedy originating from the book "Y Lam cai thac" by Vuong Thanh Nham, a famous Chinese physician of the Thanh Dynasty, to treat blood stasis. The remedy includes different drugs used in traditional medicine with the effect of activating blood and enhancing blood circulation, however, little toxicological information is available regarding its safety and scientific validity.Therefore, we conducted this research to evaluate the acute and subchronic toxicity of "Huyet phu truc u hoan" in experimental animals.

II. MATERIALS AND METHODS

1. The preparation of Huyet phu truc u

"Huyet phu truc u hoan" was manufactured by the Department of Pharmacy – Military Institute of Traditional Medicine. "Huyet phu truc u hoan" was formulated in form of dripping pill with 8.5 g per mixture of drought herbal medicine extract. Each pill contained *Semen Persicae* 0.72g; *Radix Bupleuri* 0.18g; *Radix Archiranthis bindantae* 0.54g; *Radix Rehmaniae* 0.54g; *Flos Carthami* 0.54g; *Fructus Citri aurantii* 0.36g; *Radix Platycodonis* 0.26g; *Rhizoma Ligustici wallichii* 0.26g; *Radix Angenicae sinensis* 0.54g; *Radix Glycyrrhizae* 0.18g; *Radix Paeoniae rubra* 0.36g. These materials were in compliance with the standards of Vietnamese Pharmacopeia IV. The recommended dose in human was two pills per day.

2. Experimental animals

Wistar rats (170 ± 30 g) and *Swiss* mice (20 ± 2 g) of both genders were used in this study. The animals were housed in cages (groups of ten rats or mice/cage) in a room with access to a standard certified rodent diet and water ad libitum. They were acclimated to housing for 5 - 7 days before the experiment at Department of Experimental Research - Military Institute of Traditional Medicine.

3. Acute toxicity study

Acute toxicity experiment was carried out according to the World Health Organization Guidance and determined LD_{50} by Litchfield - Wilcoxon method.[8,9] Group of mice (10 per group) were fasted for 12h and orally administered with HPTUH at ascending doses that mice could be tolerated. General health condition and signs of toxicity such as vomiting, convulsions, agitation, abnormal excretion ... and the mortality for the first critical 4 hours within 72 hours after giving HPTUH were recorded. All dead mice were operated on to assess macroscopic lesions. The oral median lethal dose (LD_{50}) was calculated as the geometric mean of dose that caused 0% and 100% mortality respectively. Animals that survived 24 hours were further observed for seven days for signs of delayed toxicity.

4. Subchronic toxicity study

Subchronic toxicity study was carried out according to WHO Guidance.[8] Thirty *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 HPTUH at 2.38 g/kg/day (equivalent to the human recommended dose, conversion ratio 7);

- Group 3: was administered orally HPTUH at 7.14 g/kg/day (3 times as high as the dose at group 2).

Animals were given the oral administration of distilled water and with the volume 10 mL/kg b.w daily for eight consecutive weeks, once per day at 8 a.m, and observed daily for general signs of toxicity and mortality. The pills were dissolved with distilled water (the solvent of HPTUH) daily before giving orally to rats.

- Rats in all groups were weighed

- Hematopoietic function: red blood cells (RBC), hemoglobin (HGB), hematocrit, total

white blood cells (WBC), WBC differentials, platelet count (PLT).

- Serum biochemistry test: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, creatinine, and ure levels.

The parameters were checked at before treatment, four weeks and eight weeks after treatment. At the end of the experiment, all animals were subjected to a full gross necropsy. The livers and kidneys of 30% of rats of each group were subjected to histopathology examinations. The micro-histological examination was carried out at Department of Pathology - Military Institute of Traditional Medicine.

4. Statistical analysis

Data were analyzed using Microsoft Excel software version 2016. The significance levels between the experimental groups and the control group were made using the student's t-test and Avant-après test. The experimental results were expressed as the Mean ± standard deviation (SD). All data were considered significant at $p < 0.05$.

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, treated animals showed no mortality at the highest dose level 50g/kg within 24 h and for additional seven days. Also, animals did not show acute toxicity signs such as piloerection, lacrimation, or changes in locomotion and respiration. Orally, 50 g/kg of HPTUH was well tolerated in mice even after 7 days. (Table1)

Table 1. Acute toxicity study of HPTUH

Group	n	Dose (g/kg)	The proportion of deaths (%)	Other abnormal signs
Group 1	10	Control	0	No
Group 2	10	10	0	No
Group 3	10	20	0	No
Group 4	10	30	0	No
Group 5	10	40	0	No
Group 6	10	50	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 compared to the control group.

Body weight changes

Figure 1 showed that after four weeks and eight weeks, the body weight at all groups increased slightly as compared with the time point "Before treatment". No significant difference in body weight was observed between the control and the treated groups ($p > 0,05$).

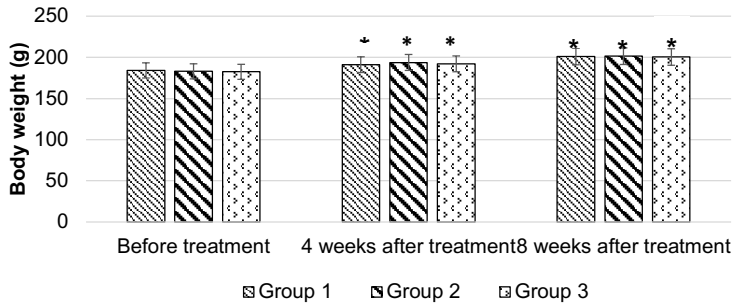


Figure 1. The effect of "Huyet phu truc u hoan" on body weight changes

* $p < 0.05$ as compared with the time point "Before treatment"

The effect of "Huyet phu truc u hoan" on the hematological system

There was no significant difference in red blood cell count, hematocrit, MCV, hemoglobin level, platelet count, total WBC count, lymphocytes, and neutrophils between the treated groups and the control group ($p > 0.05$)

Table 2. The effect of "Huyet phu truc u hoan" on hematological profile

Parameters	Group	Before treatment	After treatment	
			Four weeks	Eight weeks
Red blood cells count (T/L)	Group 1	8.18 ± 0.42	7.65 ± 0.76	7.40 ± 0.51
	Group 2	8.11 ± 0.83	8.03 ± 0.69	7.63 ± 0.84
	Group 3	7.91 ± 0.22	7.69 ± 0.86	7.59 ± 0.63
	p	> 0.05	> 0.05	> 0.05
Hemoglobin level (g/dL)	Group 1	13.38 ± 1.53	12.96 ± 0.98	12.55 ± 1.05
	Group 2	13.04 ± 1.16	13.00 ± 1.19	12.61 ± 1.00
	Group 3	13.58 ± 1.25	12.41 ± 1.42	12.57 ± 1.49
	p	> 0.05	> 0.05	> 0.05
Hematocrit (%)	Group 1	37.58± 4.63	36.83 ± 2.24	36.38 ± 2.40
	Group 2	37.28 ± 5.22	36.88 ± 2.24	36.49 ± 3.83
	Group 3	37.13 ± 4.83	36.10 ± 3.63	35.55 ± 2.61
	p	> 0.05	> 0.05	> 0.05
MCV (fl)	Group 1	48.68 ± 3.47	47.35± 3.48	47.39 ± 3.91
	Group 2	48.43± 2.72	47.66± 4.02	47.58± 3.36
	Group 3	48.97± 3.58	47.57± 1.59	46.79± 2.94
	p	> 0.05	> 0.05	> 0.05

Parameters	Group	Before treatment	After treatment	
			Four weeks	Eight weeks
Platelet count (G/L)	Group 1	368.38 ± 97.34	33890 ± 87.25	325.11 ± 91.80
	Group 2	314.00 ± 91.03	362.10 ± 91.49	370.10 ± 98.42
	Group 3	337.00 ± 68.31	321.50 ± 87.93	359.90 ± 90.64
	p	> 0.05	> 0.05	> 0.05
Total WBC count (G/L)	Group 1	12.72 ± 3.84	10.37 ± 3.28	11.38 ± 2.24
	Group 2	12.07 ± 4.55	11.68 ± 2.44	11.91 ± 4.53
	Group 3	12.64 ± 3.67	11.36 ± 4.71	10.04 ± 3.05
	p	> 0.05	> 0.05	> 0.05
Lymphocytes (%)	Group 1	67.04 ± 5.56	64.95 ± 8.60	61.94 ± 8.38
	Group 2	62.43 ± 8.86	62.02 ± 6.21	61.71 ± 5.94
	Group 3	65.63 ± 6.26	65.76 ± 5.01	63.57 ± 6.55
	p	> 0.05	> 0.05	> 0.05
Neutrophils (%)	Group 1	27.29 ± 3.56	26.36 ± 4.37	29.24 ± 3.82
	Group 2	31.35 ± 9.23	31.21 ± 6.00	31.61 ± 5.54
	Group 3	27.87 ± 5.85	27.40 ± 5.08	29.93 ± 5.89
	p	> 0.05	> 0.05	> 0.05

MCV = Mean Corpscular Volume, p: compared with the control group and "Before treatment"

The effect of "Huyet phu truc u hoan"on liver functions

There was no significant difference in aspartate aminotransferase (AST), alanine aminotransferase (ALT) level, and total bilirubin between the HPTUH treated groups and the control group (p > 0.05). The results are shown in Table 3.

Table 3. The effect of "Huyet phu truc u hoan" on liver functions

Parameters	Group	Before treatment	After treatment	
			Four weeks	Eight weeks
AST level (UI/L)	Group 1	160.92 ± 31.76	164.68 ± 44.43	162.29 ± 30.76
	Group 2	161.07 ± 34.94	159.99 ± 30.47	158.51 ± 23.44
	Group 3	161.89 ± 27.42	158.58 ± 35.22	161.71 ± 26.70
	p	> 0.05	> 0.05	> 0.05

Parameters	Group	Before treatment	After treatment	
			Four weeks	Eight weeks
ALT level (UI/L)	Group 1	39.85 ± 10.11	41.33 ± 10.72	42.12 ± 7.22
	Group 2	39.34 ± 11.14	38.83 ± 7.49	39.55 ±11.85
	Group 3	38.22 ± 12.36	37.52 ± 11.11	39.81 ± 13.25
	p	> 0.05	> 0.05	> 0.05
Total bilirubin (µmol/L)	Group 1	29.87 ± 4.65	30.93 ± 8.71	29.86 ± 7.59
	Group 2	29.80 ± 8.85	28.35 ± 8.42	30.40 ± 4.36
	Group 3	29.63 ± 7.48	28.63 ± 7.92	30.72 ± 5.08
	p	> 0.05	> 0.05	> 0.05

p: compared with the control group and the time point "Before treatment"

The effect of "Huyet phu truc u hoan" on kidney functions

Table 4 demonstrated that after two weeks and four weeks of treatment, HPTUH caused no significant difference in serum creatinine level between the control group and the two treated groups (p > 0.05).

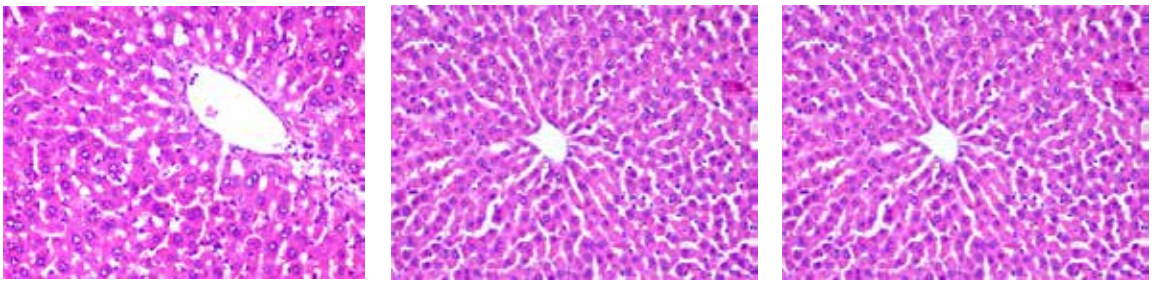
Table 4. The effects of "Huyet phu truc u hoan" on serum creatinine and ure level

0	Group	Before treatment	After treatment	
			Four weeks	Eight weeks
Creatinin (µmol/L)	Group 1	87.67 ± 9.01	88.15 ± 5.26	86.82± 7.17
	Group 2	86.38 ± 7.63	85.84 ± 7.80	87.34± 6.27
	Group 3	85.82 ± 7.94	86.64 ± 8.69	87.48± 10.01
	p	> 0.05	> 0.05	> 0.05
Ure (mmol/L)	Group 1	4.65 ± 0.83	5.07 ± 0.81	5.13 ± 0.84
	Group 2	4.60 ± 0.92	4.58 ± 0.73	4.81 ± 0.82
	Group 3	4.76 ± 0.95	4.66 ± 0.58	4.65 ± 0.94
	p	> 0.05	> 0.05	> 0.05

Histopathological examination

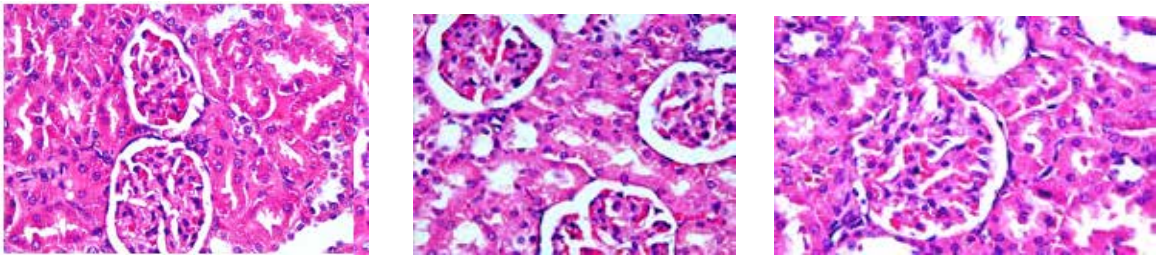
No gross lesion or change in size was observed when all experimental rats had a full gross necropsy, which examined the hearts, livers, lungs, kidneys, and abdominal cavities.

There were no significant difference in histopathological examinations of livers and kidneys between the HPTUH treated rats and the control group after eight weeks of treatment (Figure 2 and 3).



Group 1 Group 2 Group 3

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



Group 1 Group 2 Group 3

Figure 3. Histopathological images of kidneys (HE × 400)

IV. DISCUSSION

According to the WHO guidelines, all drugs of herbal or chemical origin must be evaluated for acute and subchronic toxicity before clinical trials.[8] 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 provide guidelines for selecting 'safe' therapeutic doses in humans.[10] Mice were given the drug with increasing doses from 10.0 g/kg body weight to 50.0 g/kg, approximately 27.12 times as high as the recommended human dose. This is the maximum amount that mice can drink and no sign of toxicity, and no mortality was observed for seven consecutive days, so the LD50 of HPTUH in mice has not been determined orally according to the Litchfield-Wilcoxon method. The median acute toxicity value (LD50) was estimated to be ≤ 50 g/kg (orally), indicating safety and a wide margin between the effective and toxic dose. HPTUH is therefore safe for oral use for the management of several diseases.

A subchronic toxicity study provided information on the effects of repeated oral exposure and indicated the need for longer-term studies. Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of animals' average life span, such as rodents. Specifically, they provide information on target organ toxicity. [11]

The parameters used to evaluate the toxicity of HPTUH are analyzed in detail as follows:

The body weight changes serve as a sensitive indication of the general health status

of animals. In addition to, the body weight changes are the most basic index to reflect toxicity to organs and systems and reflect the combined effects of xenobiotics on the body. For all experimental animals, general signs should be observed daily, and body weight should be measured periodically. It can be stated that HPTUH did not interfere with animals' normal metabolism as corroborated by the non-significant difference from animals using the distilled water as the control group.

The blood circulatory system has crucial functions, for example, delivering oxygen to all body tissues, maintaining vascular integrity, providing necessary immune factors for host defense reaction. The hematopoietic system is one of the most sensitive targets of toxic compounds and is an essential parameter for humans and animals' physiological and pathological status.[12] After four weeks and eight weeks of the treatment, the general lack of significant change in blood indices is an indication of safety of HPTUH. The daily administration of HPTUH for eight weeks did not produce any significant change in hematological parameters of the treated rats when compared to control group. This suggests that the extract may have no toxicological effect on the hemopoietic system.

Researching the effects of drugs on liver functions is essential when studying the safety of a drug. One of the methods to assess the degree of liver cell damage is to quantify the concentration of enzymes originating from the liver in serum.[13] In this study, we quantified the activities of ALT and AST enzymes in the serum of experimental mice. ALT is the most abundant enzyme in the liver, while in cells ALT is only found in the cytoplasm. When there is an agent that damages and destroys liver cells, even just changing the permeability of liver cell membranes, ALT activity increases. AST is localized in both the cytoplasm and mitochondria. When there is cell damage, AST in the blood increases, there is a correlation between the level of cell damage and the isoenzyme of AST entering the blood. Therefore, in hepatitis in general, ALT activity is often higher than AST. The non-significant change in ALT and AST in rats at all doses (Table 3) indicates that the extract had no deleterious effect on liver function.

Liver and kidney function analysis is very important in the toxicity evaluation of drugs and plant extracts as they are both necessary for the survival of an organism. The liver participates in the processes of degrading hemoglobin into bilirubin and excreting bilirubin in bile. Quantitative testing of serum bilirubin concentration to probe the liver's excretion and bile metabolism function. The results of this study showed that the total bilirubin concentration in the blood of mice in 2 treatment groups did not change after 4 weeks and 8 weeks of taking the test drug at a dose of 2.38g/kg/day and a dose of 7.14g/kg/day. Continuous oral administration for 8 weeks in mice did not affect the liver's excretion and bile metabolism function.

The kidney is a urinary organ, playing an important role in ensuring homeostasis. When the drug is introduced into the body, it can cause kidney damage and affect kidney function., Renal dysfunction can be assessed by concurrent measurements of urea, and creatinine and their normal levels reflect at reduced likelihood of renal problems. Our research results show that after 4 weeks and 8 weeks of continuously taking the test drug, plasma urea and creatinine concentrations in the 2 treatment groups did not change compared to before taking the drug and compared to the control group. Thus, it is proven that HPTUH taken continuously for 8

weeks does not affect the filtering function of the glomerulus.

There was no morphological damage when the mouse's organs were observed macroscopically. There was no structural damage when the liver and kidneys of mice were observed microscopically. Thus, HPTUH does not damage the microscopic structure of the liver and kidneys of mice.In various organs, the liver and kidney are vital for the drug's affinity and conducive to eliminating the drug and having a particular role in the accumulation. The histopathological examination would revealed any alteration in cell structure under the light microscope. Further histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of HPTUH. Our study showed no significant difference in histopathological examinations of the livers and kidneys between the HPTUH treated groups and the control group.

Studies have shown that *Radix Angenicae sinensis* is a Chinese herbal medicine traditionally used in prescriptions for replenishing blood, treating abnormal menstruation, and other women's diseases that indicated the toxicity is very limited and oral use is safe.[14] Besides, Xi SY et al reported that the toxic components of *Semen Persicae* are clear-cut, and its toxicity is related closely with the methods of medication. it is safe when taken it orally under the condition of accurate syndrome differentiation and reasonable dosage.[15]

The product has had the dose of each medicine adjusted to serve the clinical treatment process based on the ancient remedy Blood Palace to expel stasis in the book "Y Lam Cai Thac" by famous physician Vuong Thanh Nham, are non-toxic herbs, present in traditional remedies and have been used safely for thousands of years.[16]

V. CONCLUSION

No signs of acute toxicity and no mortality were observed in "Huyet phu truc u hoan" treated mice at the dose of 50 g/kg. Oral LD50 of "Huyet phu truc u hoan" was not determined in *Swis*s mice.

For eight consecutive weeks, "Huyet phu truc u hoan" at the oral route with doses of 2.38 g/kg/day and 7.14 g/kg/day did not make any toxic signs or symptoms of subchronic toxicities in *Wistar* rats.

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