THE EVALUATION OF ACUTE AND SUBCHRONIC TOXICITIES OF AN PHU KHANG CAPSULES IN EXPERIMENTAL ANIMALS

Ha Thi Yen, Tran Thanh Tung and Dang Thi Thu Hien✉

Hanoi Medical University

The purpose of this research was to evaluate the acute and subchronic toxicities of An Phu Khang 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 in Wistar rats at these doses of 0.54 g/kg b.w/day (equal to recommended human dose) and 1.62 g/kg b.w/day (3 times as high as recommended human dose) in 4 consecutive weeks. As a result, An Phu Khang capsules at the highest dose used for mice (36.29 g/kg b.w) did not show acute toxicity in mice. In terms of the subchronic toxicity test, after oral administration of An Phu Khang capsules, hematological parameters, hepato-renal functions, and microscopic images of liver and kidney at both doses were unchanged compared with the control group. In conclusion, An Phu Khang with both doses 0.54 g/kg b.w/ day and 1.62 g/kg b.w/day did not produce acute and subchronic toxicities in Swiss mice and Wistar rats.

Keywords: An Phu Khang capsules, acute toxicity, subchronic toxicity, polyherbal medicine, experimental animals.

I. INTRODUCTION

Herbal medicine is recognized as the most common form of alternative medicine. The World Health Organization (WHO) estimates that 80% of the world's population relies on these "alternative" plant-based medicines as their primary medical intervention, especially in the developing and in developed countries where modern medicines are predominantly used (Ogbonnia et al., 2008).[1] Over the years, the use of herbs in the treatment of illnesses has been very successful, and its historical usage has been helpful in drug discovery development. Herbal remedies are safer and less damaging to the human body than synthetic drugs. Although herbal supplements may be considered safe, some are known to be toxic at high doses, and others may have potentially adverse effects after prolonged use. However, the lack of evidence-based approaches and toxicological profiling of herbal preparations form the biggest concern of medicinal plant use. Thus, the evaluation of their toxicity plays a vital role to recognize, characterize and evaluate their risk for humans, and to propose measures to mitigate the risk, particularly in early clinical trials.[2]

An acute toxicity test is used to evaluate any adverse effects appearing within a short time after a single large dose of the test substance or after multiple doses given within 24h. A subchronic toxicity study is typically conducted from 1 to 3 months because some substances that do not cause immediate toxicity may cause toxic effects after repeated exposure. Subchronic systemic toxicity is defined as adverse effects occurring after a test sample's repeated or continuous administration for up to 12 weeks or not exceeding 10% of the

Corresponding author: Dang Thi Thu Hien
Hanoi Medical University
Email: thuhien@hmu.edu.vn
Received: 13/10/2021
Accepted: 30/11/2021

animal's lifespan. The objective of subchronic toxicity studies is to determine the possible clinical adverse reactions caused by the substance, including the nature and degree of harm, the dose-response and time-response relationships, the effects on target organs or tissues, and the reversibility, and then predict the safe dose range for repeated drug use.[3–5]

An Phu Khang capsule is a herbal medicine. It was prepared from eight medicinal herbs and two synthetic ingredients, including *Crinum latifolium* L.*, Celastrum hindsii*, *Panax pseudoginseng*, *Cyperus rotundus* L., *Leonurus japonicus*, *Artemisia vulgaris*, Curcumin nano, Pregnenolone. The constituents of An Phu Khang capsules have been studied extensively.[6-9] So far, no report have been available on the safety of a blend product from these components. Therefore, this study aimed to evaluate the acute and subchronic toxicities of An Phu Khang capsules on experimental animals.

II. METHODS

1. The preparation of An Phu Khang capsules

An Phu Khang was manufactured by Phuong Dong Trading and Pharmaceutical Company Limited. It was formulated in capsule form, and each capsule is a combination of six medicinal herbs and two synthetic ingredients. Ingredient for each 0.5 g capsule includes: 350 mg *Crinum latifolium* L.*,* 30 mg *Celastrum hindsii*, 15 mg *Panax pseudoginseng*, 25 mg *Cyperus rotundus* L., 25 mg *Leonurus japonicus*, 25 mg *Artemisia vulgaris*, 15 mg Curcumin nano, 15 mg Pregnenolone.

The usual dose in humans: 3 times per day, 03 capsules each time (equivalent to 4.5 g/day).

2. Experimental animals

Healthy *Wistar* rats of both sexes weighing between 180 - 220 g were provided by Dan Phuong Experimental Animal Center.

Healthy *Swiss* mice of both sexes weighing between 18 - 22 g were provided by the National Institute of Hygiene and Epidemiology.

The animals were housed in cages (groups of ten rats or mice per cage) in a room with access to a standard certified rodent diet and water. They were allowed to acclimatize for seven days to the laboratory conditions at the Department of Pharmacology - Hanoi Medical University.

3. Method

Acute toxicity study

Acute toxicity studies were carried out according to the Organization for Economic Cooperation and Development (OECD) guidelines 423 (OECD guideline) and WHO Guidance.[10,11]

Group of mice (10 per group) were fasted for 12 hours and orally administered with An Phu Khang at ascending doses that mice could be tolerated. 30 capsules were mixed with water to a final volume of 31 mL. This is the most concentrated solution that can be given to *Swiss* mice with a curved, ball-tipped stainless steel feeding needle. The general symptoms of toxicity and the mortality in each group were observed within 72h. The median lethal dose (LD50) was detected by the Litchfield Wilcoxon method.[12] Animals that survived after 24 hours were further observed for seven days for signs of delayed toxicity.[10]

Subchronic toxicity study

Subchronic toxicity studies were carried out according to WHO Guidance and OECD guidelines.[10,11]

Wistar rats were divided into three groups (10 per group):

- Group 1 (control group) was administered distilled water;
- Group 2 was administered orally An Phu

Khang at the dose of 0.54 g/kg body weight/day (equivalent to the human recommended dose, conversion ratio 6);

- Group 3 was administered orally An Phu Khang at the dose of 1.62 g/kg b.w/day (3 times as high as the dose at group 2).

Distilled water and An Phu Khang were administered using a curved, ball-tipped stainless steel feeding needle with the volume of 10 mL/kg b.w daily for 28 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 An Phu Khang) before giving orally to rats.

The signs and parameters were checked during the study, including general conditions, mortality, and clinical signs.

- The body weights of the animals were evaluated weekly and recorded using a sensitive balance (OECD).[10]

- 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, albumin, total cholesterol, and creatinine levels.

The parameters were checked at the time points: before treatment and two weeks, four weeks after treatment. All animals were subjected to a complete gross necropsy at the end of the experiment. The livers and kidneys of 30% of rats of each group will be taken for histopathology examinations. The micro-histological examination was carried out at the Center for Research and Early Detection of Cancer (CREDCA). Assoc. Prof. Le Dinh Roanh, Director of CREDCA, gave results of pathological image analysis.

4. Statistical analyses

Data were analyzed using Microsoft Excel software version 2010. The results are expressed as mean ± standard error of the mean (SEM). Avant-après test was employed for between and within-group comparison while student's t-test was used for paired comparison. A 95% level of significance ($p \leq 0.05$) was used for the statistical analysis.

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, the groups were administered An Phu Khang from 30 mL/kg to maximum doses of 75 mL/kg (equivalent to 36.29 g/kg b.w). An Phu Khang treated animals showed no mortality at the highest dose level within 24h and for seven 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 An Phu Khang capsules

Group	n	Dose (ml/kg)	Dose (g/kg body weight)	The proportion of deaths (%)	Other abnormal signs
Group 1	10	30	14.51	0	No
Group 2	10	45	21.77	0	No
Group 3	10	60	25.03	0	No
Group 4	10	75	36.29	0	No

2. Subchronic toxicity study

General condition

The general condition, food, and water consumption were assessed. Animals had normal locomotor activities and good feedings. There was no change in the treated group's appearance, activity, or excrement compared with the control group during the subchronic toxicity test.

Body weight changes

Figure 1 showed that the body weight in all groups increased significantly after two weeks and four weeks, compared with the time point "Before treatment". No significant differences were observed between the An Phu Khang capsules treated and the control groups ($p > 0.05$). These results demonstrated that An Phu Khang capsules exhibit no marked effects on body weight in rats.

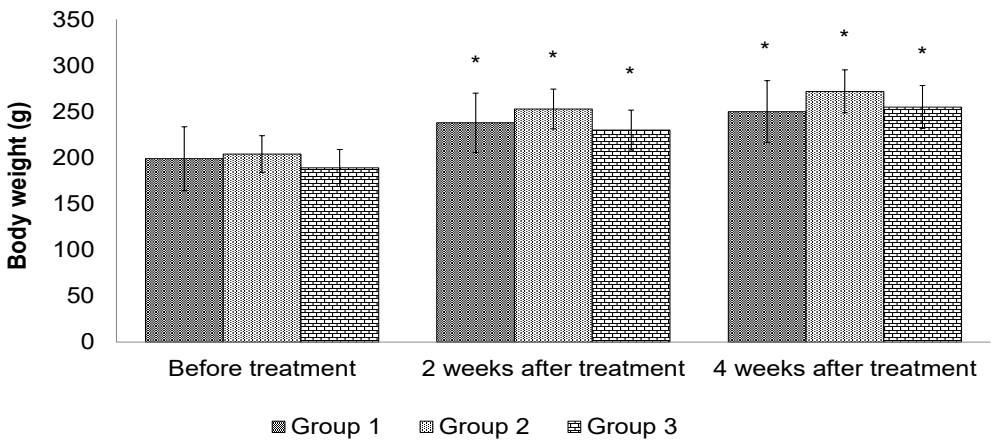


Figure 1. The effect of An Phu Khang capsules on body weight changes

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

The effect of An Phu Khang capsules on the hematological system

There were no significant differences in red blood cell count, hematocrit, hemoglobin level, platelet count, total WBC count, and WBC between An Phu Khang capsules treated groups and control group ($p > 0.05$) (Table 2 and Table 3).

Table 2. The effect of An Phu Khang capsules on hematopoietic function

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Red blood cells count (T/L)	Group 1	8.99 ± 1.13	9.93 ± 1.19	8.91 ± 0.83
	Group 2	9.17 ± 1.20	10.09 ± 1.66	9.73 ± 1.16
	Group 3	8.28 ± 1.14	9.19 ± 0.97	8.27 ± 1.19
Hemoglobin level (g/dL)	Group 1	13.79 ± 1.13	14.41 ± 1.48	14.14 ± 1.46
	Group 2	14.45 ± 0.90	14.80 ± 2.40	15.20 ± 1.71
	Group 3	13.60 ± 1.52	14.71 ± 1.60	13.56 ± 1.73

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Hematocrit (%)	Group 1	46.67 ± 4.16	49.54 ± 7.13	46.50 ± 4.25
	Group 2	47.80 ± 4.53	50.58 ± 5.72	49.02 ± 5.67
	Group 3	47.09 ± 4.25	51.42 ± 5.45	42.81 ± 6.84
MCV (fl)	Group 1	53.50 ± 2.12	54.20 ± 1.87	52.30 ± 1.16
	Group 2	54.60 ± 3.13	54.10 ± 1.91	54.10 ± 1.91
	Group 3	54.60 ± 1.90	54.80 ± 1.99	52.40 ± 3.06
Platelet count (G/L)	Group 1	623.30 ± 125.25	680.30 ± 190.71	570.60 ± 141.62
	Group 2	592.30 ± 116.28	650.70 ± 143.38	545.60 ± 88.48
	Group 3	613.70 ± 55.76	635.40 ± 97.38	694.00 ± 139.14

MCV = Mean Corpscular Volume

Table 3. The effects of An Phu Khang capsules on WBC

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
Total WBC count (G/L)	Group 1	8.61 ± 2.75	8.95 ± 2.11	9.07 ± 2.29
	Group 2	9.67 ± 2.21	9.25 ± 2.91	9.65 ± 2.08
	Group 3	9.93 ± 1.40	10.40 ± 2.10	9.84 ± 2.26
Lymphocytes (%)	Group 1	81.02 ± 3.34	79.87 ± 4.60	79.35 ± 3.84
	Group 2	80.04 ± 6.79	80.86 ± 7.39	76.48 ± 7.41
	Group 3	79.68 ± 6.09	81.32 ± 3.62	6.12 ± 2.25
Neutrophils (%)	Group 1	5.42 ± 1.02	6.12 ± 1.97	6.35 ± 1.63
	Group 2	4.90 ± 1.63	4.93 ± 1.64	6.12 ± 2.25
	Group 3	5.14 ± 1.76	4.88 ± 1.05	6.90 ± 1.90

The effect of An Phu Khang capsules on liver functions

Table 4. The effect of An Phu Khang capsules on liver functions

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
AST level (UI/L)	Group 1	98.20 ± 17.64	99.70 ± 18.02	86.50 ± 18.08
	Group 2	108.50 ± 31.73	92.30 ± 24.95	100.70 ± 13.32*
	Group 3	98.70 ± 25.52	102.90 ± 22.52	109.00 ± 23.80*

Parameters	Group	Before treatment	After treatment	
			2 weeks	4 weeks
ALT level (UI/L)	Group 1	39.90 ± 8.62	35.80 ± 7.42	34.90 ± 8.36
	Group 2	42.10 ± 8.71	36.90 ± 12.78	44.90 ± 11.82*
	Group 3	37.40 ± 6.17	36.40 ± 7.40	45.60 ± 10.06*
Total bilirubin (mmol/L)	Group 1	13.25 ± 0.25	13.48 ± 0.52	13.43 ± 0.19
	Group 2	13.41 ± 0.34	13.34 ± 0.57	13.29 ± 0.19
	Group 3	13.38 ± 0.33	13.38 ± 0.42	13.54 ± 0.38
Albumin concentration (g/dL)	Group 1	3.21 ± 0.35	3.45 ± 0.27	3.19 ± 0.45
	Group 2	3.24 ± 0.38	3.47 ± 0.54	3.35 ± 0.17
	Group 3	3.13 ± 0.18	3.56 ± 0.56	3.00 ± 0.25
Total cholesterol concentration (mmol/L)	Group 1	1.50 ± 0.12	1.48 ± 0.30	1.30 ± 0.36
	Group 2	1.62 ± 0.27	1.69 ± 0.25	1.50 ± 0.17
	Group 3	1.64 ± 0.34	1.70 ± 0.21	1.41 ± 0.28

**p < 0.05 as compared with the control group*

The liver functions of groups treated with An Phu Khang capsules were within the normal physiological range. The medicated groups and the control group exhibited no significant difference when compared with the time point "Before treatment" (Table 4; p > 0.05). AST and ALT in groups 2 and 3 were significantly increased compared with the control group (p < 0.05); however, this change was within normal ranges. The data suggested that An Phu Khang capsules treatment exerted no discernable effect on liver functions.

The effect of An Phu Khang capsules on kidney functions

Figure 2 demonstrated that after two weeks and four weeks of treatment, blood creatinine of rats of both treatment and control groups showed that the drug with a dose of 0.54 g/kg b.w/day and 1.62 g/kg b.w/day remained almost the same as that of the control (p > 0.05).

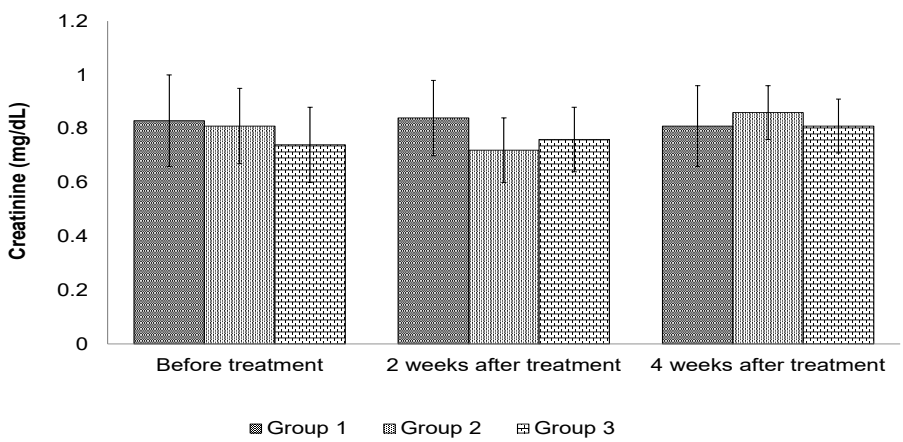
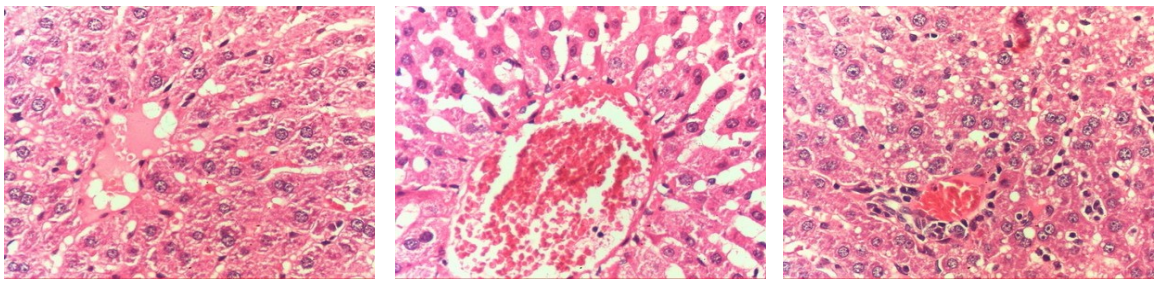


Figure 2. The effects of An Phu Khang capsules on serum creatinine level

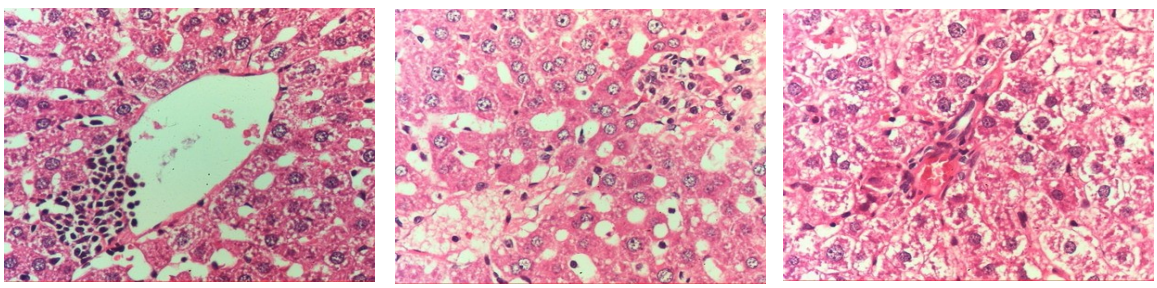
Histopathological examination



Rats no.201
Group 1

Rats no.221
Group 2

Rats no.211
Group 3

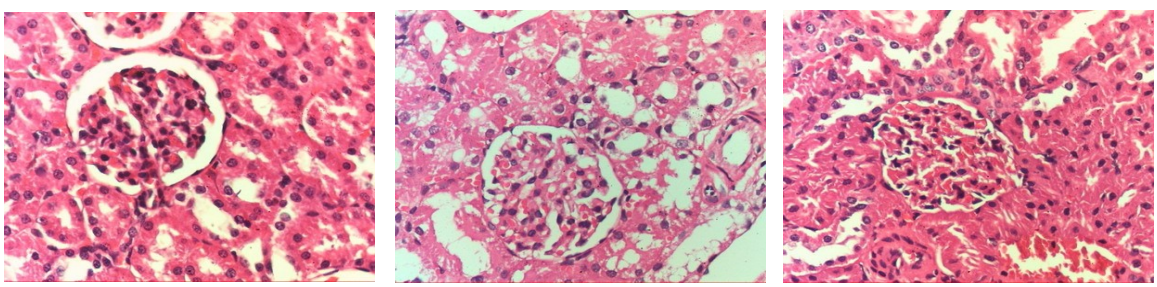


Rats no.208
Group 1

Rats no.227
Group 2

Rats no.213
Group 3

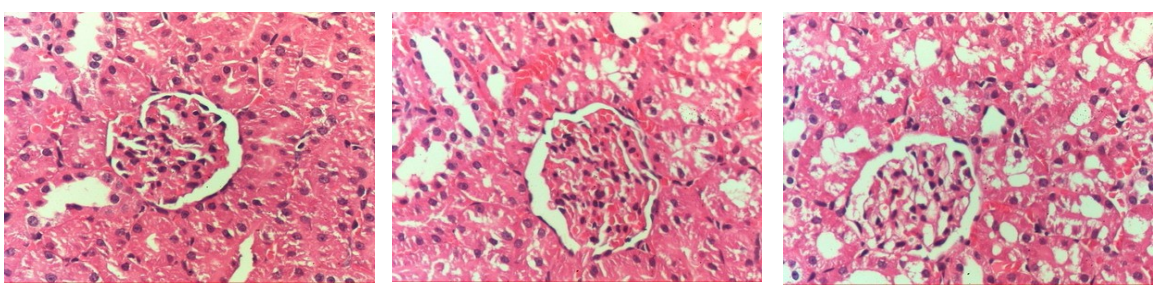
Figure 3. Histopathological morphology of liver (HE × 400)



Rats no.201
Group 1

Rats no.221
Group 2

Rats no.211
Group 3



Rats no.202
Group 1

Rats no.222
Group 2

Rats no.214
Group 3

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

No gross lesion or change in size were observed when all experimental rats were subjected to a complete gross necropsy, which was examined. All experimental rats were subjected to a complete gross necropsy, which examined the hearts, livers, lungs, kidneys, and abdominal cavities.

After four weeks of treatment, the kidney was normal in all the treatment groups. In the histopathological examination, no change in liver morphology were seen at a dose of 0.54 g/kg b.w/day compared to the control group, whereas congestion with mild inflammation was observed due to infiltration neutrophils in the liver with 1.62 g/kg b.w/day (Figure 3 and 4).

IV. DISCUSSION

In the present study, acute oral toxicity test showed that An Phu Khang capsules were tolerated up to 36.29 g/kg b.w (approximately 67.2 times as high as recommended human dose). Moreover, no signs of toxicity and no mortality were observed for seven continuous days. As a result, oral LD50 of An Phu Khang capsules was not determined in mice. As defined by WHO, An Phu Khang capsules were safe herbal medicine.

Traditional medicine use is popular in developing countries. According to the World Health Organization (WHO), up to 80% of developing country populations use traditional medicine for their primary health care. However, the safety of herbal medicine use has recently been questioned due to reports of herbal medicine's toxicity.[13–15] Although many traditional herbal medicines are available; clinical trials have verified only a few. 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. A subchronic toxicity study provides information on target organ toxicity with the long-term use of herbal medicine.[10,11]

Changes in body weight, food, and water ingestion are generally used as indicators of drugs and chemicals' harmful and unusual metabolism effects. No significant difference was observed between the An Phu Khang capsules treated and the control groups ($p > 0.05$). Thus, the findings of this study suggested that different doses of An Phu Khang capsules (0.54 g/kg b.w and 1.62 g/kg b.w) orally administered to rats for four weeks had no significant effects on general behavior, mental state, or food intake.

The hematopoietic system is one of the most sensitive targets of toxic compounds and is an essential physiological and pathological status parameter in humans and animals.[10,11] 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 two weeks and four weeks of the treatment, there was no significant difference in total red blood cells, hematocrit, hemoglobin level, platelet count, total WBC count. Moreover, WBC differentials were found between the An Phu Khang treated groups with the control group. So, it can be concluded that the An Phu Khang capsules do not affect the hematological system.

The liver and kidneys are frequent targets of drug action because the liver is the primary organ for drug biotransformation, and the kidneys are the primary organs for drug excretion. Alanine amino transaminase (ALT) and Aspartate amino transaminase (AST) is used mainly to assess liver damage by drugs or any other hepatotoxin.[16] However, ALT is more specific to the liver and is thus a better parameter for detecting liver injury.[17] The level

of ALT and AST in the research was within the normal physiological range, suggesting that An Phu Khang capsules formula may not possess a hepatotoxic effect. Total protein measurements can reflect the nutritional status and may screen for and help diagnose kidney and liver diseases and many other conditions. There were no significant changes in total protein in rats treated with An Phu Khang capsules, suggesting no sign of impaired renal function and liver function. The insignificant decrease observed in the total cholesterol level in groups treated with An Phu Khang capsules may be attributed to hypolipidemic agents in the polyherbal drug. Similarly, the drug had no adverse effect on the concentration of creatinine. This suggests no kidney damage specifically by renal filtration mechanism or probably indicates that An Phu Khang capsules did not interfere with the renal capacity to excrete these metabolites. Therefore, it was evident that the drug at doses employed did not cause renal impairment or kidney damage. In the histopathalogical examination no changes in liver and kidney morphology were seen at dose of 0.54 g/kg b.w/day, suggests that the product is more appropriate to be prescribed at this dose.

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

V. CONCLUSION

No sign of toxicity and no mortality were observed in An Phu Khang treated mice at a dose of 36.29 g/kg b.w (approximately 67.2 times as high as recommended human dose). Oral LD50 of An Phu Khang capsules was not determined in *Swiss* mice.

For four continuous weeks, An Phu Khang capsules at doses 0.54 g/kg b.w/day and 1.62 g/kg b.w/day did not make any toxic signs or symptoms of subchronic toxicities in *Wistar* rats.

REFERENCES

1. Ogbonnia S, Adekunle AA, Bosa MK, Enwuru VN. Evaluation of acute and subacute toxicity of Alstonia congensis Engler (Apocynaceae) bark and Xylopia aethiopica (Dunal) A. Rich (Annonaceae) fruits mixtures used in the treatment of diabetes. *Afr J Biotechnol*. 2008;7(6). doi:10.4314/ajb.v7i6.58507.

2. World Health Organization. *WHO Global Report on Traditionnal and Complementary Medicin*. World Health Organization; 2010. Accessed September 14, 2021. https://apps.who.int/iris/handle/10665/340838.

3. Alhaji Saganuwan S. Toxicity studies of drugs and chemicals in animals: An overview. *Bulg J Vet Med*. 2017;20:291-318. doi:10.15547/bjvm.983.

4. Jong WH, Carraway J, Re G. In vivo and in vitro testing for the biological safety evaluation of biomaterials and medical devices. *Biocompat Perform Med Devices*. Published online October 1, 2012;120-158. doi:10.1016/B978-0-85709-070-6.50007-9.

5. Zhao Y-L, Su M, Shang J-H, et al. Acute and Chronic Toxicity of Indole Alkaloids from Leaves of Alstonia scholaris (L.) R. Br. in Mice and Rats. *Nat Prod Bioprospecting*. 2020;10(2):77-88. doi:10.1007/s13659-020-00237-1.

6. Loan Pham T, Huy Nguyen V, Tam Tien Ha T, Le Thu Hoang T, NghiaPhan C, Quyen Nguyen T. Evaluation of Acute Toxicity and Semi-chronic Toxicity of Extract from Celastrus hindsii Benth. *Pak J Biol Sci PJBS*. 2020;23(8):1096-1102. doi:10.3923/pjbs.2020.1096.1102.

7. Nguyen H-YT, Vo B-HT, Nguyen L-TH, et al. Extracts of Crinum latifolium inhibit the cell viability of mouse lymphoma cell line EL4 and induce activation of anti-tumour activity of macrophages in vitro. *J Ethnopharmacol*. 2013;149(1):75-83. doi:10.1016/j.jep.2013.06.002.

8. Zhang S, Chen C, Lu W, Wei L. Phytochemistry, pharmacology, and clinical use of Panax notoginseng flowers buds. *Phytother Res PTR*. 2018;32(11):2155-2163. doi:10.1002/ptr.6167.

9. Pirzada AM, Ali HH, Naeem M, Latif M, Bukhari AH, Tanveer A. Cyperus rotundus L.: Traditional uses, phytochemistry, and pharmacological activities. *J Ethnopharmacol*. 2015;174:540-560. doi:10.1016/j.jep.2015.08.012.

10. OECD. *Guidance Document on Acute Oral Toxicity Testing*. OECD. 2002. doi:10.1787/9789264078413-en.

11. World Health Organization. WHO guidelines for assessing quality of herbal medicines with reference to contaminants and residues. Published online 2007;105.

12. Litchfield JT, Wilcoxon F. A Simplified Method of Evaluating Dose-Effect Experiments. *J Pharmacol Exp Ther*. 1949;96(2):99-113.

13. Saad B, Azaizeh H, Abu-Hijleh G, Said O. Safety of traditional arab herbal medicine. *Evid-Based Complement Altern Med ECAM*. 2006;3(4):433-439. doi:10.1093/ecam/nel058.

14. Ernst E. Toxic heavy metals and undeclared drugs in Asian herbal medicines. *Trends Pharmacol Sci*. 2002;23(3):136-139. doi:10.1016/S0165-6147(00)01972-6.

15. Cosyns J-P. Aristolochic acid and “Chinese herbs nephropathy”: a review of the evidence to date. *Drug Saf*. 2003;26(1):33-48. doi:10.2165/00002018-200326010-00004.

16. Sk R. Preclinical safety assessment: current gaps, challenges, and approaches in identifying translatable biomarkers of drug-induced liver injury. *Clin Lab Med*. 2011;31(1). doi:10.1016/j.cll.2010.10.004.

17. Ozer J, Ratner M, Shaw M, Bailey W, Schomaker S. The current state of serum biomarkers of hepatotoxicity. *Toxicology*. 2008; 245(3):194-205. doi:10.1016/j.tox.2007.11.021.