EVALUATION OF ACUTE AND SUBCHRONIC TOXICITY OF AN NGUYET KHANG TABLETS IN EXPERIMENTAL ANIMALS

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The research evaluated the acute and subchronic toxicities of An Nguyet Khang tablets in experimental animals. Acute toxicity was defined by the method of Litchfield Wilcoxon in Swiss mice. The subchronic toxicity was evaluated by WHO and OECD's recommendation in Wistar rats with oral doses of 0.65 g/kg/day (equal to recommended human dose) and 1.95 g/kg/day (3 times as high as recommended human dose) in fourconsecutive weeks. We found that An Nguyet Khang tablet at the highest dose used for mice (35.15 g/kg) did not express acute toxicity in mice. Regarding the subchronic toxicity test, after oral administration of An Nguyet Khang tablets, hematological parameters, hepato-renal functions, and microscopic images of the liver and kidney were unchanged compared with the control group. In conclusion, An Nguyet Khang tablets did not produce acute and subchronic toxicities in Swiss mice and Wistar rats.

Keywords: An Nguyet Khang tablet, acute toxicity, subchronic toxicity, experimental animals.

I. INTRODUCTION

Nature has been a source of medicinal agents from ancient times, and medicinal plants was formulated into a wide variety of traditional medicines used in various countries worldwide.[1] 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.[2] Most natural products in traditional remedies have reliable scientific evidence for their biological activity. However, the lack of information or evidence concerning the possible toxic side effect derived form herbal medicine represented the highest concern of using medicinal plants. Thus, evaluating their toxicity plays a vital role in recognizing and characterizing these toxic effects, as well as assessing their risk for human, and proposing measures to mitigate the risk, particularly in early clinical trials.[2]

Toxicity refers to unwanted effects on biological systems. To evaluate biological toxicity, it is crucial to choose the correct system since no effect may otherwise be seen. Toxicity of a substance can be impacted by many factors, such as the route of exposure (skin absorption, ingestion, inhalation, or injection); the time of exposure (a brief, acute, subchronic, or chronic exposure); the number of exposures (a single dose or multiple doses); the physical form of the toxin (solid, liquid, or gas); the organ system involved (cardiovascular, nephro-, hemo-, nervous-, or hematopoietic-system); and even the genetic makeup and robustness of the target cells or organisms.[3] Subchronic systemic toxicity is defined as adverse effects occurring after the repeated or continuous administration of a test sample for up to 12 weeks or not exceeding 10% of the animal's lifespan.[4,5]

Traditional medicine has been widely used in the treatment of gynecological diseases. Historically, these natural plants, such as

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Rhizoma Cyperi, *Radix Angelicae Sinens*,... have been used since ancient times and in folklore to treat dysmenorrhea. An Nguyet Khang tablet was prepared from 10 natural materials, including *Rhizoma Cyperi, Radix Angelicae Sinensis, Fructus Evodiae Rutaecarpae, Radix Pacomiae Lactiflorae, Herba Artemisiae vulgaris, Radix Astragalus membranacei, Rhizoma Ligustici wallichii, Radix Dipsaci, Radix Rehmanniae glutinosae praeparat*, and *Cortex Cinnamomi*. Although the effects of these herbs in treating dysmenorrhea have been mentioned in some studies, there have been no report available on the safety of a combination product from these components. Therefore, this study was conducted to investigate the acute and subchronic toxicities of An Nguyet Khang tablets in experimental animals.

II. MATERIALS AND METHODS

1. The preparation of An Nguyet Khang tablets

An Nguyet Khang tablet was manufactured by the Viet Nam University of Traditional Medicine. An Nguyet Khang tablet was formulated as tablets, and one tablet contained 600 mg of dry extract from 10 natural materials. The recommended dose in human was nine tablets per day.

2. Experimental animals

Wistar rats (170 ± 30 g) and *Swiss* mice (20 ± 2 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 a standard certified rodent diet and water ad libitum. They were acclimated to housing for 5 – 7 days at the Department of Pharmacology, Hanoi Medical University prior to the experiment.

3. Acute toxicity study

The study was performed according to the WHO Guidance and Organization for Economic Co-operation and Development guidelines (OECD guidelines).[6,7]

Groups of mice (10 per group)were fasted for 12 hours and orally administered with An Nguyet Khang at ascending doses that mice could be tolerated. The general symptoms of toxicity and mortality in each group were observed within 24 hours. The median lethal dose (LD50) was detected by the Litchfield Wilcoxon method.[8] 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 the WHO Guidance and OECD guidelines.[6,7]

The study was carried out in the course of four consecutive weeks. *Wistar* rats were divided into three groups of ten animals:

- Group 1 (control group) was given distilled water;

- Group 2 was administered orally An Nguyet Khang at 0.648 g/kg/day (equivalent to the human recommended dose, conversion ratio 6);

- Group 3 was administered orally An Nguyet Khang at 1.944 g/kg/day (3 times as high as Group 2 dose).

Animals were orally administered distilled water and An Nguyet Khang at 10 mL/kg b.w daily for four consecutive weeks and observed once daily to detect clinical signs and time points for laboratory tests. The tablets were dissolved in distilled water (the solvent of An Nguyet Khang) daily before given orally to rats.

The signs and parameters were checked during the study, including general conditions, 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 test: aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, total cholesterol, and creatinine levels.

The parameters were checked at before treatment, two weeks, and four 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 the rats of each group will be taken for histopathology examinations. The micro-histological examination was conducted at the Department of Pathology, Duc Giang Hospital.

5. Statistical analysis

Data were analyzed using Microsoft Excel software version 2016. The significant levels between the experimental and control groups derived from the student's t-test and the Avant-après test. Data were shown as mean ± standard deviation. All data were considered significant at $p < 0.05$.

III. RESULTS

1. Acute toxicity study

In the oral acute toxicity test, An Nguyet Khang tablets treated animals showed no mortality at the highest dose level (35.15 g/kg body weight) within 24 hours and for additional seven days. Also, animals did not show acute toxicity signsuch as piloerection, lacrimation, or changes in locomotion and respiration.

Table 1. Acute toxicity study of An Nguyet Khang tablets

Group	n	Dose (ml/kg)	Dose (g/kg)	The proportion of deaths (%)	Other abnormal signs
Group 1	10	30	14.06	0	No
Group 2	10	45	21.09	0	No
Group 3	10	60	28.12	0	No
Group 4	10	75	35.15	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 than the control group.

Body weight changes

Figure 1 showed that after two weeks and four weeks, the body weight of all groups increased slightly compared to "Before treatment". After four weeks of treatment, , there was a significant increase in body weight in Group 3 as compared with "Before treatment" and the control group (Group 1)($p < 0.05$).

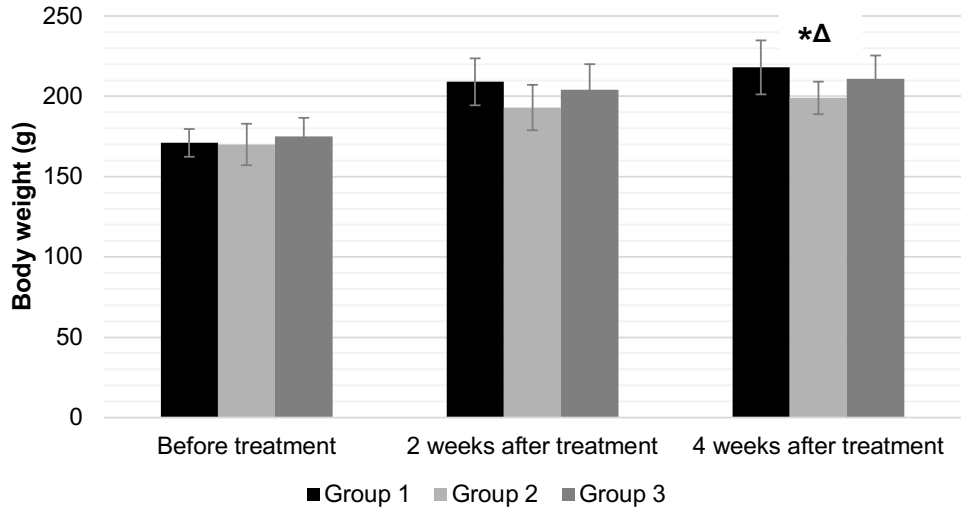


Figure 1. The effect of An Nguyet Khang tablets on body weight changes

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

Δ p < 0.05 as compared with the control group (Group 1)

The effect of An Nguyet Khang tablets on the hematological system

There were no significant difference in red blood cell count, hematocrit, MCV, hemoglobin level, platelet count, total WBC count, lymphocytes, and neutrophils between An Nguyet Khang tablets treated groups and control group (p > 0.05) (Table 2 and Table 3).

Table 2. The effect of An Nguyet Khang tablets on hematopoietic function

Parameters	Group	Before treatment	After treatment	
			Two weeks	Four weeks
Red blood cells count (T/L)	Group 1	10.91 ± 0.78	10.81 ± 1.30	11.30 ± 1.16
	Group 2	10.62 ± 0.65	11.34 ± 1.48	10.88 ± 0.72
	Group 3	11.00 ± 1.02	10.88 ± 0.72	11.02 ± 0.92
	p	> 0.05	> 0.05	> 0.05
Hemoglobin level (g/dL)	Group 1	14.01 ± 0.85	12.90 ± 1.64	13.99 ± 1.41
	Group 2	13.58 ± 1.00	13.68 ± 1.51	13.29 ± 1.05
	Group 3	13.99 ± 1.41	12.98 ± 1.94	13.82 ± 1.42
	p	> 0.05	> 0.05	> 0.05
Hematocrit (%)	Group 1	59.45 ± 5.33	56.05 ± 6.99	57.97 ± 5.24
	Group 2	56.76 ± 3.78	59.83 ± 3.93	55.63 ± 5.06
	Group 3	58.41 ± 5.55	56.97 ± 4.14	57.48 ± 5.87
	p	> 0.05	> 0.05	> 0.05

Parameters	Group	Before treatment	After treatment	
			Two weeks	Four weeks
MCV (fl)	Group 1	53.70 ± 2.31	52.20 ± 2.53	52.70 ± 3.06
	Group 2	53.10 ± 2.33	51.70 ± 1.70	51.60 ± 1.51
	Group 3	53.90 ± 2.51	52.80 ± 1.81	52.60 ± 1.96
	p	> 0.05	> 0.05	> 0.05
Platelet count (G/L)	Group 1	604.50 ± 116.32	636.90 ± 124.95	622.60 ± 137.70
	Group 2	679.40 ± 115.65	719.00 ± 107.87	698.00 ± 125.06
	Group 3	669.50 ± 112.96	694.20 ± 119.87	671.00 ± 102.11
	p	> 0.05	> 0.05	> 0.05

MCV = Mean Corpuscular Volume

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

Table 3. The effects of An Nguyet Khang tablets on WBC count and WBC differentials

Parameters	Group	Before treatment	After treatment	
			Two weeks	Four weeks
Total WBC count (G/L)	Group 1	9.49 ± 1.66	9.42 ± 2.16	8.66 ± 1.81
	Group 2	8.93 ± 1.14	8.06 ± 1.58	8.10 ± 1.86
	Group 3	9.20 ± 1.66	8.08 ± 1.35	8.99 ± 1.62
	p	> 0.05	> 0.05	> 0.05
Lymphocytes (%)	Group 1	79.86 ± 2.98	78.83 ± 4.23	76.76 ± 6.39
	Group 2	80.43 ± 6.14	81.37 ± 6.19	77.17 ± 5.78
	Group 3	81.55 ± 4.96	80.87 ± 3.46	79.24 ± 3.77
	p	> 0.05	> 0.05	> 0.05
Neutrophils (%)	Group 1	13.28 ± 3.06	12.62 ± 3.20	12.53 ± 3.53
	Group 2	12.62 ± 3.67	12.33 ± 3.81	14.17 ± 3.96
	Group 3	12.01 ± 2.80	12.39 ± 1.90	12.63 ± 3.66
	p	> 0.05	> 0.05	> 0.05

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

The effect of An Nguyet Khang tablets on liver functions

There were no significant difference in aspartate aminotransferase (AST), alanine aminotransferase (ALT) level, total bilirubin, albumin concentration, and total cholesterol concentration between An Nguyet Khang tablets treated groups and the control group (p > 0.05). The results are shown in Table 4.

Table 4. The effect of An Nguyet Khang tablets on liver functions

Parameters	Group	Before treatment	After treatment	
			Two weeks	Four weeks
AST level (UI/L)	Group 1	79.20 ± 8.05	76.90 ± 12.11	84.30 ± 12.45
	Group 2	78.00 ± 11.35	80.90 ± 12.78	83.30 ± 9.86
	Group 3	81.40 ± 9.31	81.30 ± 11.96	83.00 ± 12.40
	p	> 0.05	> 0.05	> 0.05
ALT level (UI/L)	Group 1	36.90 ± 3.60	33.80 ± 3.65	37.10 ± 4.77
	Group 2	33.60 ± 5.91	34.20 ± 4.13	34.50 ± 4.95
	Group 3	34.10 ± 4.53	35.00 ± 6.06	38.10 ± 6.42
	p	> 0.05	> 0.05	> 0.05
Total bilirubin (mmol/L)	Group 1	8.98 ± 0.67	9.55 ± 0.89	9.42 ± 0.67
	Group 2	9.09 ± 0.63	9.84 ± 0.83	9.27 ± 0.49
	Group 3	9.16 ± 0.78	8.99 ± 0.46	9.59 ± 0.91
	p	> 0.05	> 0.05	> 0.05
Albumin concentration (g/dL)	Group 1	3.30 ± 0.26	3.19 ± 0.33	3.06 ± 0.44
	Group 2	3.24 ± 0.38	3.32 ± 0.23	3.41 ± 0.30
	Group 3	3.35 ± 0.25	3.14 ± 0.31	3.33 ± 0.29
	p	> 0.05	> 0.05	> 0.05
Total cholesterol concentration (mmol/L)	Group 1	43.33 ± 5.51	42.35 ± 8.10	39.88 ± 7.96
	Group 2	39.38 ± 5.04	39.63 ± 9.06	40.60 ± 9.03
	Group 3	39.61 ± 7.14	37.69 ± 6.66	38.14 ± 5.38
	p	> 0.05	> 0.05	> 0.05

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

The effect of An Nguyet Khang tablets on kidney functions

Figure 2 demonstrated that after two weeks and four weeks of treatment, An Nguyet Khang tablets caused no significant difference in serum creatinine levels between the control and the two treated groups (p > 0.05).

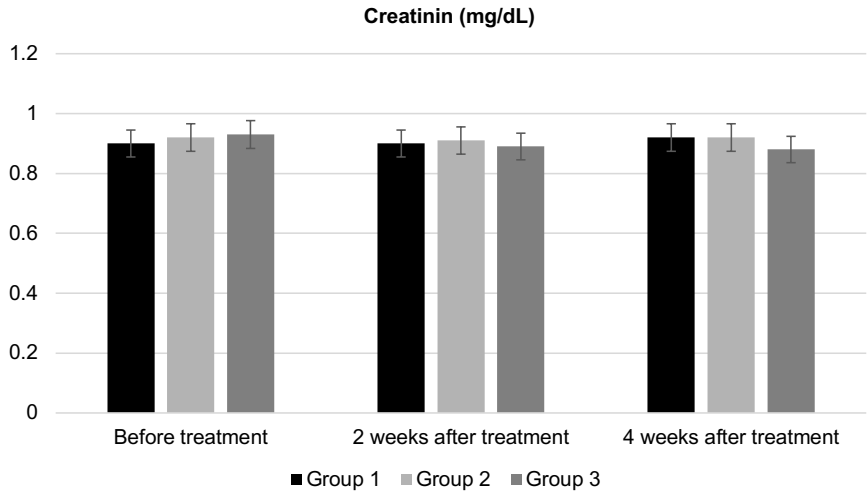
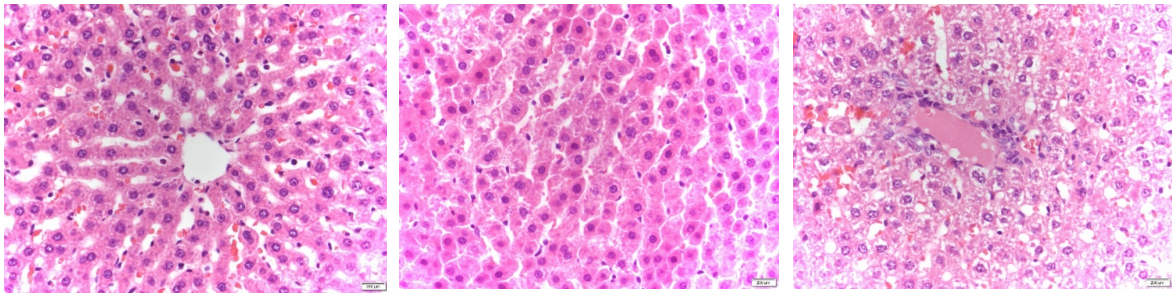


Figure 2. The effects of An Nguyet Khang tablets on serum creatinine level

Histopathological examination

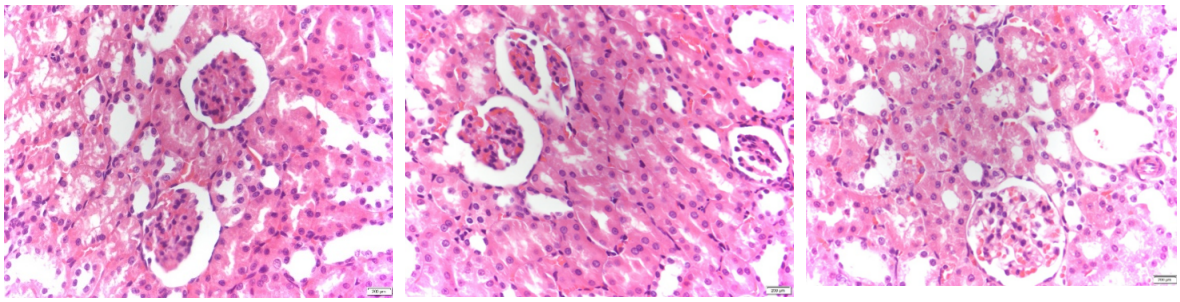
No gross lesions or changes in size were observed when subjected all experimental rats to a full gross necropsy, which examined the hearts, livers, lungs, kidneys, and abdominal cavities.

There were no significant difference in histopathological examinations of livers and kidneys between An Nguyet Khang tablets treated mice and the control group after four weeks of treatment (Figures 3 and 4).



Group 1 Group 2 Group 3

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



Group 1 Group 2 Group 3

Figure 4. Histopathological images of kidneys (HE × 400)

IV. DISCUSSION

Acute toxicity of An Nguyet Khang tablets

In this experiment, the acute oral toxicity test showed that An Nguyet Khang was tolerated up to 35.15 g/kg (approximately 27.12 times as high as recommended human dose). Moreover, no signof toxicity and no mortality were observed for seven consecutive days. As a result, oral LD50 of An Nguyet Khang tablets was not determined in mice. As defined by WHO, An Nguyet Khang is a safe herbal medicine.

Subchronic toxicity of An Nguyet Khang tablets

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.[8] 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. A subchronic toxicity study provided information on the effects of repeated oral exposure and indicated the need for longer-term studies.[6,9] Subchronic studies assess the undesirable effects of continuous or repeated exposure to plant extracts or compounds over a portion of animals' average life span, such as rodents. Specifically, they provide information on target organ toxicity.[10]

The medicinal herbs in An Nguyet Khang tablets that are effective in treating dysmenorrhea have been known in many previous studies. However, there is little information about the toxicity of these herbs. Our results were consistent with the previous report on the toxicity of *Radix Dipsaci*, a component in An Nguyet Khang tablets. According to Ji-Seok Han (2016), the *Radix Dipsaci* water extract was administered orally to rats at doses of 0, 125, 250, 500, 1000, and 2000 mg/kg for 13 weeks. During the treatment period, there were no mortality attributed to *Radix Dipsaci*. Moreover, no significant toxicity was observed concerning body weight, clinical pathology (hematology, clinical biochemistry, and urinalysis), and histopathological examination.[11] The parameters used to evaluate the toxicity of An Nguyet Khang tablets are analyzed in detail as follows.

Body weight changes serve as a sensitive indicator of the general health status of animals. In addition, body weight changes are the most basic index to reflect toxicity to organs and systems and the combined effects of xenobiotics on the body.[10] General signs should be observed daily for all experimental animals, and body weight should be measured periodically.[9] It can be stated that *An Nguyet Khang* tablets did not interfere with animals' normal metabolism, as corroborated by the non-significant difference from animals using 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, and so on. 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.[6,9] 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, and platelet count differentials between the An

Nguyet Khang treated groups with the control group. Furthermore, no significant change was observed in the neutrophils, lymphocytes, and monocytes in An Nguyet Khang, which further confirmed the above findings, so it can be concluded that the An Nguyet Khang tablets do not affect the hematological system.

Liver and kidney function analysis is critical 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 c conducted to evaluate the possible alterations in hepatic and renal functions influenced by the plant products.[12] The changes in serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents are sensitive to the degree of liver cell damage. With chronic liver injury , AST and ALT would be released from the liver cells' injury, increasing the serum level.[8] Creatinine levels can be used in describing the function of the kidneys.[9] There was no significant ALT and AST change in both male and female rats at all doses, which indicated that An Nguyet Khang had no deleterious effect on liver function. The blood biochemistry level of the control and An Nguyet Khang in treated rats at various doses presented no significant difference between the An Nguyet Khang treated groups and the control group ($p > 0.05$). This evidence shows that An Nguyet Khang tablets did not affect liver and kidney functions.

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 revealed alteration in cell structure under the light microscope.[11] As such, we suggest that further histological study could furnish additional information regarding the hepatotoxicity and nephrotoxicity of the An Nguyet Khang tablets. Our study showed no significant differencein histopathological examinations of the livers and kidneys between the An Nguyet Khang treated groups and the control group.

Overall, this study's findings indicated no significant difference- observed in blood parameters, biochemistry parameters, and histopathological observations of liver and kidney tissues between the An Nguyet Khang treated and the control groups.

Results of our study were consistent with previous reports about the component's toxicity in An Nguyet Khang tablets. According to Chen XP (2013), it was concluded that the extract from *Radix Angelica sinensis* for oral use is safe in the experiment.[13] Results from the research of Kim D (2014) showed that the subchronic no-observable-adverse-effect level for evodia fruit powder from Evodia Rutaecarp following oral administration in rats is greater than 2000 mg/kg.[14]

V. CONCLUSION

No signof acute toxicity and mortality was observed in An Nguyet Khang -treated mice at 35.15 g/kg (approximately 27.12 times as high as recommended human dose). Oral LD50 of An Nguyet Khang tablets was not determined in *Swis*s mice.

For four consecutive weeks, An Nguyet Khang tablets administered orally at 0.65 g/kg/day and 1.95 g/kg/day did not revealed any toxic sign or symptom of subchronic toxicities in *Wistar* rats.

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