Trop J Nat Prod Res, March 2021; 5(3):519-527 ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

Tropical Journal of Natural Product Research

Available online at https://www.tjnpr.org

Original Research Article

# Assessment of Acute and Sub-Chronic Toxicity of HYK Aqueous Extracts in Experimental Animals

Quang Y. Pham[1], Tien H. Vuong[2], Minh T. Doan[3], Hoang N. Nguyen[4]*

[1]*Gia Lai Traditional Medicine and Rehabilitation Hospital, 13 Dang Thuy Tram street, Hoa Lu ward, Pleiku city, Gia Lai province 61109, Vietnam*
[2]*Hanoi Medical University, 1 Ton That Tung street, Dong Da district, Hanoi 11520, Vietnam*
[3]*Vietnam University Of Traditional Medicine, 2 Tran Phu, Mo Lao, Ha Dong districtct, Hanoi 12110, Vietnam*
[4]*Vietnam Military Medical University, 160 Phung Hung, Phuc La, Ha Dong districtct, Hanoi 12108, Vietnam*

ARTICLE INFO

*Article history:*
Received 31 December 2020
Revised 28 January 2021
Accepted 03 March 2021
Published online 01 April 2021



ABSTRACT

Hoa Yên Kinh (HYK) is an oriental prescription in Vietnamese Traditional Medicine that is used to treat premenopausal syndrome in the clinical stage. This study aimed to evaluate the safety, acute and sub-chronic toxicity of aqueous extracts of HYK in *in vivo* model. In the acute toxicity study, HYK at doses ranging from 150 to 300 g /kg/day was given to Swiss mice by oral administration. Mice were observed for general behavioral changes, adverse effects, and mortality up to 7 days post-treatment to find the highest dose that did not kill mice. In sub-chronic toxicity studies, HYK was given orally with doses of 12 g and 60 g/kg/day for 12 weeks. At the end of 12 weeks, the mice were operated to observe the whole organ for histopathology. The microscopic structures of the liver, spleen, and kidney of at least 30% of mice in each group was randomly checked. The acute toxicity study in all the doses used did not cause any significant change such that no $LD_{50}$ was found. In addition, the sub-chronic toxicity study did not show any treatment-related abnormalities in the hematological and biochemical parameters. These results demonstrated that HYK aqueous extract is safe and does not appear to exert toxic effect.

***Keywords:*** Traditional Vietnamese Medicine, HYK recipe, Premenopausal syndrome, Toxicity.

## Introduction

The premenopausal syndrome is considered the third stage in the physiological development of a woman and is a completely natural process. The emergence of premenopausal disorders marks the transition from fertility to non-fertility in women. Most women at the age of 40 years will begin to have symptoms of premenopausal disorder.[1] This syndrome makes them feel tired, uncomfortable, irritable, moody that seriously affects women's health and ability to communicate with relatives or colleagues around. Symptoms that signal the onset of perimenopause are often not the same for women. Most women entering perimenopause suffer from unpleasant problems, mainly due to a decrease in the female sex hormone estrogen.[2] In Vietnam, HYK is an oriental remedy that is used for the treatment of premenopausal syndrome through the regulation of estrogen in females in the clinical stage.

This prescription was derivated from a Traditional Chinese Medicine formulation named "*Dật Kinh Thang*", an ancient prescription written in the book "*Biện Chứng Kỳ Văn - 辨證奇聞*" of author "Tiền Cảnh Hồ", living in Qing Dynasty (*Qīng cháo* -清朝 in Chinese), the last Chinese dynasty of China.[3] Originally, "*Dật Kinh Thang*" included 11 herbal ingredients as Thục địa (*Radix Rehmanniae glutinosae praeparata*) 16 g, Đương quy (*Radix Angelicae*) 10 g, Đảng sâm (*Radix Codonopsis*) 10 g, Táo nhân sao (*Semen Zizyphi*) 6 g, Đơn bì (*Cortes Mouton Radicis*) 6 g, Bạch truật (*Rhizoma Atractylodis*) 16 g, Hoài sơn (*Rhizoma Dioscoreae Popositae*) 10 g, Bạch thược *(Radix Paconiae*) 6 g, Sa sâm (*Radix Glehniae*) 6 g, Đỗ trọng (*Cortex Eucommiae*) 4 g, and Sài hồ (*Radix Bupleuri*) 4 g.[3,4] The "*Dật Kinh Thang*" recipe was used in Traditional Chinese Medicine to support ancestry, and clear the blockage in energy and blood. Especially, according to the traditional therapy, this recipe could reduce blood failure that causing headaches, dizziness, sweating, red face, dry mouth, accompanied by bad vitality, tired body, loss of appetite, sleepless, sad melancholy, fear of restlessness, irregular periods, pale pink tongue, and hypotenuse.[3,4] On the basis of the effect of this remedy and according to clinical practice, Hoa Yên Kinh (HYK) was formulated by adding three more ingredients as Câu kỷ tử (*Fructus Lycium chinense*), Hà thủ ô (*Radix Polygonum multiflorum*), and Dâm dương hoắc (*Herba Epimedium brevicornum*) to increase the effect of kidney health in the treatment of genital disorders in premenopausal women. This study aimed to evaluate the safety, acute and sub-chronic toxicity of aqueous extracts of HYK in *in vivo* model

*Corresponding author. E mail: nganvmu@gmail.com
Tel: 0382689686

Citation: Pham QY, Vuong TH, Doan MT, Nguyen HN. Assessment of Acute and Sub-Chronic Toxicity of HYK Aqueous Extracts in Experimental Animals. Trop J Nat Prod Res. 2021; 5(3):519-527. doi.org/10.26538/tjnpr/v5i3.17

Official Journal of Natural Product Research Group, Faculty of Pharmacy, University of Benin, Benin City, Nigeria.

## Materials and Methods

*General materials*

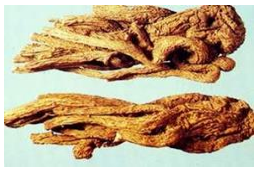
The automatic biochemical testing machine was a Biochemical Systems International model 3000 Evolution (Biochemical Systems International, Italia). The hematology testing machine was an Humancout 30TS (Human, Germany), analytical balance $10^{-4}$, model CP224S (Sartorius, Germany).

*HYK ingredients*

The ingredients of the HYK remedy were collected from April to June 2019 and shown in Table 1. The voucher specimens of *Radix Rehmanniae glutinosae praeparata* (PQY-01), *Radix Angelicae* (PQY-02), *Radix Paeoniae* (PQY-03), *Rhizoma Atractylodis* (PQY-04), *Radix Bupleuri* (PQY-05), *Semen Ziziphi* (PQY-06), *Radix*

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

**Table 1:** HYK ingredients

| Local names and pictures | Crude drug names | Family | Amount (g) |
|---|---|---|---|
| Thục địa  | *Radix Rehmanniae glutinosae praeparata* | Scrophulariaceae | 18 |
| Đương quy  | *Radix Angelicae* | Apiaceae | 10 |
| Bạch thược  | *Radix Paeoniae* | Ranunculaceae | 6 |
| Bạch truật  | *Rhizoma Atractylodis* | Asteraceae | 18 |
| Sài hồ bắc  | *Radix Bupleuri* | Apiaceae | 4 |
| Táo nhân  | *Semen Ziziphi* | Rhamnaceae | 6 |
| Đảng sâm  | *Radix Codonopsis* | Campanulaceae | 10 |
| Hoài sơn  | *Rhizoma Dioscoreae oppositae* | Dioscoreaceae | 10 |

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

| | | | |
|---|---|---|---|
| Đơn bì  | *Cortes Mouton Radicis* | Ranunculaceae | 6 |
| Đỗ trọng  | *Cortex Eucommiae* | Eucommiaceae | 4 |
| Sa sâm  | *Radix Glehniae* | Apiaceae | 6 |
| Hà thủ ô  | *Radix Polygonum multiflorum* | Polygonaceae | 6 |
| Câu kỷ tử  | *Fructus Lycium chinense* | Solanaceae | 6 |
| Dâm dương hoắc  | *Herba Epimedium brevicornum* | Berberidaceae | 6 |

*Codonopsis* (PQY-07), *Rhizoma Dioscoreae oppositae* (PQY-08), *Cortes Mouton Radicis* (PQY-09), *Cortex Eucommiae* (PQY-010), *Radix Glehniae* (PQY-011), *Radix Polygonum multiflorum* (PQY-012), *Fructus Lycium chinense* (PQY-013), and *Herba Epimedium brevicornum* (PQY-014) were deposited at the Laboratory of Pharmacology, Vietnam Military Medical University.

The medicinal materials were used in the dried form and met the Pharmacopoeia V standards established by The National Ministry of Health, Vietnam. HYK was extracted by decoction machine KTP-EP-25 (Korea Techno Pack, Korea) at the Department of Pharmacy, Vietnam Military Institute of Traditional Medicine. The aqueous extract was prepared in different concentrations, depending on the required dose for oral administration. The doses were calculated according to the dry medicinal package/kg/day or drug package/kg/day.[5] According to established guidelines, the expected dose for human use is 116 g/person/day.[5,6] According to the conversion coefficient from humans to experimental animals, the equivalent dose in rats, using a conversion coefficient of 6.2, the expected effective dose for rats is approximately 12.00 g/kg/day.[5,6] The equivalent dose in mice, using a conversion coefficient of 12.3, the expected effective dose for mice is approximately 24.00 g/kg/day.[5,6]

*Extraction*

All of the medicinal materials in this prescription were used in dried form and met the standards in the Vietnamese Pharmacopoeia V, and are processed according to the regulations of traditional medicine.[7-9] HYK was extracted with hot water at a ratio of 1:1 (1 g medicinal herbs/1 mL water). The extraction was done by decoction machine KTP-EP-25 (Korea Techno Pack, Korea) at Faculty of Pharmacy, Military Institute of Traditional

Medicine. The primary standard for HYK was used to concentrate to obtain a range of different concentrations.

*Animals*

The animals used for the acute toxicity study were 40 adult Swiss mice, irrespective of breed, with body weights at the start of the experiment ranging from 18–22 g. The animals used for the semi-long-term toxicity study were 24 adult Wistar mice, regardless of breed, with body weights at the start of the experiment ranging from 160–180 g. The animals had free access to food and water and were maintained under standard laboratory conditions, which included a 12-hour light-dark cycle and temperature of 28-30°C. Animals were acclimatized for one week prior to the study. The experimental protocol was approved by the Vietnam Military Medical University, Hanoi, Vietnam (Permission number IACUC-074/19 issued on August 08, 2019).[10]

*Acute toxicity study and the determination of the dose required for 50% Death ($LD_{50}$)*

The acute toxicity study and the determination of the $LD_{50}$ values for reagents in Swiss mice were performed based on the literature procedures.[11-13] Before conducting the experiment, the mice have fasted overnight. Mice were randomly divided into 6 groups, each containing 10 mice. The HYK samples were mixed in distilled water at different concentrations, so that the volume for each dose was 0.25 mL/10 g body weight. HYK samples were administered 3 times during a 24-hour period, with each dose at least 2 hours apart. Mice were treated with increasing doses to identify the highest dose that could be administered without causing death [0% Death Dose ($LD_0$)] and the lowest dose that killed all mice [100% Death Dose ($LD_{100}$)]. The general conditions of the mice were monitored for signs of intoxication (vomiting, convulsions, and agitation), and the number of dead mice in each group was recorded for 72 hours after the last drug administration. A linear graph was generated to determine the $LD_{50}$ dose. The overall conditions of the mice continued to be monitored (activity, eating, and excretion) in each group for 7 days after the last drug administration. Following animal death, if any, the conditions of the organs were immediately examined to determine the cause of death.[11-13]

*Sub-chronic toxicity studies*

The sub-chronic toxicity studies were performed according to the regulations of the Ministry of Health of Vietnam,[9] and the guidelines on evaluating the safety and efficacy of traditional medicine.[10-13] A total of 24 Wistar mice were randomly divided into 6 groups, each containing 10 mice. Mice were given HYK samples or distilled water (control group) daily, once a day (morning), at a volume of 10 mL/kg body weight. The dose administered to treatment group 1 was 12 g/kg/day, whereas the dose administered to treatment group 2 was 60 g/kg/day. Before and during treatments, the mice were evaluated for general condition, body weight, hematopoietic functions, based on erythrocyte numbers, average red blood cell volume, hemoglobin content, hematocrit content, white blood cell counts, and platelet counts. In addition, liver function was assessed by quantifying the following enzymes and metabolites in the blood: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, and total cholesterol, renal function was also evaluated by quantifying serum creatinine levels. All monitored parameters were evaluated before HYK administration and 4, 8 and 12 weeks after administration. To perform histopathological evaluations, 12 weeks after HYK administration, whole organs were excised from the mice for observation. The microscopic structures of the liver, spleen, and kidney from at least 30% of animals in each group were randomly checked and captured following the literature guidance.[14]

*Statistical analysis*

Data were expressed as the mean ± standard deviations (SD). Statistical significance was assessed by the two-tailed unpaired Student's t-test and *P* values less than 0.05 were considered statistically significant

## Results and Discussion

*Acute toxicity study and the determination of the dose required for 50% death ($LD_{50}$)*

Swiss albino mice were treated with HYK at different doses, ranging from 150 g/kg body weight to 300 g/kg body weight, in a volume of 0.25 mL/10 g, 3 times each day (Table 2).

The results showed that the tested concentration range did not produce any clinical signs of toxicity or cause death in mice. No dead of the mice in all research groups is recorded. In our observation, mice did not display any abnormal symptoms within 72 hours after HYK administration or during the 7 days after administration (Table 2). Even at the highest dose of 300 g/kg/day, the mice remained alive and did not display any unusual symptoms. These results demonstrated that HYK did not cause acute toxicity through oral administration. Because no mice died during this experiment, the $LD_{50}$ value for orally administered HYK could not be determined. Even at the highest dose administered was represents 12.5-fold the expected effective dose, no acute toxicity was observed. Interestingly, in this study, food intake and water consumption were found to have no effects on the administration of HYK. Additionally, the oral administration of HYK did not appear to promote appetite suppression or other damaging effects.

*Sub-chronic toxicity study results*

*The influence of HYK on mice general condition and body weight of mice during long-term administration*

All mice used in this study were monitored daily for general conditions, including motor activity, eating behavior, fur, skin, mucous membrane conditions, and secretions. Mice in the control group and in the HYK oral administration groups displayed normal activities, smooth fur, normal mucosal skin, normal eating behaviors, and the production of molded feces. When comparing the body weights of mice before and after oral HYK administration, the body weights in all three study groups increased significantly. In details, from 4 to 12 weeks oral administration, the body weights of the group 1 (12 g/kg/day**)** and 2 (60 g/kg/day**)** were increasing from 185.80 ± 5.59 g to 234.30 ± 8.56 g, and from 186.20 ± 5.25 g to 235.90 ± 10.16 g, respectively (Table 3), and the change was statistically significant ($p < 0.01$). No significant difference was observed for the weights of the mice in the two administration groups compared with the weight of the mice in the control group, at all examined time points ($p > 0.05$). Thus, HYK appeared to have no effects on mice body weight in this study at least in the range of our used concentration

*Effects of HYK on hematological parameters*

As shown in Table 4, the numbers of red blood cells and hemoglobin contents did not show significant changes following HYK administration ($p > 0.05$). With the dose 1 (12 g/kg/day), the number of red blood cells was decreased from 7.14 ± 0.84 to 7.09 ± 0.65×$10^{12}$ g/L in the period of 4 to 12 weeks after administration. In the high dose, dose 2 (60 g/kg/day), the number of red blood cells were also somewhat decreased from 7.19 ± 0.85 to 7.12 ± 0.85×$10^{12}$ g/L ($p > 0.05$). The hemoglobin content in both administration doses did not change so much as from 12 to 13 g/L ($p > 0.05$). Thus, it is possible to note that HYK, even at different doses and for different durations, did not cause any changes in red blood cell numbers or hemoglobin contents in mice blood (Table 4).As shown in Table 5, the numbers of white blood cells and platelets in mice blood did not change significantly following HYK administration ($p > 0.05$). In details, from 4 to 12 weeks, the number of white blood cells were somewhat changed from 6.89 ± 1.79 to 7.02 ± 1.85 g/L in dose 1 and increased from 6.98 ± 2.21 to 7.13 ± 1.96 g/L, these changes were accepted due to the similar levels between treated mice and in the control group. The levels of platelets were also recognized but not so many changing differences were observed ($p > 0.05$). Thus, it was possible to note that HYK with different administration doses and for different durations did not cause any changes in white blood cell or platelet counts in mice blood.

*Evaluating liver cell damage*

AST (or serum glutamic oxaloacetic transaminase, SGOT) and ALT (or serum glutamic pyruvic transaminase, SGPT) are liver enzyme indicators that can reflect liver damages. In this experiment, the AST and ALT enzyme activities did not change significantly following both two HYK administration doses. The enzyme levels were recorded from 94.77 ± 22.15 to 94.16 ± 15.32 UI/L in the dose 1, and from 94.12 ± 17.22 to 94.39 ± 16.40 UI/L in the dose 2 ($p > 0.05$) after 12 weeks administration (Table 6). Thus, even at different doses and for

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

different durations, HYK did not affect AST or ALT enzyme activity indicating that drinking this recipe might cause damage to rat liver cells.

*Assessing the effects of HYK on liver function*

Albumin is one of the most important protein constituents of serum, accounting for 58%–74% of the total serum protein content. Albumin in blood has many important functions such as maintain colloidal osmotic pressure, stopping water from leaking out of blood vessels, providing amino acids for peripheral protein synthesis, and linking and transporting certain substances produced during metabolisms, such as fatty acids, bilirubin, steroid hormones, and other active substances, throughout the body. The liver is the only organ in the body that produces albumin; thus, the detection of albumin index can reflect the functional status of the liver. Bilirubin is a degenerative product of Heme - an ingredient in the hemoglobin of red blood cells. The degenerative process takes place in retinal tissue such as the liver, spleen, and bone marrow. Bilirubin in the blood includes total bilirubin, direct bilirubin, and indirect bilirubin. Indication of bilirubins is very useful in diagnosing blood-related diseases, hematopoietic organs, hepatobiliary tract, infectious diseases, and viruses affection. In our experiment, albumin and bilirubin were evaluated both before and after HYK administration. The results in Table 7 showed that these parameters were not significantly changed, the number of albumin levels was about 22 g/L in both administration doses, meanwhile, the total bilirubin was calculated as about 54 mmol/L ($p > 0.05$). Based on these results, it is worthy to note that HYK administration did not produce any significant changes in total albumin and bilirubin levels.

*Assessing the effects of HYK on kidney function*

Blood creatinine is a catabolic product of creatine phosphate. Creatinine is exogenously derived from food and endogenously generated by the liver, kidney, and pancreas. Thus, quantitative creatinine testing in the blood can indicate many health problems, including impaired kidney function. In our experiment, creatinine levels were evaluated in mice blood both before and after oral two doses of HYK via administration. As shown in Table 8, blood creatinine concentrations were decreased from 88.23 ± 14.83 to 83.29 ± 13.68 mmol/L at dose 1 administration, and significantly changed from 89.06 ± 13.49 to 84.49 ± 11.86 mmol/L after 12 weeks comparing to the control group ($p > 0.05$). Thus, HYK might not affect creatine concentrations in the blood, regardless of the dose used or the duration of treatment.

**Table 2:** The mortality of mice after 7 days of oral HYK administration

| Groups | Number of mice | HYK dose (g/kg in 0.25 mL/10 g, 3 times/day) | Number of live/dead animals after 72 hours | Number of live/dead animals after 7 days |
|---|---|---|---|---|
| 1 | 10 | 150 | 10/0 | 10/0 |
| 2 | 10 | 180 | 10/0 | 10/0 |
| 3 | 10 | 210 | 10/0 | 10/0 |
| 4 | 10 | 240 | 10/0 | 10/0 |
| 5 | 10 | 270 | 10/0 | 10/0 |
| 6 | 10 | 300 | 10/0 | 10/0 |

**Table 3:** Effects of long-term HYK administration on mice body weights (n = 10)

| Time point | Control (1) | Dose 1 12 g/kg/day (2) | Dose 2 60 g/kg/day (3) | *P-values* |
|---|---|---|---|---|
| | | Weight (g) | | |
| Before oral administration (a) | 186.90 ± 4.82 | 185.80 ± 5.59 | 186.20 ± 5.25 | $p_{2\text{-}1} > 0.05$<br>$p_{3\text{-}1} > 0.05$<br>$p_{3\text{-}2} > 0.05$ |
| After 4 weeks (b) | 211.10 ± 9.06 | 209.20 ± 6.51 | 212.40 ± 9.67 | $p_{2\text{-}1} > 0.05$<br>$p_{3\text{-}1} > 0.05$<br>$p_{3\text{-}2} > 0.05$ |
| After 8 weeks (c) | 225.60 ± 7.29 | 223.00 ± 7.79 | 226.00 ± 9.89 | $p_{2\text{-}1} > 0.05$<br>$p_{3\text{-}1} > 0.05$<br>$p_{3\text{-}2} > 0.05$ |
| After 12 weeks (d) | 235.10 ± 10.13 | 234.30 ± 8.56 | 235.90 ± 10.16 | $p_{2\text{-}1} > 0.05$<br>$p_{3\text{-}1} > 0.05$<br>$p_{3\text{-}2} > 0.05$ |
| *P-values* | $P_{b,c,d\text{-}a} < 0.01; p_{c,d\text{-}b} < 0.01; p_{c\text{-}d} < 0.01$ | | | - |

Data represent mean + S.D. (n = 10). Significantly different from the control group ($p < 0.05$).

**ISSN 2616-0684 (Print)**
**ISSN 2616-0692 (Electronic)**

**Table 4:** Effects of HYK on the numbers of red blood cells and hemoglobin contents in mice blood (n = 10)

| Time point | Control (1) | Dose 1 12 g/kg/day (2) | Dose 2 60 g/kg/day (3) | *P-values* |
|---|---|---|---|---|
| Number of red blood cells ($\times 10^{12}$ g/L) | | | | |
| Before oral administration (a) | 7.16 ± 0.76 | 7.14 ± 0.84 | 7.19 ± 0.85 | $p_{2\text{-}1} > 0.05$ |
| After 4 weeks (b) | 7.13 ± 0.59 | 7.12 ± 0.91 | 7.15 ± 0.64 | $p_{3\text{-}2} > 0.05$ |
| After 8 weeks (c) | 7.07 ± 0.72 | 7.11 ± 1.99 | 7.13 ± 0.89 | $p_{3\text{-}1} > 0.05$ |
| After 12 weeks (d) | 7.05 ± 0.71 | 7.09 ± 0.65 | 7.12 ± 0.85 | |
| *P-values* | $P_{b,c,d\text{-}a} > 0.05$; $P_{c,d\text{-}b} > 0.05$; $p_{d\text{-}c} > 0.05$ | | | |
| Hemoglobin contents (g/L) | | | | |
| Before oral administration (a) | 13.12 ± 1.58 | 12.92 ± 1.24 | 13.10 ± 0.90 | $p_{2\text{-}1} > 0.05$ |
| After 4 weeks (b) | 12.74 ± 1.27 | 13.09 ± 1.14 | 12.80 ± 1.19 | $p_{3\text{-}2} > 0.05$ |
| After 8 weeks (c) | 12.93 ± 1.02 | 12.98 ± 1.76 | 13.02 ± 1.54 | $p_{3\text{-}1} > 0.05$ |
| After 12 weeks (d) | 13.16 ± 1.64 | 12.79 ± 1.43 | 13.08 ± 1.21 | |
| *P-values* | $P_{b,c,d\text{-}a} > 0.05$; $P_{c,d\text{-}b} > 0.05$; $P_{d\text{-}c} > 0.05$ | | | |

Data represent mean + S.D. (n = 10). Significantly different from the control group ($p < 0.05$).

**Table 5:** Effects of HYK on the numbers of white blood cells and platelets in mice blood

| Time point | Control (1) | Dose 1 12 g/kg/day (2) | Dose 2 60 g/kg/day (3) | *P-values* |
|---|---|---|---|---|
| White blood cells (g/L) | | | | |
| **Before oral administration (a)** | 6.95 ± 2.70 | 6.89 ± 1.79 | 6.98 ± 2.21 | $p_{2\text{-}1} > 0.05$ |
| **After 4 weeks (b)** | 7.02 ± 2.06 | 6.96 ± 1.67 | 7.10 ± 2.40 | $p_{3\text{-}2} > 0.05$ |
| **After 8 weeks (c)** | 6.83 ± 2.32 | 6.92 ± 1.26 | 7.06 ± 1.66 | $p_{3\text{-}1} > 0.05$ |
| **After 12 weeks (d)** | 7.06 ± 1.63 | 7.02 ± 1.85 | 7.13 ± 1.96 | |
| *P-values* | $P_{b,c,d\text{-}a} > 0.05$; $p_{c,d\text{-}b} > 0.05$; $P_{d\text{-}c} > 0.05$ | | | |
| Platelets (G/L) | | | | |
| **Before oral administration (a)** | 509.00 ± 121.24 | 503.60 ± 128.60 | 482.40 ± 207.56 | $p_{2\text{-}1} > 0.05$ |
| **After 4 weeks (b)** | 512.50 ± 90.76 | 531.90 ± 106.39 | 469.20 ± 155.31 | $p_{3\text{-}2} > 0.05$ |
| **After 8 weeks (c)** | 439.70 ± 122.02 | 484.00 ± 99.79 | 506.30 ± 171.70 | $p_{3\text{-}1} > 0.05$ |
| **After 12 weeks (d)** | 526.60 ± 98.44 | 466.10 ± 101.28 | 503.10 ± 105. 10 | |
| *P-values* | $P_{b,c,d\text{-}a} > 0.05$; $P_{c,d\text{-}b} > 0.05$; $P_{d\text{-}c} > 0.05$ | | | - |

Data represent mean + S.D. (n = 10). Significantly different from the control group ($p < 0.05$).

**Table 6:** Effects of HYK on AST and ALT activities.

| Time point | Control (1) | Dose 1 12 g/kg/day (2) | Dose 2 60 g/kg/day (3) | *P-values* |
|---|---|---|---|---|
| AST activity (UI/L) | | | | |
| Before oral administration (a) | 99.42 ± 19.99 | 94.77 ± 22.15 | 94.12 ± 17.22 | $p_{2\text{-}1} > 0.05$ |
| After 4 weeks (b) | 101.40 ± 21.42 | 96.82 ± 19.90 | 93.69 ± 16.67 | $p_{3\text{-}2} > 0.05$ |
| After 8 weeks (c) | 97.57 ± 21.50 | 95.24 ± 19.97 | 96.81 ± 19.98 | $p_{3\text{-}1} > 0.05$ |
| After 12 weeks (d) | 100.45 ± 15.77 | 94.16 ± 15.32 | 94.39 ± 16.40 | |
| *P-values* | $P_{b,c,d\text{-}a} > 0.05$; $P_{c,d\text{-}b} > 0.05$; $P_{d\text{-}c} > 0.05$ | | | - |
| ALT activity (UI/L) | | | | |
| Before oral administration (a) | 88.10 ± 20.89 | 86.91 ± 16.19 | 89.02 ± 14.72 | $p_{2\text{-}1} > 0.05$ |
| After 4 weeks (b) | 89.60 ± 18.81 | 83.28 ± 15.01 | 88.48 ± 11.62 | $p_{3\text{-}2} > 0.05$ |
| After 8 weeks (c) | 84.56 ± 13,12 | 83.16 ± 17.66 | 86.69 ± 15.89 | $p_{3\text{-}1} > 0.05$ |
| After 12 weeks (d) | 89.93 ± 17.91 | 82.62 ± 13.69 | 84.38 ± 19.12 | |
| *P-values* | $P_{b,c,d\text{-}a} > 0.05$; $P_{c,d\text{-}b} > 0.05$; $P_{d\text{-}c} > 0.05$ | | | - |

Data represent mean + S.D. (n = 10). Significantly different from the control group ($p < 0.05$)

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

| Time point | Control (1) | Dose 1 12 g/kg/day (2) | Dose 2 60 g/kg/day (3) | *P-values* |
|---|---|---|---|---|
| | | Albumin (g/L) | | |
| Before oral administration (a) | 22.44 ± 2.08 | 22.72 ± 2.36 | 22.63 ± 2.49 | $p_{2-1} > 0.05$ |
| After 4 weeks (b) | 22.56 ± 2.10 | 22.81 ± 2.26 | 22.70 ± 2.72 | $p_{3-2} > 0.05$ |
| After 8 weeks (c) | 22.24 ± 1.98 | 22.33 ± 1.84 | 22.79 ± 2.84 | $p_{3-1} > 0.05$ |
| After 12 weeks (d) | 22.39 ± 2.29 | 22.60 ± 1.99 | 22.91 ± 2.66 | |
| *P-values* | | $P_{b,c,d-a} > 0.05$; $P_{c,d-b} > 0.05$; $P_{d-c} > 0.05$ | | - |
| | | Bilirubin (μmol/L) | | |
| Before oral administration (a) | 53.57 ± 11.86 | 54.17 ± 11.84 | 54.64 ± 12.74 | $p_{2-1} > 0.05$ |
| After 4 weeks (b) | 54.56 ± 14.00 | 52.91 ± 13.30 | 53.11 ± 12.91 | $p_{3-2} > 0.05$ |
| After 8 weeks (c) | 55.38 ± 11.80 | 52.47 ± 12.35 | 52.84 ± 12.10 | $p_{3-1} > 0.05$ |
| After 12 weeks (d) | 57.13 ± 13.67 | 53.13 ± 10.12 | 53.54 ± 10.27 | |
| *P-values* | | $P_{b,c,d-a} > 0.05$; $P_{c,d-b} > 0.05$; $P_{d-c} > 0.05$ | | - |

Data represent mean + S.D. (n = 10). Significantly different from the control group ($p < 0.05$)

**Table 8:** Effects of HYK on mice blood creatinine concentrations (n = 8, ± SD).

| Time point | Control (1) | Dose 1 12 g/kg/day (2) | Dose 2 60 g/kg/day (3) | *P-values* |
|---|---|---|---|---|
| | | Creatinine (mmol/L) | | |
| Before oral administration (a) | 84.41 ± 11.76 | 88.23 ± 14.83 | 89.06 ± 13.49 | $p_{2-1} > 0.05$ |
| After 4 weeks (b) | 88.50 ± 11.81 | 89.19 ± 12.90 | 86.82 ± 12.88 | $p_{3-2} > 0.05$ |
| After 8 weeks (c) | 85.34 ± 13.03 | 86.84 ± 10.08 | 86.40 ± 11.91 | $p_{3-1} > 0.05$ |
| After 12 weeks (d) | 86.69 ± 10.75 | 83.29 ± 13.68 | 84.49 ± 11.86 | |
| *P-values* | | $p_{b,c,d-a} > 0.05$; $p_{c,d-b} > 0.05$; $p_{d-c} > 0.05$ | | - |

Data represent mean + S.D. (n = 10). Significantly different from the control group ($p < 0.05$).

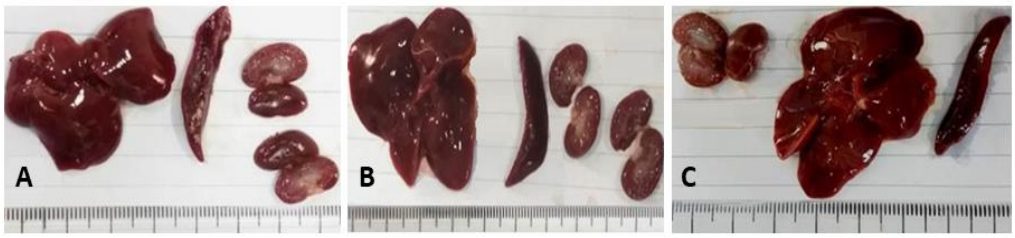



**Figure 1:** General images of the liver, spleen, and kidney from representative mice treated with control (A), dose1 (B), and dose 2 (C).

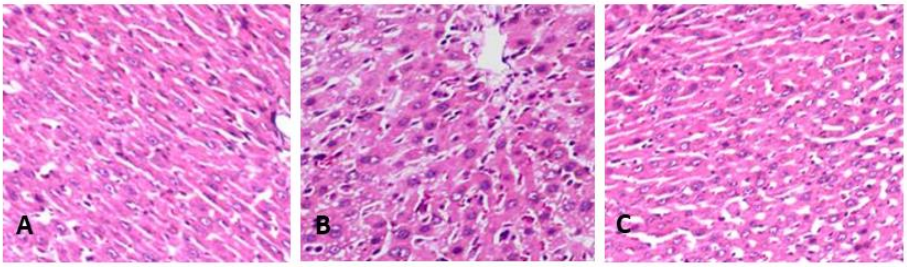



**Figure 2:** Histopathological images of representative rat livers from the control (A), dose 1 (B), and 2 (C) after 12 weeks (HE × 400).

ISSN 2616-0684 (Print)
ISSN 2616-0692 (Electronic)

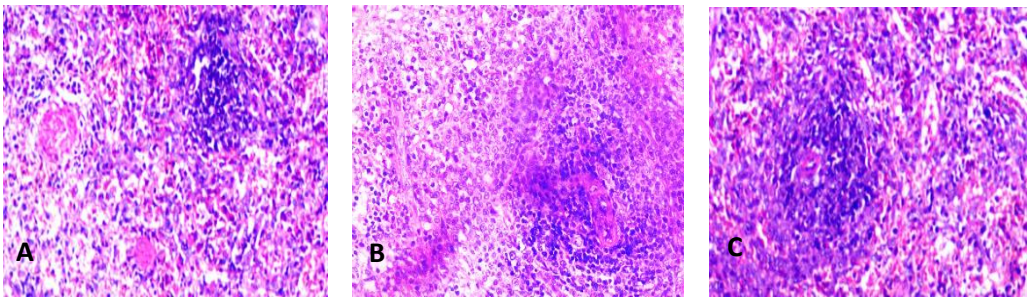



**Figure 3:** Histopathological images of representative rat spleens from the control (A), dose 1 (B), and 2 (C) after 12 weeks (HE × 400).

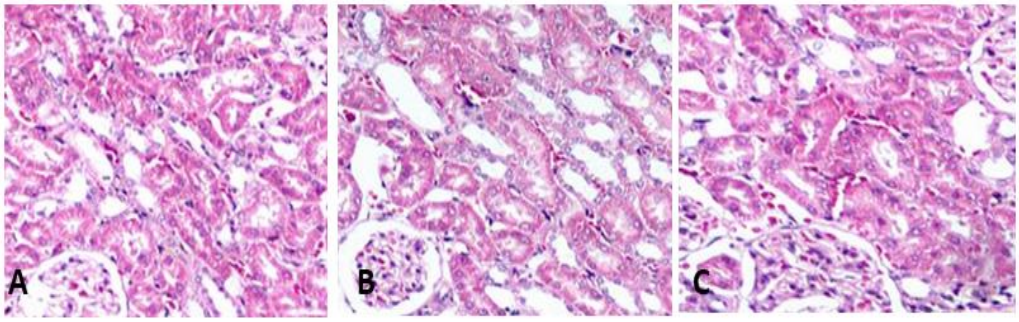



**Figure 4:** Histopathological images of representative rat kidneys from the control (A), dose 1 (B), and 2 (C) after 12 weeks (HE × 400).

*Histopathological results*

The liver, spleen, and kidney were observed by the naked eye and under a microscope, at 25×magnification. The colors and morphologies of the liver, spleen, and kidney in groups treated with dose 1 and 2 did not differ from those for the control group (Figure 1). The histopathological samples were examined at the pathological morphology level. The histopathology results for the liver, spleen, and kidney of treated mice showed that the oral administration of HYK at doses 1 and 2 continuously for 12 weeks, did not cause liver, kidney, or spleen damage in mice. In details, for the hepatic histopathology, the results in Figure 2 show that the structures of the hepatic lobe remained clear and conspicuous, without necrosis phenomena or hepatocellular degeneration, were observed in mice treated with control (A), dose 1 (B), and 2 (C) after 12 weeks Figure 3 showed histopathological images of representative rat spleens from the control (A), dose 1 (B), and 2 (C) after 12 weeks of oral administration. The spleen remained intact, demonstrating a white marrow, with large cell numbers concentrated in the large lymphoid follicles. Thickened penile arteries could be observed in the white marrow, and the red marrow, characterized by cyst sinuses, contains many red blood cells and some macrophages in normal growth stages. The intact kidneys of mice from all three groups remained clear and preserved (Figure 4). The medulla and the cortex remained unblemished, and the glomeruli are evenly distributed, with no inflammatory infection. Arteries and small arterioles have normal structures.

## Conclusion

In our study, the toxicological evaluation revealed that for both acute and sub-chronic toxicity tests, HYK extract did not produce any toxic effect in mice or mice. No morbidity or mortality was observed during the acute toxicity study. The $LD_{50}$ value of the extract was not identified suggesting that the HYK extract is essentially non-toxic and safe for the oral administration of the tested doses. During the sub-chronic toxicology test, no deaths or treatment-related symptoms were observed in any group. The histopathological inspection of the liver, kidney, and spleen of mice showed a normal situation, suggesting no microscopic changes or morphological disturbances were caused by the oral administration of HYK at two doses. Interestingly, no significant differences in food and water intake, weight gain, biochemical, or hematological parameters were observed between the control and treated groups during the oral administration period. In conclusion, the water extract of HYK was safe during either the acute or sub-chronic toxicity studies.

## Conflict of interest

The authors declare no conflict of interest.

## Authors' Declaration

The authors hereby declare that the work presented in this article is original and that any liability for claims relating to the content of this article will be borne by them.

## References

1. Silva-Bermudez LS, Toloza FJK, Perez-Matos MC, de Souza RJ, Banfield L, Vargas-Villanueva A, Mendivil CO. Effects of oral contraceptives on metabolic parameters in adult premenopausal women: a meta-analysis. Endocr Conn. 2020; 9(10):978-998.
2. Butt SA, Lidegaardi Ø, Skovlund C, Hannaford PC, Iversen L, Fielding S, Mørch LS. Hormonal contraceptive use and risk of pancreatic cancer-A cohort study among premenopausal women. PLoS One. 2018; 13(10):1-8.
3. Hoang DT and Hoang AT. Book name "*Phương Tễ Học*", Thuận Hóa Publishing House, Ha Noi, Vietnam, 2009; 20-120p.
4. Oriental Medicine Faculty Note, Hanoi Medical University, Book name "*Các Bệnh Phụ Khoa Y học Cổ Truyền*", Medical Publishing House, Ha Noi, Vietnam, 2000; 635-642p.

5. Vietnam National Ministry of Health, Circular No. 29/2018/TT-BYT on clinical trial (2018) (In Vietnamese).
6. Nguyen TNP. Book name "*Nội Tiết Sinh Sản"*, Medical Publishing House, Ha Noi, Vietnam, 2012; 201-227p.
7. Do TL. *Medicinal Plants in Vietnam*, Health Publishing House, Ha Noi, Vietnam, 2015; 507-509:721-723p.
8. Nguyen NK. Basic Theology in Vietnamese Oriental Medicines, Vietnam Education Publishing, Ha Noi, Vietnam 2011; 161-265 p.
9. Vietnam Ministry of Health. Vietnamese Pharmacopoeia, episode 2, 5th Edition, Health Publishing, Ha Noi, Vietnam. 2018; 1154-1379p.
10. Nair AB and Jacob S. A simple practice guide for dose conversion between animals and human. J Basic Clin Pharm. 2016; 7(2):27-31.
11. Schlede E, Genschow E, Spielmann H, Stropp G, Kayser D. Oral acute toxic class method: a successful alternative to the oral LD50 test. Regul Toxicol Pharmacol. 2005; 42(1):15-23.
12. Organization of Economic Co-operation and Development - OECD, The OECD Guideline for Test No. 408: Repeated Dose 90-Day Oral Toxicity Study in Rodents, Paris, France, 2018.
13. World Health Organization - WHO, General Guidelines for Methodologies on Research and Evaluation of Traditional Medicine, Geneva, Switzerland, 2012.
14. Ebbo AA, Sani D, Suleiman MM, Ahmad A, Hassan AZ. Acute and sub-chronic toxicity evaluation of the crude methanolic extract of *Diospyros mespiliformis* hochst ex a. Dc (ebenaceae) and its fractions. Toxicol Rep. 2020; 7:1138-1144.