ACUTE AND SUB-CHRONIC ORAL TOXICITIES OF DA.AMLODEPON HVD HARD CAPSULE IN EXPERIMENTAL ANIMALS

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Assessment of toxicities of DA.AMLODEPON HVD hard capsule on experimental animals. The acute toxicity of DA.AMLODEPON HVD was assessed on Swiss mice according to World Health Organization Guidance, and LD_{50} determination according to the method of Litchfield – Wilcoxon. The sub-chronic toxicity study of DA.AMLODEPON HVD at two doses (0.42 g/kg/day and 1.26g/kg/day) was conducted in rats for four consecutive weeks. After administration, general conditions and the body weight of rats were evaluated. Blood samples were collected for analyzing serum parameters before treatment (T0), second week (T1), and fourth week (T2). Histopathological analysis of livers and kidneys was observed at the end of the experiment. The results revealed that mice were taken up to a maximum dose of 39.15 g/kg with no symptoms of acute toxicity, LD_{50} of DA.AMLODEPON HVD has not been determined. The sub-chronic toxicity study at two doses did not change the body weight of rats, general conditions. The parameters for structures and functions of livers and kidneys and microscopic of the livers and kidneys are in a normal range during the study period.

Keywords: DA.AMLODEPON HVD hard capsule, acute toxicity, sub-chronic toxicity, experimental animals.

I. INTRODUCTION

Cardiovascular diseases (CVDs) are the first leading cause of death and morbidity in developed countries. An estimated 17.9 million people died from CVDs in 2019, representing 32% of all global deaths.[1] Commonly, CVD can refer to a class of diseases that involves the heart or blood vessels such as stroke, heart failure, atherosclerosis, hypertension… It was projected that CVD would be the cause of more than 23 million deaths in 2030 around the world.[2] According to the World Health Organization (WHO), over three-quarters of CVD deaths have occurred in low- and middle-income countries, which has been a growing epidemic problem in recent years.[1,3] Most prescription drugs used in primary and secondary prevention of CVD (e.g., *aspirin, beta-blockers;* statins...) are synthetic substances that cause several complications for patients.[4] Sometimes, surgical operations are required to treat CVDs as coronary artery bypass, valve repair, and replacement; heart transplantation places a heavy burden on the economics of the patients and their families.

In Vietnam, traditional medicine has a long-established history and plays an essential role in healthcare. The use of traditional medicinal plants has rapidly expanded in recent years. There is a growing interest in using natural

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Received: 08/09/2021
Accepted: 29/09/2021

products as a complementary therapy in treating various diseases, including various diseases, including CVD, considered necessary in the management, prevention, and control of this disease. Currently, herbal medicine has been researched to treat CVDs in traditional medicine systems.[5] The DA.AMLODEPON HVD is composed of restorative materials available in Vietnam which have been used as folk medicine to treat CVDs, including *Styphnolobium japonicum (0.8 g); Embryo Nelumbinis (0.6 g); Codonopsis pilosula (0.6 g); Semen Gardeniae (0.1 g); Canca edulis-Kur (0.1 g).*[6] For safe use, knowledge of adverse reactions and herb-drug interactions is necessary; however, the safety of a combination of herbal medicine in DA.AMLODEPON HVD has not been evaluated. Thus, the present study aimed to investigate the acute toxicity and sub-chronic toxicity of DA.AMLODEPON HVD hard capsule on experimental animals.

II. METHODS

1. Plant materials

DA.AMLODEPON HVD was prepared in a hard capsule form. 580 mg extract includes the ingredients: *Styphnolobium japonicum (0.8 g); Embryo Nelumbinis (0.6 g); Codonopsis pilosula (0.6 g); Semen Gardeniae (0.1 g); Canca edulis-Kur (0.1 g)* and other synthetic ingredients just enough for one hard capsule.

Number of hard capsules in a bottle: 90, Mfg Date: 02.2021; Lot.No: 001, Exp Date: 02.202. These hard capsules were manufactured by Haiduong Pharmaceutical, Medical materials joint-stock Company, and distributed by Hung Vuong Duong Pharmacy Joint Stock Company.

2. Animals

Acute toxicity experiment: *Swiss* mice (18 - 22 g) of either sex were purchased from the National Institute of Hygiene and Epidemiology.

Wistar rats of both sexes weighed 180 - 220 g provided by The Center of Experimental Animals, Danphuong, Hanoi.

The animals were acclimated to housing in the laboratory of the Department of Pharmacology, Hanoi Medical University 7 days before and during the study period; they were fed with standard food and unlimited water intake (housed in a temperature (25 ± 2^{o}C) and humidity (80% ± 10%) under a 12h light/12h dark cycle.

3. Methods

According to World Health Organization Guidance, the acute toxicity experiment was carried out and LD_{50} was determined by Litchfield - Wilcoxon method.[7,8] The mice were fasted for 18 hours before the experiment and randomly divided into different groups, each of 10 mice. DA.AMLODEPON HVD was given orally in increasing doses to determine the lowest dose causing death in 100% of the mice, and the highest dose do not cause death in mice (0% of death in mice). Mice were fed orally 3-time a day, each time at 0.25 mL/10g; Assess the general health condition of mice and signs of toxicity such as vomiting, convulsions, agitation, abnormal excretion... and the number of rats that died within 72 hours after giving DA.AMLODEPON HVD. All dead mice were operated on to assess macroscopic lesions. The lethal dose in 50% (LD_{50}) was determined by the Litchfield - Wilcoxon method. Then continue to monitor the mice for seven days after giving DA.AMLODEPON HVD.

Subchronic toxicity experiment was carried out in compliance with the guidance of the World Health Organization.[7] The DA.AMLODEPON HVD was given orally for four consecutive weeks. The animals were divided into three groups of 10 animals each group. Group I (normal control group), rats received distilled

water, group II and III received with 0.72 (low dose- equivalent to clinical dose) and 2.16 g/kg/day (high dose - 3 times-equivalent to clinical dose) of DA.AMLODEPON HVD, respectively. Animals were treated daily by the oral route of administration once a day in the morning for four consecutive weeks and observed once daily to detect signs of toxicity. Blood samples of animals were collected via saphenous vein puncture in tubes containing EDTA for hematological analysis hematology, and the non-heparinized blood was carefully collected for biochemistry analysis. At the end of the experiment, rats were operated on to observe the macroscopic of all organs, the histology samples (liver, kidney) were collected in 30% of rats to assess microscopic morphology. The micro-histological examination was carried out at the Center for Research and Early Detection of Cancer (CREDCA). Assoc.Prof. Le Dinh Roanh – the director of CREDCA.

4. Statistical analysis

Data sets were entered, edited, and analyzed using Excel 2013 software. Results were expressed as the Mean value ± Standard Deviation (SD) or the percentage (%). Appropriate statistical analysis was applied with $p < 0.05$, considered as a significant difference.

III. RESULTS

1. Acute toxicity experiment

The number of dead mice and the dose of DA. Oral administration of DA.AMLODEPON HVD hard capsule did not reveal any toxicity signs and symptoms up to the highest dose of 39.15 g/kg within the first 72h of treatment and for the next 7 seven days. Besides, animals did not show significant acute toxicity signs such as piloerection, muscle twinge, and lethargy. AMLODEPON HVD is shown in Table 1.

Table 1. Acute toxicity of DA.AMLODEPON HVD

Group	n	Dosage (ml/kg b.w)	Dosage (g/kg b.w)	Mortarity rates (%)
Group 1	10	45	23.49	0
Group 2	10	60	31.32	0
Group 3	10	75	39.15	0

2. Sub-chronic toxicity experiments

Effect on the general condition and the body weight

The sub-chronic oral administration of DA.AMLODEPON HVD (0.42 g/kg and 2.16 g/kg) groups produce no behavior change, toxic signs, or mortality during the experimental period.

The body weight: After four weeks of the experiment, the body weight of rats in all groups (control group and two treatment groups) was significantly increased compared to that of the control group before the experiment. The difference had no statistical significance between the control and treatment groups ($p > 0.05$), as has shown in Figure 1.

There was no change in feeding and water consumption of all groups during the experimental periods by observation.

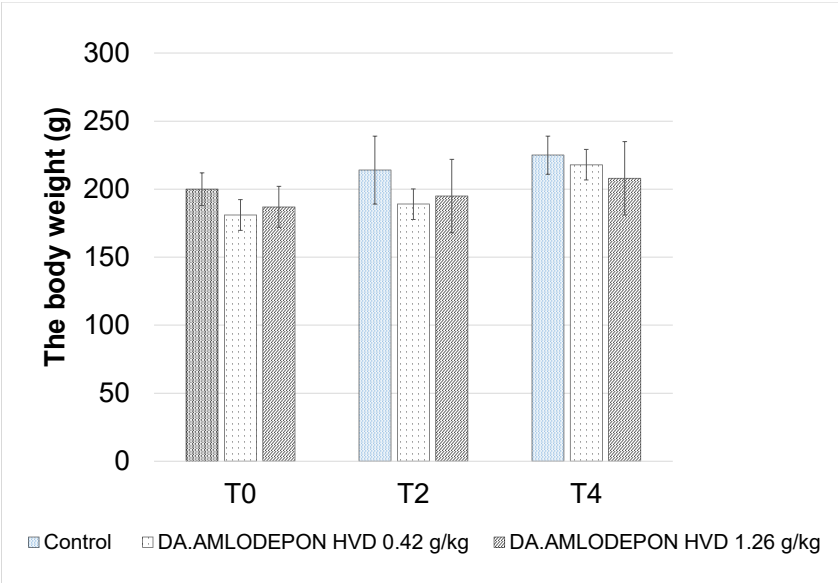


Figure 1. Effect of DA.AMLODEPON HVD on rat's body weight.
Data are expressed as Mean ± SD (n = 10)

Effect of DA.AMLODEPON HVD on hematological parameters in rats

In the sub-chronic toxicity study, the blood count in both group I (control group), group II (DA. AMLODEPON HVD at the dose of 0.42 g/kg/day), and group III (DA.AMLODEPON HVD at the dose of 2.16 g/kg/day) had no significantly changed among groups, and there was no significantly different when compared between the before and after the experiment (p > 0.05) as shown in Table 2 and Table 3.

Table 2. Effect of DA.AMLODEPON HVD on rat's hematological parameters

Parameters	Groups (n = 10)	T0 $\bar{X}$ SD)	T2 $\bar{X}$ SD)	T4 $\bar{X}$ SD)
Red blood cells (T/l)	Control	9.79 ± 1.43	10.29 ± 1.35	9.15 ± 1.25
	DA.AMLODEPON HVD 0.42 g/kg	10.09 ± 1.08	10.64 ± 0.87	9.21 ± 1.36
	DA.AMLODEPON HVD 2.16 g/kg	10.03 ± 1.24	10.60 ± 1.58	9.43 ± 0.87
p (before – after)		> 0.05	> 0.05	> 0.05
Hemoglobin (g/dl)	Control	13.81 ± 1.38	14.18 ± 1.80	12.53 ± 1.50
	DA.AMLODEPON HVD 0.42 g/kg	13.94 ± 1.67	14.57 ± 1.19	13.27 ± 1.34
	DA.AMLODEPON HVD 2.16 g/kg	14.28 ± 1.38	14.61 ± 1.82	13.00 ± 2.04
p (before – after)		> 0.05	> 0.05	> 0.05

Parameters	Groups (n = 10)	T0 ($\bar{X}$ SD)	T2 ($\bar{X}$ SD)	T4 ($\bar{X}$ SD)
Hematocrit (%)	Control	53.35 ± 4.97	53.97 ± 4.37	48.70 ± 6.24
	DA.AMLODEPON HVD 0.42 g/kg	52.43 ± 6.82	54.22 ± 5.09	48.75± 6.55
	DA.AMLODEPON HVD 2.16 g/kg	53.86 ± 5.52	54.45 ± 4.52	49.63 ± 5.07
MCV(fl)	Control	54.30 ± 1.89	55.20 ± 1.87	53.00 ± 1.25
	DA.AMLODEPON HVD 0.42 g/kg	52.70 ± 2.31	54.70 ± 2.21	51.80 ± 2.70
	DA.AMLODEPON HVD 2.16 g/kg	52.40 ± 3.06	53.90 ± 3.07	51.90 ± 1.85
p (before – after)		> 0.05	> 0.05	> 0.05
White blood cells (G/l)	Control	8.44 ± 1.56	10.18 ± 2.22	7.99 ± 1.82
	DA.AMLODEPON HVD 0.42 g/kg	9.82 ± 2.27	10.44 ± 2.87	8.80 ± 1.37
	DA.AMLODEPON HVD 2.16 g/kg	9.16 ± 2.26	10.26 ± 1.81	9.55 ± 2.08
p (before – after)		> 0.05	> 0.05	> 0.05
Platelet (G/l)	Control	561.80 ± 117.77	473.20 ± 113.79	516.60 ± 88.81
	DA.AMLODEPON HVD 0.42 g/kg	557.00 ± 82.85	483.20 ± 87.33	492.60 ± 91.09
	DA.AMLODEPON HVD 2.16 g/kg	475.30 ± 91.24	526.40 ± 84.39	468.20 ± 97.46
p (before – after)		> 0.05	> 0.05	> 0.05

Table 3. The effects of hard capsules on neutrophils and lymphocytes in control and treated rats of subchronic toxicity study

Time	Group I (n = 10)		Group II (n = 10)		Group III (n = 10)	
	Lym (%)	Neu (%)	Lym (%)	Neu (%)	Lym (%)	Neu (%)
Before treatment	76.12 ± 4.93	11.76 ± 3.07	75.76 ± 3.35	11.30 ± 2.64	71.69 ± 7.60	14.08 ± 3,98
2 weeks after treatment	76.53 ± 3.64	11.19 ± 3.07	75.50 ± 3.47	11.82 ± 2.52	72.47 ± 6.28	13.13 ± 3.30
p (before - after)	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Time	Group I (n = 10)		Group II (n = 10)		Group III (n = 10)	
	Lym (%)	Neu (%)	Lym (%)	Neu (%)	Lym (%)	Neu (%)
4 weeks after treatment	75.73 ± 6.25	12.18 ± 2.89	75.11 ± 4.44	11.72 ± 2.25	72.62 ± 4.24	14.92 ± 3.33
p (before - after)	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05	> 0.05

Effect of DA.AMLODEPON HVD on serum biochemical parameters

The liver functions: The effects of sub-chronic administration DA.AMLODEPON HVD to rats on serum biochemical parameters at the termination of treatment are shown in Table 4. The results of serum biochemical indices indicate that DA.AMLODEPON HVD hard capsules did not cause significant difference (p > 0.05) in the liver functions between the treated groups and the control group. There was no significant difference comparing the before and after treatment (p > 0.05).

Table 4. Effect of DA.AMLODEPON HVD on rat's plasma biochemical parameters for liver function

Parameters	Groups (n = 10)	T0 ($\bar{X}$ ± SD)	T2 ($\bar{X}$ ± SD)	T4 ($\bar{X}$ ± SD)
Total Albumin (g/dL)	Control	2.99 ± 0.46	3.30 ± 0.27	2.74 ± 0.23
	DA.AMLODEPON HVD 0,42 g/kg	2.90 ± 0.25	3.09 ± 0.26	2.92 ± 0.20
	DA.AMLODEPON HVD 2,16 g/kg	3.08 ± 0.33	3.13 ± 0.31	2.93 ± 0.29
p (before – after)			> 0.05	> 0.05
Total Cholesterol (mmol/L)	Control	1.43 ± 0.24	1.40 ± 0.17	1.29 ± 0.16
	DA.AMLODEPON HVD 0,42 g/kg	1.39 ± 0.15	1.45 ± 0.16	1.24 ± 0.12
	DA.AMLODEPON HVD 2,16 g/kg	1.45± 0.20	1.59 ± 0.25	1.32 ± 0.20
p (before – after)			> 0.05	> 0.05
Total bilirubin (mmol/L)	Control	13.21 ± 0.38	13.38 ± 0.43	13.36 ± 0.60
	DA.AMLODEPON HVD 0,42 g/kg	13.32 ± 0.26	13.37 ± 0.27	13.45 ± 0.41
	DA.AMLODEPON HVD 2,16 g/kg	13.41 ± 0.38	13.43 ± 0.38	13.38 ± 0.29
p (before – after)			> 0.05	> 0.05

The levels of liver cells destruction: The results of tests to assess the level of liver cells destruction (AST, ALT activities in rat blood) are shown in Figure 2. Both treatment group 1 and

treatment group 2 did not have a significant difference compared with the control group and compared between two times before and after taking DA.AMLODEPON HVD (p > 0.05).

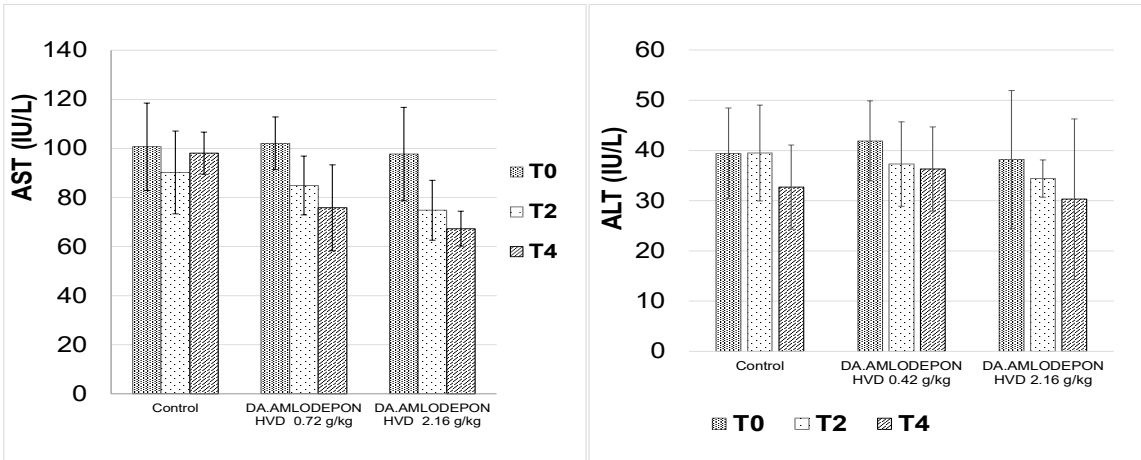


Figure 2. Effects of DA.AMLODEPON HVD on rat's plasma biochemical parameters for level of liver cells destruction. Data are expressed as Mean ± SD (n = 10)

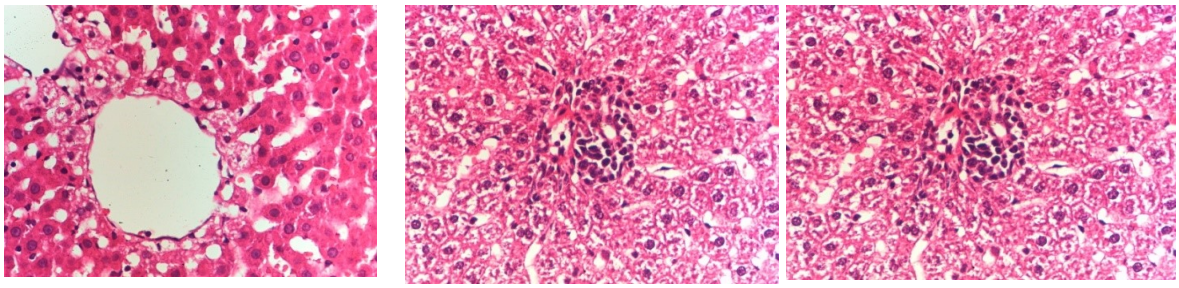
Kidney functions: There were no significant difference in the concentration of serum markers of kidney functions compared with a control group. The results are shown in Table 5.

Table 5. Effect of DA.AMLODEPON HVD on rats plasma biochemical parameters for kidney function

Parameter	Groups (n = 10)	T0 ($\bar{X}$ ± SD)	T2 ($\bar{X}$ ± SD)	T4 ($\bar{X}$ ± SD)
Creatinine (mg/dL)	Control	0.76 ± 0.14	0.84 ± 0.19	0.84 ± 0.17
	DA.AMLODEPON HVD 0,42 g/kg	0.83 ± 0.15	0.86 ± 0.14	0.84 ± 0.13
	DA.AMLODEPON HVD 2,16 g/kg	0.81 ± 0.16	0.89 ± 0.11	0.83 ± 0.16
p (before – after)			> 0.05	> 0.05

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

Effect of DA.AMLODEPON HVD on experimental animal histopathology: In all experiment rats (control group and DA.AMLODEPON HVD treated groups), no pathological change in the macroscopic of all organs was observed: heart, lung, liver, spleen, pancreas, kidney, and digestive system. The effect of DA.AMLODEPON HVD on the histopathology of the liver and kidney at the treatment termination is shown in Figures 3 - 4. At the end of the treatment period, the livers appeared normal with preserved hepatic structure, normal hepatocytes. Eosinophilic cytoplasm and central nucleic are observed during autopsy in the control group. Results showed that DA.AMLODEPON HVD did not affect the morphology of the kidney and liver in the experimental animals.

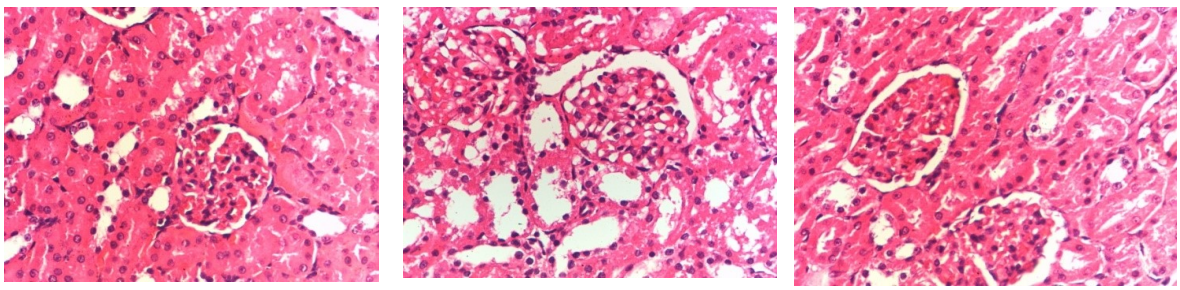


a. Control group

b. DA.AMLODEPON HVD 0.42 g/kg

c. DA.AMLODEPON HVD 2.16 g/kg

Figure 3. Liver sections of control rats (a) and rats treated daily with DA.AMLODEPON HVD at two doses of 0.42 g/kg (b), 2.16 g/kg (c).



a. Control group

b. DA.AMLODEPON HVD 0.42 g/kg

c. DA.AMLODEPON HVD 2.16 g/kg

Figure 4. Kidney sections of control rats (a) and rats treated daily with DA.AMLODEPON HVD at two doses of 0.42 g/kg (b), 2.16 g/kg (c).

IV. DISCUSSION

Herbal medicine has been used as a medical treatment since the beginning of human pharmacotherapy. Some derivatives (such as digitalis, reserpine) have been used in patients with congestive heart failure, atherosclerosis,. Continuing research is necessary to elucidate the pharmacological activities of the many herbal remedies now being used to treat cardiovascular diseases. Although the effect of these herbs, in general, has been proven, a safety assessment is essential before prescribing them for treatment. It is also a necessary process for preclinical dose determination in drug discovery and development. Toxicity study results provide accurate information on potentially relevant adverse effects for the substance being evaluated.[8]

In the present acute toxicity study, the DA.AMLODEPON HVD hard capsules up to 39.5 g/kg (the highest possible dose given to mice) 46.8 times the expected clinical dose showed no mortality, no clinical signs indicating the increase of decrease in temperature, change of skin color, general appearance, diarrhea, or sedation. Therefore, the LD50 of the hard capsule could not be determined because the most significant dose recommended in the study did not show any toxicity symptoms. Thus, it can be concluded from this study that DA.AMLODEPON HVD hard capsules were included in the unclassified criteria. According

to other researches, the principal alkaloid of CHCl extracts, neferine (1200 gram of embryos of the seeds of *Nelumbo nucifera* can extract 2.99 g neferine), dose-dependently inhibited locomotor activity in mice. Dose *Nelumbo nucifera embryos* of in DA.AMLODEPON HVD is 100mg/kg per day did not show toxicity in clinical symptoms.[9] On another hand, the toxicity of *Codonopsis* has not been reported in the scientific literature.[10]

As a continuation of the acute toxicity study, the subchronic toxicity study evaluated for four consecutive weeks with two doses of 0.72 g/kg and 2.16 g/kg/day was conducted. No toxicity or mortality was observed in all the treated groups after 28 days of oral administration of DA.AMLODEPON HV; food, water consumption, and body weight were not affected. These indications show that the hard capsules did not affect the growth of the rats. The blood is affected by all of the organs but at the same time is also affected and reflects the specific state of the hematopoietic system. The hard capsules induced no treatment-related adverse effects concerning hematological parameters. Hepatic and renal function is crucial, with one being used for the metabolism of ingestion and the other for excretion of the waste product, respectively. In order to evaluate the toxicity of any herbal medicine, it is necessary to know the state of these two vital organs, which can be verified by biochemical estimation.[8] Similarly, histological examination findings have not changed from all the groups. This information showed that the DA.AMLODEPON HV is not toxic at the doses studied.

In previous research, Jing-Yu He et al. (2015) proved hepatoprotective activity of *Codonopsis* alcohol-induced hepatic injury in mice. The medicinal properties of the plant are due to the presence of adenosine, adiponectin, and saponins. In addition, the saponin compound in *Codonopsis* had effects on the blood system by inhibited erythrocyte hemolysis, decreases MDA, serum creatinine, and blood urea nitrogen levels.[10]

Currently, there is limited research on the toxicity of the medicinal herbs in DA.AMLODEPON HVD. According to a study by Hwan-Suck Chung et al. (2012) reported that in the safety evaluation studies, *Nelumbo nucifera* seed was shown to be safe up to a dose of 4000 mg/kg/day over 13 weeks of administration in rats and up to 2000 mg/kg/day over four weeks of administration in the dog.[11] Besides, *Nelumbo nucifera* seeds have hepatoprotective and free radical scavenging effects on carbon tetrachloride (CCl4) and aflatoxin B1 (AFB1)-induced hepatocyte toxicity models.[12] The results of this examination support the survival data obtained, indicating that poly-herbal formulation tablets were not harmful. The administration of poly-herbal formulation tablets at a dose up to 4.032 mg/kg in rats for 91 consecutive days did not present any signs of toxicity, either on clinical observation, laboratory and microscopic examination of internal organs, or microscopically.

V. CONCLUSION

The DA.AMLODEPON HV hard capsules were administered by gavage on mice. Mice were given a maximum dose of 39.5 g/kg with no manifestations of acute oral toxicity, LD_{50} of DA.AMLODEPON HVD has not been determined.

DA.AMLODEPON HVD was oral administration on rats during four consecutive weeks with a dose equivalent to the clinical dose and three times the clinical dose did not cause sub-chronic oral toxicity.

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