# EVALUATION OF SUB-CHRONIC TOXICITY OF TD.MV HARD CAPSULE IN EXPERIMENTAL RATS

**Trinh Vinh Quang[1], Nguyen Thi Huong Lien[2], Nguyen Thi Hong Van[2], Luu Van Quynh[2], Bui Minh Anh[2], Dinh Thi Thu Hang[1] and Pham Thi Van Anh[1,✉]**

*[1]Hanoi Medical University, Hanoi, Vietnam*

*[2]Sao Thai Duong Joint Stock Company*

*TD.HK01 (TD.MV) is prescribed for preventive treatment of cardiovascular disease (CVD) collectively, and myocardial infarction (MI) individually. However, the safety of this product upon long-term consumption has never been reported. In this study, we focus on determining the sub-chronic toxicity of TD.MV hard capsules in Wistar rats, paving the way for clinical trials in the near future. The method used in this study follows the guidance of the World Health Organization and Organization for Economic Co-operation and Development. Rats were treated at both dosage of 0.18 grams and 0.54 grams per kilogram body weight for a period of 90 consecutive days. Results showed that TD.MV hard capsules caused no significant dose-related changes in general health status and body weight. We also found no notable changes in hematological parameters, no renal and hepatic damage through both biochemical tests and histological images. In conclusion, TD.MV hard capsules at two doses, 0.18 and 0.54 g/kg/day, did not cause sub-chronic toxicity in rats.*

**Keywords: TD.HK01, TD.MV, cardiovascular disease, sub-chronic toxicity.**

## I. INTRODUCTION

In the recent decades, the rising in the number of patients with cardiovascular disease, especially myocardial infarction, have threatened the global healthcare system because of its high death rate[1], and thus required more efficient with less adverse effects treatment. The treatment of myocardial infarction included acute management and long-term preventive management, is vital and recommended for all patients with high CVD risks.[2–4] Most of prescription drugs use in primary and secondary prevention of CVD (e.g. aspirin, statins) are synthetic substances with several complications for patients.[2,3] There is a growing interest in using natural products as complementary therapy in the treatment of a variety of diseases included CVD.[5–8] TD.MV hard capsule is a combination of herbal ingredients, which H. medicinalis, nattokinase, are the major components. Using separately, the above herbal products have reliable effects in CVD treatment,[5–8] reducing symptoms, recurrence and also complications for patients, promising one more efficient complementary therapy in the near future when used in combination. To date, there are no systematic scientific studies to evaluate its toxicity on experimental animal. Therefore, we decided to investigate the subchronic toxicity of TD.MV hard capsules in rats through this study.

*Corresponding author: Quang Vinh Trinh*
*Hanoi Medical University*
*Email: quangtv1511@gmail.com*
*Recieved day: 18/09/2020*
*Accepted day: 13/10/2020*

## II. SUBJECTS AND METHODS

### 1. Plant materials

Each capsule TD.HK01 (TD.MV) contains 200mg Nattokinase, 200mg Hirudo medicinalis, 100 mg Medicinal plants dry extract (containing: Paeoniae alba, Glycyrrhizae uralensis,

Codonopsis pilosula, Eucommiae ulmoides Oilv., Angelicae pubescentis,Angenicae sinensis, Archiranthis bidentae, Ledebouriella divaricate, Ramulus cinamomi, Rehmannia glutinosa Libosch, Panasis notoginseng F., Ramunlus loranthi, Gentiana macrophylla, Herba Asari sieboldi, Rhizoma Ligustici wallichii); Excipients are (calcium carbonate, talc, magnesium stearate, sodium benzoate).

TD.HK01 (TD.MV) was supplied by Sao Thai Duong Joint Stock company. This capsule is dissolved in water before oral administration.

**2. Experimental animals**

Healthy Wistar albino rats weighing between 180 and 200 grams were obtained

the animal center of Dan Phuong, Ha Noi. The animals were acclimatized for 7 days to laboratory conditions prior to the initiation of the study. They were maintained for 12 hours in light and dark cycle in a well ventilated cage, with free access to food and water ad libitum.

**3. Experimental design**

Sub-chronic toxicity study were carried out according to the guidance of the World Health Organization and Organization for Economic Co-operation and Development 9.

A total of thirty Wistar albino rats were divided into three groups of ten animals:

- Group 1 (control group): orally administered 1 ml sterile distilled water per 100g body weight daily;

- Group 2 (treated group): orally administered TD.MV at 0.18 grams per 1kg bodyweight daily;

- Group 3 (treated group): orally administered TD.MV at 0.54 grams per 1kg bodyweight daily

TD.MV were orally administered daily for a period of 90 consecutive days by oral gavages.

Blood with EDTA was used immediately for the determination of hematological parameters (total red blood cells, hematocrit, hemoglobin concentration, total white blood cells and platelet count). Standardized diagnostic kits of Hospitex Diagnostics (Italy) and DIALAB GmbH (Austria), were used for the determination of the following biochemical parameters: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin, albumin, total cholesterol and creatinine. All serum biochemistry was performed using biochemical analyzer Erba Chem. Hematological analysis was performed using automatic hematological analyzer Exigo – Boule Medical AB.

At the end of the experiment, after blood sample collection, kidney and liver were removed, cleaned with saline solution and preserved in 10% formalin for histopathology examinations.

**4. Statistical analysis**

Data were analyzed using Microsoft Excel software version 2016. The data were expressed as the mean ± standard deviation (SD) and statistical analysis was carried out employing student's T-test. The p-value < 0.05 was considered to be statistically significant.

## III. RESULTS

**1. Effect on general status**

After the experiment period, all rats were still alive. All rats were healthy at time of sacrifice. There was no adverse effect observed; no notable change in vital signs, skin, fur and daily behavior.

**2. Effect on total body weight**

As showed in Table 1, after 30, 60, 90 days of treatment, repeated daily oral administration of TD.MV at oral doses of 0.18 grams/kg/day and 0.54 grams /kg/day did not cause any abnormal

changes in total body weight, no significant difference observed when comparing the increase in total body weight among three groups.

**Table 1. Effect of TD.MV on total body weight**

| Days | Total body weight (gram) | | |
|---|---|---|---|
| | Group 1 | Group 2 | Group 3 |
| Before | 156.15 ± 15.16 | 161.92 ± 19.95 | 159.62 ± 13.91 |
| After 30 days | 183.08 ± 27.80 | 186.15 ± 18.05 | 196.92 ± 17.50 |
| p (before – after) | < 0.05 | < 0.05 | < 0.05 |
| After 60 days | 203.08 ± 33.51 | 190.77 ± 28.42 | 196.92 ± 28.69 |
| p (before – after) | < 0.05 | < 0.05 | < 0.05 |
| After 90 days | 207.69 ± 42.26 | 195.38 ± 27.87 | 196.92 ± 23.59 |
| p (before – after) | < 0.05 | < 0.05 | < 0.05 |

**3. Effect on hematological parameters**

**Table 2. Effect of TD.MV on total red blood cells**

| Days | Total red blood cells (T/L) | | |
|---|---|---|---|
| | Group 1 | Group 2 | Group 3 |
| Before | 8.41 ± 0.69 | 8.96 ± 1.89 | 8.30 ± 0.42 |
| After 30 days | 8.24 ± 1.01 | 7.99 ± 0.64 | 8.12 ± 0.86 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |
| After 60 days | 8.07 ± 1.09 | 7.95 ± 0.49 | 8.53 ± 0.65 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |
| After 90 days | 8.48 ± 0.79 | 8.74 ± 0.52 | 8.32 ± 0.90 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |

As shown in table 2, after 90 days of treatment, the total Red blood cells of both treated group (Group 2 & 3) have no significant difference when comparing to the control group (Group 1) (p > 0.05)

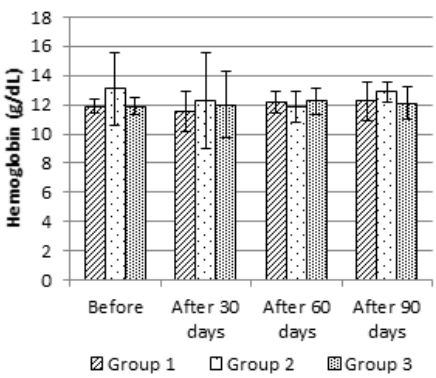


**Figure 1. Effect on hemoglobin concentration**

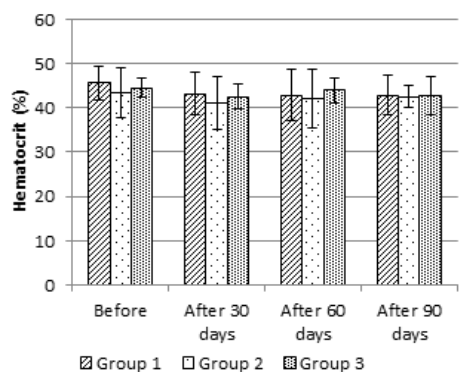


**Figure 2. Effect on hematocrit levels**

Figure 1 & 2 express the hematocrit and hemoglobin of three groups. There are not significantly different in hematocrit, hemoglobin concentration among all three groups (p > 0.05)

**Table 3. Effect of TD.MV on total white blood cells**

| Days | Total white blood cells (G/L) | | |
|---|---|---|---|
| | Group 1 | Group 2 | Group 3 |
| Before | 7.44 ± 1.44 | 7.30 ± 1.02 | 7.45 ± 0.95 |
| After 30 days | 7.92 ± 2.11 | 8.09 ± 1.65 | 7.42 ± 1.58 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |
| After 60 days | 8.88 ± 2.05 | 8.04 ± 1.32 | 8.30 ± 1.57 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |
| After 90 days | 8.17 ± 1.46 | 8.78 ± 2.33 | 8.62 ± 2.74 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |

As summarized in Table 3, white blood cells value of groups treated with TD.MV showed no difference when comparing to the control group (p > 0.05).

**Table 4. Effect of TD.MV on platelet count**

| Days | Platelet count (G/L) | | |
|---|---|---|---|
| | Group 1 | Group 2 | Group 3 |
| Before | 580.77 ± 136.82 | 550.15 ± 125.51 | 586.15 ± 110.31 |
| After 30 days | 577.00 ± 113.31 | 558.85 ± 143.79 | 615.38 ± 139.98 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |
| After 60 days | 672.08 ± 155.87 | 625.46 ± 172.97 | 605.15 ± 105.46 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |
| After 90 days | 665.08 ± 141.80 | 607.46 ± 78.23 | 655.15 ± 116.90 |
| p (before – after) | > 0.05 | > 0.05 | > 0.05 |

As in table 4, there were no significantly different in the platelet count between groups that were treated with TD.MV at 0.18 grams/kg/day and 0.54 grams/kg/day and the control group (p >0.05).

**4. Effect on liver damage**

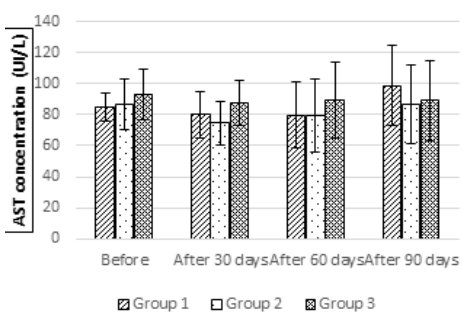


**Figure 3. Effect on AST concentration**

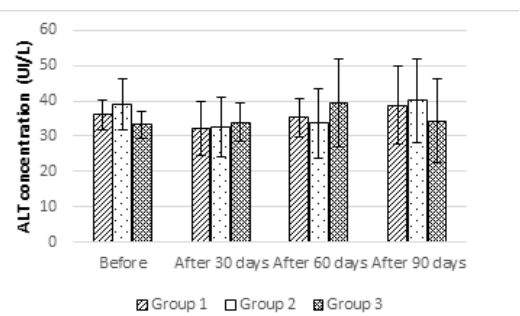


**Figure 4. Effect on ALT concentration**

As show in Figure 3 & 4, there were not significant difference in AST, ALT value between the treated groups and the control group ($p > 0.05$).

**5. Effect on liver function**

**Table 5. Effect of TD.MV on total bilirubin, albumin and total cholesterol**

| Parameters | Days | Group 1 (1) | Group 2 (2) | Group 3 (3) |
|---|---|---|---|---|
| Total bilirubin (mmol/l) | Before (a) | 13.35 ± 0.66 | 13.55 ± 0.45 | 13.38 ± 0.52 |
| | After 30 days (b) | 13.52 ± 0.37 | 13.52 ± 0.39 | 13.58 ± 0.35 |
| | After 60 days (c) | 13.30 ± 0.53 | 13.40 ± 0.32 | 13.54 ± 0.26 |
| | After 90 days (d) | 13.39 ± 0.50 | 13.47 ± 0.45 | 13.38 ± 0.44 |
| | $p_{2-1} > 0.05$, $p_{3-1} > 0.05$, $p_{b-a} > 0.05$, $p_{c-a} > 0.05$, $p_{d-a} > 0.05$ | | | |
| Albumin (g/dL) | Before (a) | 3.54 ± 0.23 | 3.74 ± 0.19 | 3.67 ± 0.34 |
| | After 30 days (b) | 3.49 ± 0.26 | 3.55 ± 0.37 | 3.55 ± 0.26 |
| | After 60 days (c) | 3.35 ± 0.41 | 3.45 ± 0.51 | 3.60 ± 0.36 |
| | After 90 days (d) | 3.38 ± 0.40 | 3.52 ± 0.42 | 3.44 ± 0.44 |
| | $p_{2-1} > 0.05$, $p_{3-1} > 0.05$, $p_{b-a} > 0.05$, $p_{c-a} > 0.05$, $p_{d-a} > 0.05$ | | | |
| Total cholesterol (mmol/L) | Before (a) | 1.66 ± 0.37 | 1.62 ± 0.32 | 1.67 ± 0.28 |
| | After 30 days (b) | 1.65 ± 0.29 | 1.53 ± 0.27 | 1.58 ± 0.26 |
| | After 60 days (c) | 1.53 ± 0.35 | 1.51 ± 0.32 | 1.78 ± 0.31 |
| | After 90 days (d) | 1.73 ± 0.28 | 1.60 ± 0.31 | 1.64 ± 0.36 |
| | $p_{2-1} > 0.05$, $p_{3-1} > 0.05$, $p_{b-a} > 0.05$, $p_{c-a} > 0.05$, $p_{d-a} > 0.05$ | | | |

As shown in table 5, serum levels of total bilirubin, albumin, total cholesterol of the treated groups using TD.MV at 0.18 grams/kg/day and 0.54 grams/kg/day were not statistically different comparing to the control group ($p > 0.05$).

**6. Effect on kidney function**

**Table 6. Effect of TD.MV on creatinine**

| Parameters | Days | Group 1 (1) | Group 2 (2) | Group 3 (3) |
|---|---|---|---|---|
| Total bilirubin (mmol/l) | Before (a) | 13.35 ± 0.66 | 13.55 ± 0.45 | 13.38 ± 0.52 |
| | After 30 days (b) | 13.52 ± 0.37 | 13.52 ± 0.39 | 13.58 ± 0.35 |
| | After 60 days (c) | 13.30 ± 0.53 | 13.40 ± 0.32 | 13.54 ± 0.26 |
| | After 90 days (d) | 13.39 ± 0.50 | 13.47 ± 0.45 | 13.38 ± 0.44 |
| | $p_{2-1} > 0.05$, $p_{3-1} > 0.05$, $p_{b-a} > 0.05$, $p_{c-a} > 0.05$, $p_{d-a} > 0.05$ | | | |

| Parameters | Days | Group 1 (1) | Group 2 (2) | Group 3 (3) |
|---|---|---|---|---|
| Albumin (g/dL) | Before (a) | 3.54 ± 0.23 | 3.74 ± 0.19 | 3.67 ± 0.34 |
| | After 30 days (b) | 3.49 ± 0.26 | 3.55 ± 0.37 | 3.55 ± 0.26 |
| | After 60 days (c) | 3.35 ± 0.41 | 3.45 ± 0.51 | 3.60 ± 0.36 |
| | After 90 days (d) | 3.38 ± 0.40 | 3.52 ± 0.42 | 3.44 ± 0.44 |
| | $p_{2-1} > 0.05$, $p_{3-1} > 0.05$, $p_{b-a} > 0.05$, $p_{c-a} > 0.05$, $p_{d-a} > 0.05$ | | | |
| Total cholesterol (mmol/L) | Before (a) | 1.66 ± 0.37 | 1.62 ± 0.32 | 1.67 ± 0.28 |
| | After 30 days (b) | 1.65 ± 0.29 | 1.53 ± 0.27 | 1.58 ± 0.26 |
| | After 60 days (c) | 1.53 ± 0.35 | 1.51 ± 0.32 | 1.78 ± 0.31 |
| | After 90 days (d) | 1.73 ± 0.28 | 1.60 ± 0.31 | 1.64 ± 0.36 |
| | $p_{2-1} > 0.05$, $p_{3-1} > 0.05$, $p_{b-a} > 0.05$, $p_{c-a} > 0.05$, $p_{d-a} > 0.05$ | | | |

The effect on creatinine, which is the most essential parameter indicating the kidney function, of TD.MV is expressed in table 6. Successive daily administration of TD.MV at both oral doses 0.18 and 0.54 grams/kg/day did not cause notable changes ($p > 0.05$) comparing to the control group.

**7. Histopathological examination**

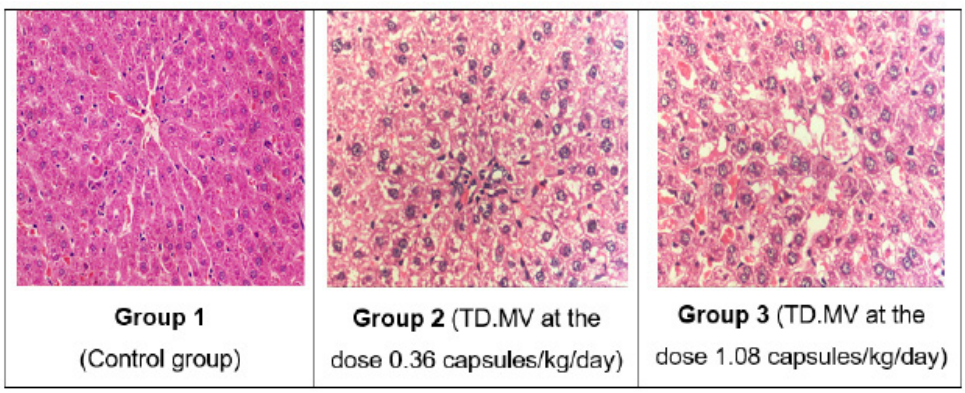


**Figure 5. Histopathological images of liver (HE × 400)**

**Macroscopic images**: We did not observe any pathological change on the major organs: brain, heart, liver, spleen, pancreas, kidney and gastrointestinal tract in all 3 groups.

**Micrographs of liver & kidney**

Histopathological examination of the control group and both treated groups indicated no significant damage on kidney normal structure without any gross pathological lesion (figure 6). Histological images of livers in the control group showed that one out of three samples have vacuoles inside the cells at medium level. The TD.MV group at 0.18 g/kg/day, 1/3 of the samples was at medium level and 2/3 at mild level. The TD.MV group at 0.54 g/kg/day, 2/3 was at medium level and 1/3 at mild level (figure 5).

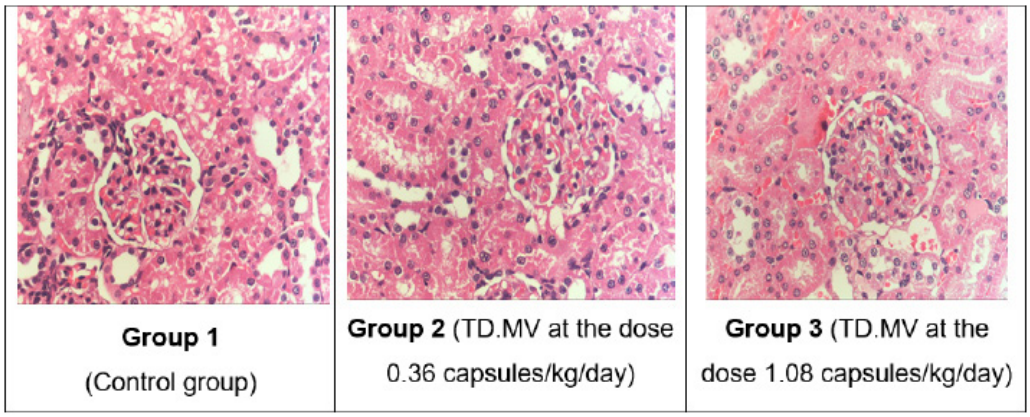


**Figure 6. Histopathological images of kidney (HE × 400)**

## IV. DISCUSSION

A sub-chronic toxicity study provides evidences about the safety on long-term use of drugs. The information on the effects of repeated oral exposure may indicate the necessity for further longer term studies.[9]

The body weight changes serve as a sensitive indication of the general health status of animals.[9] All the weight gains of animals in this study were observed that there is no abnormal change among three groups. The histopathology examination also indicates normal micro-structure of the two major organs: liver & kidney. It can be suggested that TD.MV did not cause any interference with the normal metabolism of experimental animals used in this study.

The hematologic system is one of the most susceptible organs to toxicity of compounds because of the higher proliferation. In addition, information observed from hematological tests is also important for physiological and pathological evaluation[9]. After 90 consecutive days of treatment, no significant difference in total red blood cells, hematocrit, hemoglobin concentration, total white blood cells and platelet count value indicated that the administration of TD.MV did not affect the hematological profile and blood formation process.

The liver plays a crucial role in almost metabolic pathways, not only itself but also of other tissues. The biochemical parameters, as evidence from this study, expressed non-significant changes in ALT, AST, total bilirubin, albumin and total cholesterol in both gender of experimental animals at both doses. It can be concluded that TD.MV had no deleterious effect on liver function and damage.The histology images of all three groups showed vacuoles inside the cells and some are apoptosis, which explained that the 90 days experiment was significant to develop degeneracy in normal liver cells because the experimental rats at the beginning of the study already reached their maturity. Literally, in the normal control group, one out of three liver samples showed vacuoles inside the cells at medium level. The same results observed in the two groups treated with TD.MV, the result was also 1/3 and 2/3 respectively; other samples did show the vacuoles at mild level. Furthermore, only 3 out of 10 rats were sacrificed for histology,

it could partly but not fully prove the accuracy of the assessment that one need to evaluate the histological examination simultaneously with biochemical results. These results are in accordance with ones from biochemical tests, so the above conclusion is reliable.

Kidney function analysis is essential in the determination of toxicity of drugs and compounds. Concentration of creatinine can be used as an useful index to evaluate the function of kidneys.[9] Creatinine levels observed from all three groups of animals are not significantly different. Moreover, the histological study can furnish more information related to nephrotoxicity of TD.MV. There are all normal-structure without any gross pathological lesions in kidneys indicating that TD.MV did not affect the kidney function.

In conclusion, our study determinate that no significant difference was observed concerning about blood profile and biochemistry parameters. The results were strongly confirmed by histopathological sections. Recently, there is no research worldwide about the chronic toxicity of TD.MV but several studies also have similar results: The study of Hausatu Babayi et al (2018) was conducted to investigate the sub-chronic toxicity of H. medicinalis in rats, showed that sub-chronic administration of H. medicinalis extract in 28 consecutive days at different doses 25, 50 ,100 mg/kg/day did not affect the normal behavior, mental status, body weight and other hematological parameters.[10] In addition, the study of Grafskaia et al (2019) also confirms the safety of the peptides derived from H. medicinalis.[11] In another study, B. J. Lampe and J. C. English assessed the toxicity of nattokinase derived from Bacillus subtilis var. natto in 2016. From that study, the authors concluded that the nattokinase product was well tolerated at dose level of 10 mg/kg for a duration of 4 weeks, the product was not genotoxic in vitro and did not cause adverse effects in male and female SD rats following oral administration of repeated doses of 1000 mg/kg-day.[12] Furthermore in the study, the male and female rat NOAEL were orally exposed in 90-day to nattokinase derived from B. subtilus natto at 1000 mg/kg per day, which may be biologically scaled to a human equivalent NOAEL of approximately 250 mg/kg-day 12. In addition, the study of Fu et al (2012) also has the same conclusion that after 30 days using nattokinase at dose 4 g/kg/day, there were no adverse effect and toxicity observed.[13]

## V. CONCLUSION

Based on this study, we properly conclude that TD.MV hard capsule at doses 0.18 grams/kg/day and 0.54 grams/kg/day did not produce any adverse events or evident symptoms at sub-chronic oral administration.

### Acknowledgements

The authors acknowledge the support of Professor Le Dinh Roanh, Director of the Center for Research and Early Detection of Cancer, with the histopathological section.

We also thank to Sao Thai Duong company for appropriate funding of this study.

## REFERENCES

1. Roth GA, Johnson C, Abajobir A, et al. Global, Regional, and National Burden of Cardiovascular Diseases for 10 Causes, 1990 to 2015. *J Am Coll Cardiol*. 2017;70(1):1-25.

2. Ojha N, Dhamoon AS. Myocardial Infarction. In: *StatPearls*. StatPearls Publishing; 2020.

3. Stewart J, Manmathan G, Wilkinson P. Primary prevention of cardiovascular disease: A review of contemporary guidance and literature. *JRSM Cardiovasc Dis*. 2017;6.

4. Skinner JS, Cooper A, Feder GS. Secondary prevention for patients after a myocardial infarction: summary of NICE guidance. *BMJ*. 2007;334(7603):1112-1113.

5. Chen H, McGowan EM, Ren N, et al. Nattokinase: A Promising Alternative in Prevention and Treatment of Cardiovascular Diseases. *Biomark Insights*. 2018;13.

6. Salzet M. Anticoagulants and inhibitors of platelet aggregation derived from leeches. *FEBS Lett*. 2001;492(3):187-192.

7. Abdualkader AM, Ghawi AM, Alaama M, Awang M, Merzouk A. Leech Therapeutic Applications. *Indian J Pharm Sci.* 2013;75(2):127-137.

8. Corral-Rodríguez MA, Macedo-Ribeiro S, Pereira PJB, Fuentes-Prior P. Leech-derived thrombin inhibitors: from structures to mechanisms to clinical applications. *J Med Chem*. 2010;53(10):3847-3861.

9. Pacific WHORO for the W. *Research Guidelines for Evaluating the Safety and Efficacy of Herbal Medicines*. Manila : WHO Regional Office for the Western Pacific; 1993.

10. Babayi H, Praise OO, Ajoke LF, Olayemi IK, Baba E et al. Evaluation of the effects of Leech Salivary Extract (LSE) on Haematological parameters in Rats. *J Hematol Clin Res*. 2018;2:006-014.

11. E. N. Grafskaia et al. Medicinal leech antimicrobial peptides lacking toxicity represent a promising alternative strategy to combat antibiotic-resistant pathogens. *European Journal of Chemistry*. 2019;180:143-153.

12. Lampe BJ, English JC. Toxicological assessment of nattokinase derived from Bacillus subtilis var. natto. *Food Chem Toxicol Int J Publ Br Ind Biol Res Assoc*. 2016;88:87-99.

13. Fu Y-S, Li Y-L, Zhang Y. Toxicological safety assessment on safety of nattokinase capsule. 2012;19:1714-1716.