SUBCHRONIC TOXICITY ASSESSMENTS OF SILYMAX COMPLEX CAPSULES IN WISTAR RATS

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It has recently become obvious that the prevalence of adults with diagnosed liver diseases has been rapidly increasing in Vietnam, as well as other countries, over the past two decades. There has been a current trend for researchers to discover new natural ingredients which were safe and still effective in the treatment of liver diseases. Silymax Complex was a herbal-derived product (Carduus marianus, Extractum Herba Phyllanthi urinariae, Extractum Fructus Schisandrae, Extractum Herba Adenosmatis caerulei and Curcuma longa L.) used as an oral medication. So far, the safety of this product has not been reported in Vietnam. Thus, this study was designed to assess the subchronic toxicity of Silymax Complex capsules in Wistar rats. The method used in this study followed the guidance of World Health Organization and Organization for Economic Co-operation and Development in rats with 2 oral doses of 316.8 mg/kg b.w/day and 950.4 mg/kg b.w/day for 12 consecutive weeks. As a result, Silymax Complex capsules caused no significant changes in general condition, hematological indexes, hepato-renal functions and microscopic images of livers and kidneys. In light of our findings, Silymax Complex capsules did not cause subchronic toxicity in experimental rats. Moreover, this also revealed partly the safety of Silymax Complex capsules in clinical practice.

Keywords: Silymax Complex capsules, subchronic toxicity, Wistar rats.

I. INTRODUCTION

Liver disease accounts for approximately 2 million deaths per year worldwide, 1 million due to complications of cirrhosis and 1 million due to viral hepatitis and hepatocellular carcinoma. Cirrhosis is currently the 11st most common cause of death globally and cirrhosis is within the top 20 causes of disability-adjusted life years and years of life lost, accounting for 1.6% and 2.1% of the worldwide burden.[1] Pharmacological therapy is central to the management of cirrhosis and its complications. However, these synthetic drugs caused numerous undesirable effects such as bloating, flatulence, diarrhea…[2] Therefore, one of the most urgent mission of research was the discovery of novel drugs derived from herbs which not only exhibited anti-obesity effect but also limited the side effects.

Nature has been a source of medicinal agents from the ancient times and medicinal plants, especially, have formed the basis of the wide variety of traditional medicines used in various countries worldwide.[3] The exclusive use of herbal drugs for the management of variety of ailments continues due to easy access, better compatibility and economic reasons. According to the World Health Organization (WHO), up to 80% of developing country populations

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uses traditional medicine for their primary health care. However, lack of evidence-based approaches and lack of toxicological profiling of herbal preparations form the largest concern of medicinal plants use. Thus, the evaluation of their toxicity plays a vital role in recognizing the effects, to evaluate their risk for human, and in proposing measures to mitigate the risk particularly in early clinical trials.[4]

Toxicity refers to unwanted effects on biological systems. In order to evaluate biological toxicity, it is very important to choose the correct system, since no effects 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 over a period of time); the physical form of the toxin (solid, liquid, or gas); the involved organ system (cardiovascular, nephro-, hemo-, nervous-, or hematopoietic-system); and even the genetic makeup and robustness of the target cells or organisms.[5] Subchronic systemic toxicity is defined as adverse effects occurring after the repeated or continuous administration of a test sample for up to 90 days or not exceeding 10% of the animal's lifespan.[6]

Silymax Complex capsules was a herbal-derived product including *Carduus marianus*, *Extractum Herba Phyllanthi urinariae*, *Extractum Fructus Schisandrae*, *Extractum Herba Adenosmatis caerulei* and *Curcuma longa* L. However, to date, there has been no report available on the toxicity of a combination product from these components. Therefore, the purpose of this study was to validate the subchronic toxicity of Silymax Complex capsules in experimental animals.

II. MATERIALS AND METHODS

1. The preparation of Silymax Complex capsules

Silymax Complex was manufactured by Mediplantex National Pharmaceutical Joint Stock Company, distributing by Hung Viet Trading And Pharmaceutical Joint Stock Company. Each capsule contained 140 mg dried extract of *Carduus marianus* (corresponding to 70 mg silymarin), 200 mg dried extract of *Extractum Herba Phyllanthi urinariae* (corresponding to 1400 mg *Extractum Herba Phyllanthi urinariae*), 25 mg dried extract of *Extractum Fructus Schisandrae* (corresponding to 150 mg *Extractum Fructus Schisandrae*), 50 mg dried extract of *Extractum Herba Adenosmatis caerulei* (corresponding to 714.5 mg *Extractum Herba Adenosmatis caerulei*) and 25 mg dried extract of curcuminoids from *Curcuma longa L.* This product was prepared and offered in form of capsules. The human recommended dosage was 2 capsules each time, 2 – 3 times per day.

2. Chemicals and laboratory equipments

Kits for testing enzymes and metabolites in blood: ALT (alanine aminotransferase), AST (aspartate aminotransferase), total bilirubin, albumin, total cholesterol, creatinine kits from Hospitex Diagnostics (Italy) và DIALAB GmbH (Austria) were used for Screen Master machine of Hospitex Diagnostics (Italy). Blood-testing solutions ABX Minidil LMG of ABX Diagnostics were used for Vet abcTM Animal Blood Counter. Chemicals for tests and histopathological examination.

3. Experimental animals

Healthy *Wistar* rats (180 ± 20 g) were used in this study. The animals were housed in cages (groups of ten rats/cage) under the standard conditions (temperature 25^0C ± 2^0C and relative humidity 80% ± 10%), 12 hours

dark/light time. We fed the mice with standard animal feed and allowed free access to water. They were acclimated to housing for at least 1 week prior to investigation at the Department of Pharmacology, Hanoi Medical University.

4. Subchronic toxicity study

Subchronic toxicity study were carried out according to WHO Guidance and OECD guidelines.[7,8]

The study was carried out in a continuous 12-week period. *Wistar* rats were divided into three groups of ten animals:

- Group 1 (control) was administered 1 mL distilled water/100 g b.w/day;

- Group 2 was administered Silymax Complex capsules at the dose of 316.8 mg/kg b.w/day (equivalent to human recommended dose, conversion ratio 6);

- Group 3 was administered Silymax Complex capsules at the dose of 950.4 mg b.w/kg/day (3 times as high as the dose at group 2).

Animals were treated daily by oral once a day in the morning for 12 consecutive weeks and were observed once daily to detect signs of toxicity.

The signs and indexes were checked during the study including:

- General condition consists of 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: aspartate amino transferase (AST), alanine amino transferase (ALT), total bilirubin, albumin, total cholesterol and creatinine levels.

The parameters were checked at these following times: before treatment, 4 weeks after treatment, 8 weeks after treatment and 12 weeks after treatment. At the end of experiment, all animals were subjected to a full gross necropsy. Liver and kidney of 30% rats of each group will be removed for histopathology examinations. The micro-histological examination was carried out at Center for Research and Early Detection of Cancer (CREDCA). Assoc. Prof. Le Dinh Roanh, Director of CREDCA gave results of pathological image analysis.

5. Statistical analysis

Data were analyzed using Microsoft Excel software version 2010. The levels of significance between the experimental groups and the control group were made using student's t-test and Avant-après test. Data was shown as mean ± standard deviation. All data were considered significantly at $p < 0.05$.

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ compared with the control group.

$^{\Delta}p < 0.05$, $^{\Delta\Delta}p < 0.01$, $^{\Delta\Delta\Delta}p < 0.001$ compared with the time point "before treatment"

III. RESULTS

1. 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 when compared with the control group.

2. Body weight changes

Table 1. The effect of Silymax Complex capsules on body weight changes

Time	Body weight (g)		
	Group 1	Group 2	Group 3
Before treatment	200.0 ± 49.4	204.0 ± 15.1	209.0 ± 28.8
4 weeks after treatment	232.0 ± 26.6	209.0 ± 25.1	226.0 ± 20.7
8 weeks after treatment	215.0 ± 42.0	197.0 ± 41.6	237.0 ± 30.2$^{\Delta}$
12 weeks after treatment	227.0 ± 43.5	195.0 ± 41.4	235.0 ± 24.2$^{\Delta}$

* p < 0.05, ** *p < 0.01, *** p < 0.001 compared with control group (group 1)*

$^{\Delta}$ p < 0.05, $^{\Delta\Delta}$ *p < 0.01,* $^{\Delta\Delta\Delta}$ *p < 0.001 compared with the time point "before treatment*"

Table 1 showed that after 4 weeks, 8 weeks and 12 weeks of treatment, there was an upward trend at group 1 (control group) and group 3 as compared with the time point "before treatment", but no significant difference was observed (p > 0.05). At group 2, there was no statistical change in body weight as compared with the time point "before treatment" (p > 0.05). No significant difference was observed between groups treated with Silymax Complex capsules and the control group (group 1)(p > 0.05).

3. Effect on hematological examination

Table 2. Effect of Silymax Complex capsules on hematopoietic function

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Red blood cells count (T/L)	Group 1	9.5 ± 1.0	8.4 ± 1.8	8.7 ± 1.1	8.6 ± 1.1
	Group 2	9.2 ± 1.9	7.8 ± 1.5	8.5 ± 0.7	8.4 ± 2.0
	Group 3	8.8 ± 1.0	7.9 ± 1.1	8.6 ± 0.4	7.8 ± 1.3
Hemoglobin level (g/dL)	Group 1	12.7 ± 1.4	11.7 ± 1.2	11.4 ± 1.5	11.5 ± 1.6
	Group 2	13.4 ± 1.8	11.3 ± 2.7	12.4 ± 1.0	11.4 ± 2.7
	Group 3	12.3 ± 1.3	11.1 ± 1.6	12.0 ± 0.6	10.8 ± 1.9
Hematocrit (%)	Group 1	47.8 ± 7.3	41.8 ± 9.2	42.6 ± 7.6	43.1 ± 3.9
	Group 2	48.5 ± 11.8	39.4 ± 7.1	42.1 ± 4.2	43.2 ± 10.9
	Group 3	45.4 ± 9.1	39.2 ± 5.4	43.1 ± 1.9	39.5 ± 5.0
MCV (fL)	Group 1	53.8 ± 2.1	51.7 ± 4.3	50.6 ± 4.9	52.8 ± 2.9
	Group 2	55.4 ± 1.8	52.5 ± 5.1	52.1 ± 5.8	53.0 ± 3.6
	Group 3	55.5 ± 3.0	52.7 ± 4.6	52.3 ± 5.0	53.2 ± 2.7
Platelet count (G/L)	Group 1	505.2 ± 124.3	501.6 ± 119.1	427.7 ± 99.8	528.0 ± 138.2
	Group 2	550.5 ± 83.8	519.3 ± 146.4	549.9 ± 194.9	447.7 ± 144.7
	Group 3	523.7 ± 130.3	537.2 ± 97.7	507.0 ± 85.9	442.5 ± 154.9

MCV: Mean corpuscular volume

* p < 0.05, ** *p < 0.01, *** p < 0.001 compared with control group (group 1)*

$^{\Delta}$ p < 0.05, $^{\Delta\Delta}$ *p < 0.01,* $^{\Delta\Delta\Delta}$ *p < 0.001 compared with the time point "before treatment*"

There was no significant difference in red blood cells count, hematocrit, hemoglobin level, MCV and platelet count between groups treated with Silymax Complex capsules and group 1 (p > 0.05)(Table 3).

Table 3. Effects of Silymax Complex capsules on total WBC count and WBC differentials

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Total WBC count (G/L)	Group 1	9.9 ± 2.5	8.3 ± 2.1	7.8 ± 2.3	9.3 ± 1.9
	Group 2	8.1 ± 2.1	6.5 ± 2.1	6.2 ± 2.0	8.9 ± 2.0
	Group 3	8.2 ± 1.3	6.7 ± 1.8	7.2 ± 1.4	7.9 ± 1.7
Lymphocytes (%)	Group 1	64.4 ± 6.0	67.7 ± 7.7	64.0 ± 9.1	68.1 ± 7.2
	Group 2	70.6 ± 8.9	66.0 ± 11.8	66.8 ± 7.5	62.6 ± 11.7
	Group 3	70.1 ± 7.2	67.7 ± 14.6	68.7 ± 6.7	66.0 ± 10.9
Neutrophils (%)	Group 1	25.2 ± 6.0	20.8 ± 3.6	22.7 ± 5.8	20.1 ± 7.4
	Group 2	20.0 ± 5.1	21.7 ± 7.0	23.9 ± 6.7	18.0 ± 5.8
	Group 3	19.1 ± 7.3	21.5 ± 7.7	21.9 ± 5.9	14.9 ± 5.2

WBC: white blood cells

* p < 0.05, ***p < 0.01, *** p < 0.001 compared with control group (group 1)*

$^{\Delta}$ p < 0.05, *$^{\Delta\Delta}$p < 0.01, $^{\Delta\Delta\Delta}$ p < 0.001 compared with the time point "before treatment*"

Table 3 demonstrated that at all time points, there was no significant difference in total WBC count, lymphocytes and neutrophils at groups treated Silymax Complex capsules as compared with group 1 and the time point "before treatment" (p > 0.05).

4. Effect on liver parameters

There were no significant diference in aspartate amino transferase (AST) level and alanine amino transferase (ALT) level, total bilirubin, albumin concentration and total cholesterol concentration between groups treated Silymax Complex capsules and group 1 (p > 0.05). The results were shown in table 4.

Table 4. Effects of Silymax Complex capsules on liver parameters

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
AST level (UI/L)	Group 1	77.9 ± 16.9	67.4 ± 10.6	67.5 ± 7.4	63.7 ± 14.6
	Group 2	71.0 ± 25.5	67.8 ± 24.4	72.3 ± 14.0	60.9 ± 21.3
	Group 3	67.9 ± 14.0	70.2 ± 14.2	77.8 ± 14.7	84.1 ± 28.6
ALT level (UI/L)	Group 1	46.2 ± 16.0	35.5 ± 5.6	42.0 ± 10.1	34.0 ± 11.6
	Group 2	34.9 ± 10.7	29.9 ± 9.5	44.3 ± 11.5	31.0 ± 9.8
	Group 3	36.5 ± 3.1	35.1 ± 6.7	43.1 ± 11.3	36.0 ± 11.7

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Total bilirubin (mmol/L)	Group 1	13.5 ± 0.3	13.4 ± 0.4	13.5 ± 0.5	13.3 ± 0.3
	Group 2	13.4 ± 0.3	13.4 ± 0.3	13.4 ± 0.5	13.1 ± 0.6
	Group 3	13.3 ± 0.3	13.3 ± 0.4	13.5 ± 0.5	13.3 ± 0.6
Albumin concentration (g/dL)	Group 1	2.8 ± 0.1	2.6 ± 0.4	2.5 ± 0.5	2.5 ± 0.4
	Group 2	3.0 ± 0.4	2.7 ± 0.4	2.6 ± 0.5	2.6 ± 0.5
	Group 3	2.7 ± 0.2	2.7 ± 0.4	2.5 ± 0.4	2.5 ± 0.4
Total cholesterol concentration (mmol/L)	Group 1	1.1 ± 0.2	1.0 ± 0.2	1.2 ± 0.6	1.2 ± 0.1
	Group 2	1.3 ± 0.1	1.1 ± 0.3	1.1 ± 0.3	1.2 ± 0.3
	Group 3	1.2 ± 0.1	1.0 ± 0.3	1.2 ± 0.5	1.1 ± 0.3

* $p < 0.05$, ** *$p < 0.01$, *** $p < 0.001$ compared with control group (group 1)*

$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}$ *$p < 0.01$,* $^{\Delta\Delta\Delta}$ *$p < 0.001$ compared with the time point "before treatment*"

5. Effect on kidney function

Table 5 illustrated that Silymax Complex capsules caused no significant difference in serum creatinine level between groups treated with Silymax Complex capsules and group 1 ($p > 0.05$).

Table 5. Effects of Silymax Complex capsules on serum creatinine level

Days	Creatinine level (mg/dl)		
	Group 1	*Group 2*	*Group 3*
Before treatment	0.79 ± 0.15	0.84 ± 0.17	0.82 ± 0.15
4 weeks after treatment	0.82 ± 0.19	0.80 ± 0.16	0.86 ± 0.15
8 weeks after treatment	0.85 ± 0.21	0.86 ± 0.21	0.83 ± 0.17
12 weeks after treatment	0.84 ± 0.20	0.82 ± 0.18	0.71 ± 0.10

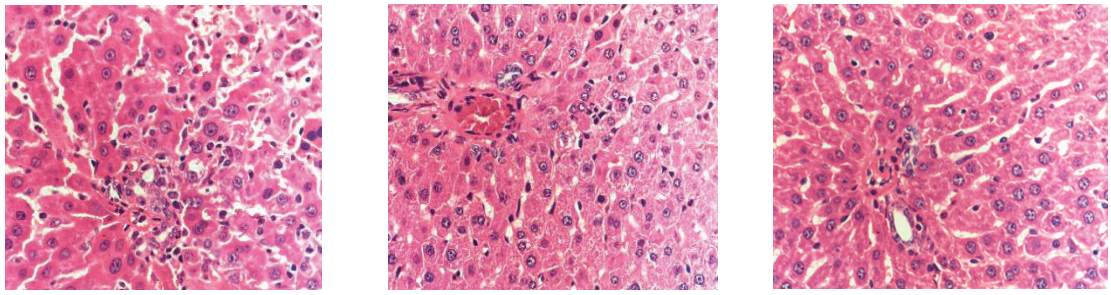
* $p < 0.05$, ** *$p < 0.01$, *** $p < 0.001$ compared with control group (group 1)*

$^{\Delta}$ $p < 0.05$, $^{\Delta\Delta}$ *$p < 0.01$,* $^{\Delta\Delta\Delta}$ *$p < 0.001$ compared with the time point "before treatment*"

6. Histopathological examination

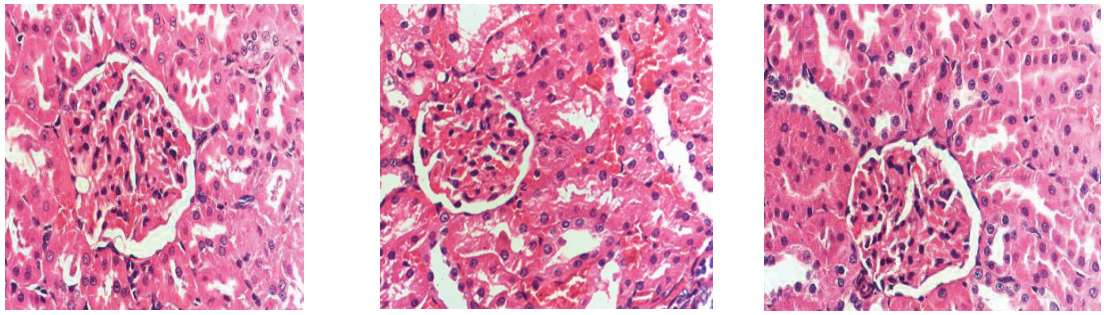
No gross lesion or change in size was observed when subjected all experimental rats to a full gross necropsy which examination of the hearts, livers, lungs, kidneys and abdominal cavities.

There was no significant difference in histopathological examination of liver and kidney between groups treated with Silymax Complex capsules and control group after 12 weeks of treatment (figure 1 and 2).



Group 1 Group 2 Group 3

Figure 1. Histopathological images of liver (HE × 400)



Group 1 Group 2 Group 3

Figure 2. Histopathological images of kidney (HE × 400)

IV. DISCUSSION

Subchronic toxicity of Silymax Complex capsules

Toxicity is the degree to which a substance can harm human or animals. Toxicity can refer to the effect on a cell (cytotoxicity), an organ (e.g. renal or liver toxicity), or the whole organism. To determine the safety of drugs and plant products for human use, toxicological evaluation is conducted in various experimental animal models to predict toxicity and to provide guidelines for selecting 'safe' therapeutic doses in humans. A subchronic toxicity study provides information on the effects of repeated oral exposure and can indicate the need for further longer term studies.[7,10] Subchronic studies assess the undesirable effects of continuous or repeated exposure of plant extracts or compounds over a portion of the average life span of experimental animals, such as rodents. Specifically, they provide information on target organ toxicity.[11]

The body weight changes serve as a sensitive indication of the general health status of animals.[11] Weights were observed in all animals treated with Silymax Complex capsules. It can be stated that Silymax Complex *capsules* did not interfere with the normal metabolism of animals as corroborated by the nonsignificant difference from animals in the distilled water control group.

The hematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in man and animals. 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.[7,10] After 4 weeks, 8 weeks and 12 weeks of the treatment, there were no significantly differences in total red blood cells, hematocrit, hemoglobin level, platelet count, total WBC count and WBC differentials between groups treated Silymax Complex capsules with control group, so it can be concluded that the administration of Silymax Complex capsules did not affect the hematological profile and blood formation process.

Analysis of kidney and liver functions is very important 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 carried out to evaluate the possible alterations in hepatic and renal functions influenced by the plant products.[12] The liver releases AST, ALT and an elevation in plasma concentration of these enzymes is an indicator of liver damage.[7] There was no subtantial change in AST level and ALT level between the group treated Silymax Complex capsules and the control group. These results indicated that Silymax Complex capsules had no deleterious effect on liver function.

Creatinine level can be used in describing the function of the kidneys.[10] No significantly differences were observed in blood biochemical parameters between control group and groups treated Silymax Complex capsules at various dose levels ($p > 0.05$). Thus, Silymax Complex capsules did not affect the liver and kidney function.

The histopathological examination revealed the alteration in cell structure when viewed under the light microscope. Further histological study could furnish more information regarding the hepatotoxicity and nephrotoxicity of Silymax Complex capsules. Our study showed that there was no significant difference in histopathological examination of the liver and kidney between groups treated Silymax Complex capsules and the control group.

Overall, the findings of this study indicated that no significant differences were observed in blood profile, biochemistry parameters and histopathological observations of liver and kidney tissues between groups treated Silymax Complex capsules and the control group.

Our study was consistent with the previous report about the toxicity of some components in Silymax Complex capsules. In the 2-years study, silymarin was administered orally 570, 1180, and 2520 mg/kg for male rats and 630, 1300, and 2750 mg/kg for female. There were no significant changes in mean body weights, and no major toxicity was occurred.[13] Extract of *Curcuma longa* on repeated oral administration in either sex of the rats for 90 days did not demonstrate any significant toxic or adverse effects.[14]

V. CONCLUSIONS

Silymax Complex capsules at doses of 316.8 mg/kg b.w/day and 950.4 mg/kg b.w/day administered orally during continuous 12 weeks did not produce any toxic signs or evident symptoms of subchronic toxicity in Wistar rats.

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