SUBCHRONIC TOXICITY EVALUATION OF DIATARIN IN EXPERIMENTAL ANIMALS

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Diatarin was a product of the Ipharm Vietnam Pharmaceutical Joint Stock Company. So far, the safety of Diatarin has not been reported yet. Thus, this study was to investigate the subchronic oral toxicity of Diatarin at doses of 163.2 mg/kg b.w/day and 489.6 mg/kg b.w/day in rats during consecutive 12 weeks following the recommendation of WHO and OECD. The results revealed that after 12 weeks of exposure, Diatarin had no deleterious effects on body weight change, hematological parameters, hepato-renal functions, and micro-histopathological images of livers and kidneys. In conclusion, oral administration of Diatarin for 12 weeks did not induce the subchronic toxicity regarding to body weight, hematological and biochemical parameters, histopathology of livers and kidneys.

Keywords: Diatarin, subchronic toxicity, rats.

I. INTRODUCTION

Nature has been a source of medicinal agents from ancient times. Medicinal plants were used as therapeutic alternatives, safer choices, or in some cases, as the only effective treatment.[1] The exclusive use of herbal drugs for the management of variety of ailments continues due to easy access, better compatibility and for economic reasons. According to the World Health Organization (WHO), up to 80% of developing country populations use traditional medicine for their primary health care. However, the lack of evidence-based approaches and lack of toxicological profiling of herbal preparations form the biggest concern of medicinal plant use. Thus, the evaluation of their toxicity plays a vital role in recognizing these effects, in helping to characterize them, to evaluate their risk for human, and in proposing measures to mitigate the risk, particularly in early clinical trials. [2]

Toxicity refers to unwanted effects on biological systems. To evaluate biological toxicity, it is 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), the physical form of the toxin (solid, liquid, or gas), the organ system involved (cardiovascular, nephro-, hemo-, nervous-, or

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hematopoietic-system) and even the genetic makeup and robustness of the target cells or organisms.[3] Subchronic systemic toxicity is defined as adverse effects occurring after the repeated or continuous administration of a test sample for up to 12 weeks or not exceeding 10% of the animal's lifespan. [4,5]

Diatarin was a product applied third generation nanotechnology with the main component which was the targeted delivery system [GA (berberin - curcumin)] (the system contained berberin and curcumin attaching glycyrrhizic acid molecules to create liver cell – targeted delivery) with nanoparticles with diameters of 50-70 nm. Berberin was isolated from Coscinium fenestratum (Gaertn.) Colebr and curcumin was isolated from Curcuma longa L. Besides, Diatarin also contained rutin and quercetin isolated from Styphnolobium japonicum L. and Lagerstroemia speciosa L. Despite the widespread use of these plants in traditional medicine, there have been no report available of the safety of a combined preparation in human as well as in animals. Therefore, to ensure the safety of these combined components in a preparation, the present study aimed to evaluate the subchronic toxicity of Diatarin in experimental animals.

II. SUBJECTS AND METHODS

1. The preparation of Diatarin

Diatarin was provided by the Ipharm Vietnam Pharmaceutical Joint Stock Company. It was formulated in form of capsules and each capsule contained 250 mg the targeted delivery system [GA (berberin - curcumin] with berberin isolated from Coscinium fenestratum (Gaertn.) Colebr and curcumin isolated from Curcuma longa L, 75 mg total flavonoid (75% rutin and 25% quercetin) isolated from Styphnolobium japonicum L. and 15 mg Lagerstroemia speciosa L.

In patients with diabetes, the recommended dosage of Diatarin was 2 capsules each time, 2 - 3 times per day.

2. Experimental animals

Healthy Wistar rats of either sex, weighing 160 ± 20 grams were purchased from The Center of Experimental Animals, Dan Phuong, Ha Tay. The animals were housed in cages (groups of ten rats/cage) in a room with access to standard certified rodent diet and water ad libitum. They were acclimated to housing in the laboratory of the Department of Pharmacology, Hanoi Medical University for 5 – 7 days before the study period.

3. Methods

Subchronic toxicity study was carried out according to WHO Guidance6 and OECD guidelines.[5,6]

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 applied 1 ml distilled water/100g b.w/day by oral route of administration.

- Group 2 was applied Diatarin at the dose of 163.2 mg/kg b.w/day (equivalent to the human recommended dose, conversion ratio 6);

- Group 3 was applied Diatarin at the dose of 489.6 mg/kg b.w/day (3 times as high as the dose at group 2).

Animals were treated daily by the oral route of administration once a day in the morning for successive twelve weeks and observed once daily to detect signs of toxicity.

The signs and some parameters were examined during the study including: General condition consists of mortality and clinical signs; body weight changes; Hematopoietic functions: red blood cells (RBC), hemoglobin (HGB), hematocrit, total white blood cells (WBC), WBC differentials, platelet count; Serum biochemistry:

aspartate aminotransferase (AST), alanine aminotransferase (ALT), total bilirubin, albumin, total cholesterol, glucose level, and creatinine levels.

The parameters were checked at the time points: before treatment, four weeks after treatment, eight weeks after treatment, and twelve weeks after treatment. At the end of the experiment, all animals were subjected to a full gross necropsy. Livers and kidneys of 30% rats in each group will be collected for histopathology examinations.

4. Statistical analysis

Data were analyzed using Microsoft Excel software version 2010 and statistical analysis was carried out employing student's t-test and Avant-après test. Data are shown as mean ± standard deviation.

III. RESULTS

1. General condition

During the experiment, animals at three groups had normal locomotor activities, good feedings, bright eyes, smooth feathers, and dry feces.

2. Bodyweight changes

Table 1. The effect of Diatarin on body weight changes

Time	Body weight (g)		
	Group 1	**Group 2**	**Group 3**
Before treatment	184.00 ± 24.59	184.00 ± 36.58	187.00 ± 34.66
4 weeks after treatment	225.00 ± 46.67	229.00 ± 42.54	218.00 ±27.41
p (before - after)	**< 0.05**	**< 0.01**	**< 0.01**
8 weeks after treatment	245.00 ± 52.76	253.00 ± 58.13	244.00 ± 26.75
p (before - after)	**< 0.001**	**< 0.01**	**< 0.001**
12 weeks after treatment	278.00 ± 38.24	296.00 ± 54.81	285.00 ± 31.71
p (before - after)	**< 0.001**	**< 0.001**	**< 0.001**

Table 1 shows that after 4, 8 and 12 weeks of treatment, the bodyweight of rats at three groups increased significantly as compared with the bodyweight before treatment. At all time points, there was no significant change in body weight between groups treated Diatarin and the control group.

3. Effect on hematological parameters

There were no significant differences in the number of red blood cells, hematocrit, hemoglobin level, MCV, platelet count, total WBC count, and WBC differentials between groups treated Diatarin and control group, between time points "before treatment" and "after treatment" ($p > 0.05$) **(Table 2 and Table 3).**

Table 2. Effect of Diatarin 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	9.75 ± 0.71	9.84 ± 0.83	9.87 ± 0.95	9.60 ± 0.87
	Group 2	9.27 ± 0.80	10.31 ± 1.37	10.24 ± 1.38	9.50 ± 1.24
	Group 3	9.48 ± 0.59	9.77 ± 0.69	10.07 ± 0.68	9.51 ± 0.78
	p	> 0.05	> 0.05	> 0.05	> 0.05
Hemoglobin level (g/dL)	Group 1	13.50 ± 0.85	13.31 ± 1.37	12.74 ± 1.06	12.69 ± 1.45
	Group 2	14.15 ± 1.25	13.88 ± 0.90	13.23 ± 2.41	12.93 ± 4.40
	Group 3	14.13 ± 0.93	13.11 ± 0.77	13.52 ± 1.53	13.03 ± 1.51
	p	> 0.05	> 0.05	> 0.05	> 0.05
Hematocrit (%)	Group 1	51.86 ± 2.26	49.50 ± 3.19	49.76 ± 4.17	48.77 ± 4.33
	Group 2	51.46 ± 3.00	48.72 ± 7.24	51.76 ± 8.39	49.20 ± 4.23
	Group 3	51.83 ± 3.72	48.10 ± 3.47	52.80 ± 6.64	47.75 ± 3.84
	p	> 0.05	> 0.05	> 0.05	> 0.05
MCV (fL)	Group 1	53.60 ± 1.17	52.40 ± 2.76	51.40 ± 3.57	51.60 ± 4.36
	Group 2	53.70 ± 2.16	51.00 ± 2.79	51.00 ± 2.79	51.10 ± 3.45
	Group 3	52.88 ± 4.03	50.60 ± 1.71	50.50 ± 1.84	50.20 ± 3.22
	p	> 0.05	> 0.05	> 0.05	> 0.05
Platelet count (G/L)	Group 1	567.20 ± 09.16	584.90 ± 96.38	654.90 ± 102.76	628.10 ± 110.43
	Group 2	624.60 ± 59.69	664.60 ± 134.46	600.70 ± 165.03	610.40 ± 119.4
	Group 3	587.60± 137.32	672.70 ± 110.81	676.10 ± 85.78	645.10 ± 119.02
	p	>0.05	>0.05	>0.05	> 0.05

Table 3. Effect of Diatarin 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	9.43 ± 2.37	9.85 ± 2.22	9.24 ± 2.30	9.81 ± 1.10
	Group 2	9.74 ± 1.59	11.45 ± 2.31	9.89 ± 2.20	10.94 ± 2.20
	Group 3	10.84 ± 2.17	11.81 ± 2.25	11.11 ± 1.80	10.06 ± 1.68
	p	> 0.05	> 0.05	> 0.05	> 0.05
Lymphocytes (%)	Group 1	76.89 ± 6.63	76.34 ± 8.84	73.44 ± 5.39	74.09 ± 6.68
	Group 2	76.86 ± 4.88	72.31 ± 4.73	72.05 ± 7.62	74.94 ± 7.09
	Group 3	75.11 ± 5.45	70.56 ± 6.22	72.33 ± 5.58	76.11 ± 2.89
	p	> 0.05	> 0.05	> 0.05	> 0.05

Parameters	Group	Before treatment	4 weeks after treatment	8 weeks after treatment	12 weeks after treatment
Neutrophils (%)	Group 1	8.11 ± 1.85	8.56 ± 2.90	9.30 ± 3.00	9.59 ± 3.09
	Group 2	8.59 ± 2.35	10.36 ± 1.40	11.12 ± 3.24	8.34 ± 2.86
	Group 3	9.74 ± 3.24	10.65 ± 2.23	10.50 ± 2.58	7.84 ± 1.42
	p	> 0.05	> 0.05	> 0.05	> 0.05

4. Effect on liver parameters

There were no significant differences in total bilirubin, albumin concentration, total cholesterol concentration, and glucose level between groups treated Diatarin and the group 1 (p > 0.05).

In terms of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) level, at the dose, there was no significant difference between the group of treated Diatarin at the dose of 489.6 mg/kg/day and the control group, between time points before treatment and after treatment. After four weeks and 8 weeks of treatment, AST level at group treated Diatarin at the dose of 163.2 mg/kg/day decreased substantially as compared with the time point “before treatment”. However, the AST level returned to normal after 12 weeks of treatment. The results were shown in **Table 4 and Table 5**.

Table 4. Effect of Diatarin 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	105.80 ± 29.1	87.40 ± 22.16	90.20 ± 19.15	81.50 ± 23.02
	Group 2	110.40 ± 4.22	87.70 ± 8.61**	72.90 ± 4.45***$^{\Delta}$	93.70 ± 22.23
	Group 3	108.40 ± 4.81	94.60 ± 26.20	87.60 ± 21.46	94.80 ± 25.50
ALT level (UI/L)	Group 1	49.30 ± 12.37	49.60 ± 18.73	49.70 ± 14.90	39.40 ± 12.19
	Group 2	49.90 ± 8.31	44.50 ± 4.99	42.50 ± 5.32	41.70 ± 6.06
	Group 3	50.30 ± 12.96	49.90 ± 22.26	44.90 ± 10.30	42.80 ± 10.25
Total bilirubin (mmol/L)	Group 1	13.34 ± 0.54	13.42 ± 0.40	13.40 ± 0.41	13.40 ± 0.54
	Group 2	13.34 ± 0.38	13.36 ± 0.35	13.34 ± 0.28	13.29 ± 0.60
	Group 3	13.46 ± 0.39	13.58 ± 0.44	13.44 ± 0.35	13.38 ± 0.41
Albumin concentration (g/dL)	Group 1	3.59 ± 0.24	3.43 ± 0.32	3.39 ± 0.30	3.33 ± 0.29
	Group 2	3.56 ± 0.29	3.32 ± 0.43	3.29 ± 0.50	3.60 ± 0.31
	Group 3	3.46 ± 0.30	3.29 ± 0.23	3.21 ± 0.35	3.48 ± 0.27
Total cholesterol concentration (mmol/L)	Group 1	1.81 ± 0.22	1.65 ± 0.23	1.64 ± 0.38	1.86 ± 0.39
	Group 2	1.83 ± 0.34	1.54 ± 0.19	1.67 ± 0.34	1.85 ± 0.32
	Group 3	1.64 ± 0.32	1.66 ± 0.18	1.64 ± 0.41	1.60 ± 0.24

Table 5. Effect of Diatarin on glucose level after 12 weeks of treatment

Group	Glucose level (mmol/l)	p compared with group 1
Group 1	3.72 ± 0.66	
Group 1	4.29 ± 0.82	> 0.05
Group 2	4.21 ± 0.69	> 0.05

5. Effect on kidney function

Table 6 demonstrates that after treatment, Diatarin caused no significant differences in serum creatinine levels between the control group and the group of treated Diatarin (p > 0.05).

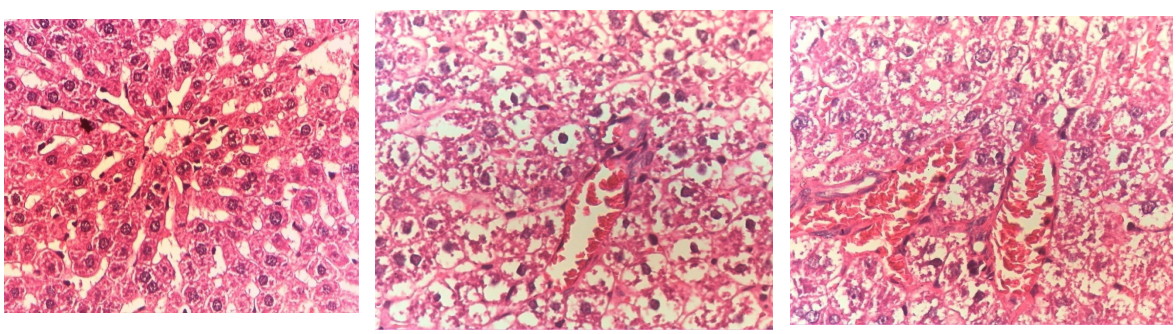
Table 6. Effect of RA on serum creatinine level

Days	Creatinine (mg/dl)			p (t- test Student)
	Group 1	Group 2	Group 3	
Before treatment	0.81 ± 0.22	0.72 ± 0.14	0.83 ± 0.18	> 0.05
After 4 weeks	0.88 ± 0.18	0.82 ± 0.14	0.88 ± 0.15	> 0.05
p (before – after)	> 0.05	> 0.05	> 0.05	
After 8 weeks	0.72 ± 0.11	0.75 ± 0.14	0.81 ± 0.17	> 0.05
p (before - after)	> 0.05	> 0.05	> 0.05	
After 12 weeks	0.74 ± 0.13	0.76 ± 0.12	0.76 ± 0.10	> 0.05
p (before – after)	> 0.05	> 0.05	> 0.05	

6. Histopathological examination

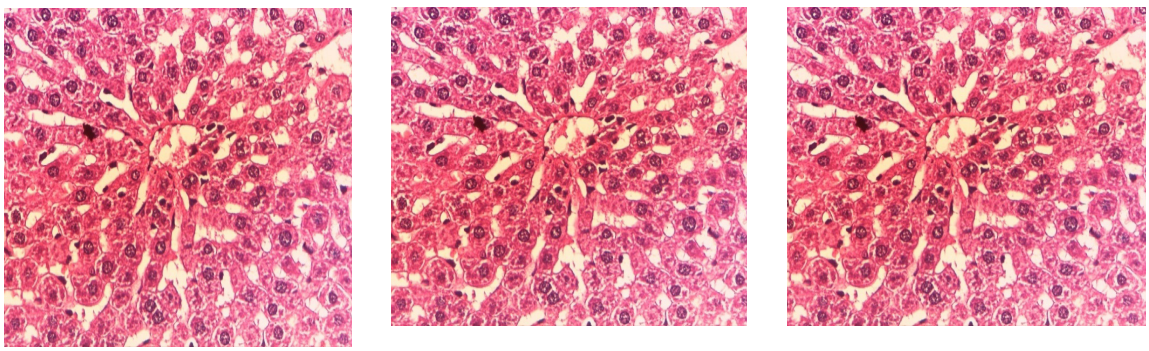
No gross lesions or changes in size observed when subjected all experimental rats to a full gross necropsy, which examined the hearts, livers, lungs, kidneys and abdominal cavities.

In terms of the micro-histopathological study of liver and kidney, there was no significant difference between the group of treated Diatarin and the control group after twelve weeks of treatment **(Figures 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 Diatarin

Toxicity is the degree to which a substance can harm humans or animals. Toxicity can refer to the effect on a cell (cytotoxicity), an organ (e.g. renal or liver toxicity), or the whole organism.[8] To determine the safety of drugs and plant products for human use, toxicological evaluation is carried out in various experimental animal models to predict the 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.[6,9] 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.[10]

The changes in bodyweight are the most basic index to reflect toxicity to organs and systems and also reflect the combined effects of xenobiotics on the body.[10] For all experimental animals, general signs should be observed daily and body weight should be measured periodically.[9] It can be stated that Diatarin did not interfere with the normal metabolism of animals as corroborated by the nonsignificant difference from animals in the control group (group 1).

The blood circulatory system performs important functions, for example, delivering oxygen to all body tissues, maintaining vascular integrity, providing necessary immune factors for host defense reaction, and so on. The hematopoietic system is one of the most sensitive targets of toxic compounds and is an important index of physiological and pathological status in humans and animals.[6,9] After 4 weeks, 8 weeks, and 12 weeks of treatment, there were no significant changes in the number of blood cells and platelet; hematocrit, hemoglobin level and WBC differentials between groups treated Diatarin and the control group. So Iit can be concluded that the administration of Diatarin did not affect the hematological profile and blood formation process.

Analysis of the kidney and liver plays a vital role 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.[11] The changes of serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) contents is a sensitive index to reflect the degree of liver cell damage. When the chronic liver injury happened, AST and ALT would be released from the injury of the liver cells, resulting in an increase in the serum.[8] Creatinine levels can be used in describing the function of the kidneys.[9] At the group treated Diatarin at the dose of 163.2 mg/kg/day, AST level declined substantially as compared with the time point "before treatment" but the AST level was still in the normal range. Nonsignificant differences in other blood biochemistry parameters at the control group and groups treated Diatarin were presented between groups treated Diatarin and control group. Thus, Diatarin did not affect the liver and kidney function in the rats.

In various organs, the liver and kidney are have strong for the drug's affinity and are conducive to the elimination of the drug, but also have a certain role in the accumulation. The histopathological examination would reveal anyed the alteration in cell structure when viewed under the light microscope.[11] Our study showed that there was no alteration in the cell structure of the liver and kidney betweengroup treated Diatarin and the control group when viewed under the light microscope.

These results were consistent with the study from previous reports about the toxicity of components in Diatarin. The repeated administration of curcuminoid-essential oil complex for 90 days in Wistar rats at a dose of 1,000 mg/kg body weight did not induce any observable toxic effects, compared with control animals.[12] Synthetic curcumin did not cause mortality or toxic effects in a 90-day repeated-dose oral toxicity study at daily doses of 250, 500, or 1000 mg/kg b.w/day.[13] Besides, another study demonstrated that there are no toxicologically significant effects on 28 days of repeated oral administration of Lagerstroemia Speciosa and Allium Sativum at the doses of 62.5, 125, and 250 mg/kg body weight for a period of 28 days.[14]

V. CONCLUSION

In light of these findings, for continuous 12 weeks, Diatarin at the doses 163.2 mg/kg b.w/day and 489.6 mg/kg b.w/day did not have deleterious effects on the body weight, histopathological signs, hepato-renal function, and micro-histopathological images of liver and kidney. Further studies to ascertain the effects of Diatarin on other organs including immunotoxicity, genotoxicity, carcinogenicity and reproductive toxicity should be encouraged to fully explore the safety profile of this product.

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