A SUBCHRONIC TOXICITY STUDY OF RA LIQUID EXTRACT IN EXPERIMENTAL ANIMALS

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Arachis hypogaea L. extract is the main ingredient of RA liquid extract, a product of Military Institute of Traditional Medicine. So far, the safety of RA liquid extract has not been reported yet. Thus, this study aimed to investigatethe subchronic oral toxicity of RA at doses of 14 g/kg b.w/day and 42 g/kg b.w/day in rats within 90 consecutive days following the recommendation of WHO and OECD. The results revealed that after 90 days of exposure, RA extract had no deleterious effects on body weight change, hematological indexes, hepato-renal functions and micro-histopathological images of kidney. Compared with control group, microscopic images of liver were slightly injured at both groups treated RA. Conclusively, oral administration of RA liquid extract for 90 days did not induce the subchronic toxicity with regard to body weight, hematological analysis, biochemical parameters and renal histopathology. RA liquid extract, however, caused light changes in hepatic structure and this is dose - dependent toxicity.

Key words: RA liquid extract, subchronic toxicity, rats.

I. INTRODUCTION

Nature has been a source of medicinal agents from the 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 uses traditional medicine for their primary health care. However, lack of evidence - based approaches and lack of toxicological profiling of herbal preparations form the biggest concern of medicinal plants 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. 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 organ system involved (cardiovascular, nephro - , hemo - , nervous - , or hematopoietic

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- 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 90 days or not exceeding 10% of the animal's lifespan.[4]

RA liquid extract was prepared from the natural material (Arachis hypogaea L.). Arachis hypogaea L., commonly known as peanut and a member of the legume family contains oil, proteins, minerals, vitamins, compounds of medicinal importance. Peanuts are rich in unsaturated fatty acids that contribute to beneficial health effects with regards to metabolic and cardiovascular disease conditions.[5] Despite the widespread use of Arachis hypogaea L. in traditional medicine, adequate characterization about the effects of this plant has not yet been done and there have been no reports available of the safety of this component in human as well as in animals. Therefore, in order to ensure the safety of Arachis hypogaea L., the present study aimed to evaluate the subchronic toxicity of RA liquid extract in experimental animals.

II. METHODS

1. The preparation of RA liquid extract

Arachis hypogaea L. was collected at Dong Thai commune, Ba Vi district, Hanoi. This herbal material was washed, cut into pieces, dried at 650C – 700C and then extracted 2 times: 1st time within 3 hours, 2nd time within 1.5 hours (starting from boiling time). The combined extract settled naturally for 24 hours, filtrated to collect the supernatant. The supernatant was decocted into the liquid extract with the porpotion 1:1 and bottled 250 ml. Before bottling, the liquid extract achieved Standard Basis from Hanoi Drug Cosmetic Food Quality Control Centre. The preparation of RA was produced at Pharmacy Department, Military Institute of Traditional Medicine.

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 Guidance and OECD guidelines.[6,7]

The study was carried out in a continuous 90 - day 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 RA at the dose of 14 g/kg b.w/day (equivalent to human recommended dose, conversion ratio 7);
- Group 3 was applied RA at the dose of 42 g/kg b.w/day (3 times as high as the dose at group 2).

Animals were treated daily by oral route of administration once a day in the morning for successive 90 days and 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 functions: red blood cells (RBC), hemoglobin (HGB), hematocrit, total white blood cells (WBC), WBC differentials, platelet count; Serum biochemistry: aspartate amino transferase (AST), alanine amino

transferase (ALT), total bilirubin, albumin, total cholesterol and creatinine levels.

The parameters were checked at the time points: before treatment, 30 days after treatment, 60 days after treatment and 90 days after treatment. At the end of experiment, all animals were subjected to a full gross necrospy. 30% rats of each group will be removed livers and kidneys for histopathology examinations.

4. Statistical analysis

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

III. RESULTS

1. General condition

During the experiment, animals at 3 groups had normal locomotor activities, good feedings, bright eyes, smooth feathers and dry feces.

2. Body weight changes

Table 1. The effect of RA liquid extract on body weight changes

Time	Body weight (g)		
	Group 1	**Group 2**	**Group 3**
Before treatment	143.00 ± 13.78	144.00 ± 22.21	154.50 ± 14.99
30 days after treatment	160.00 ± 14.91	170.00 ± 21.60	183.00 ± 20.58*
p (before - after)	**< 0.05**	**< 0.01**	**< 0.01**
60 days after treatment	148.00 ± 25.30	205.00 ± 34.72**	207.00 ± 26.69***
p (before - after)	**> 0.05**	**< 0.01**	**< 0.001**
90 days after treatment	165.00 ± 27.99	209.00 ± 33.48**	223.30 ± 35.28**
p (before - after)	**< 0.05**	**< 0.001**	**< 0.001**

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

Table 1 shows that after 90 days of treatment, body weight of rats at 3 groups increased significantly as compared with the body weight before treatment. After 60 days and 90 days of treatment, there was a significant growth in body weight between groups treated RA and control group.

3. Effect on hematological examination

There was no significant difference in red blood cells count, hematocrit, hemoglobin level, MCV, platelet count, total WBC count and WBC differentials between groups treated RA and group 1, between timepoint before treatment and after treatment (p > 0.05) (Table 2 and Table 3).

Table 2. Effect of RA liquid extract on hematopoietic function

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
Red blood cells count (T/L)	Group 1	8.02 ± 2.00	8.79 ± 1.28	7.57 ± 1.21	8.44 ± 0.70
	Group 2	7.16 ± 1.43	8.18 ± 0.70	8.27 ± 1.83	8.05 ± 0.93
	Group 3	7.71 ± 0.61	8.39 ± 0.78	8.99 ± 2.16	8.58 ± 1.31
	p	> 0.05	> 0.05	> 0.05	> 0.05
Hemoglobin level (g/dL)	Group 1	11.09 ± 1.96	10.32 ± 1.61	9.96 ± 1.54	11.59 ± 1.27
	Group 2	11.85 ± 1.67	11.33 ± 1.42	10.70 ± 1.89	12.05 ± 1.09
	Group 3	11.92 ± 1.22	11.29 ± 0.58	11.28 ± 2.48	11.59 ± 1.54
	p	> 0.05	> 0.05	> 0.05	> 0.05
Hematocrit (%)	Group 1	39.30 ± 3.32	42.11 ± 4.89	36.97 ± 5.93	40.37 ± 3.60
	Group 2	39.68 ± 5.36	41.76 ± 4.85	40.29 ± 7.30	39.77 ± 3.71
	Group 3	42.02 ± 3.98	42.91 ± 4.18	40.59 ± 4.92	41.55 ± 6.28
	p	> 0.05	> 0.05	> 0.05	> 0.05
MCV (fL)	Group 1	52.10 ± 2.18	51.20 ± 4.39	49.98 ± 3.15	49.87 ± 3.29
	Group 2	53.00 ± 2.49	51.00 ± 3.80	51.31 ± 1.52	50.90 ± 1.40
	Group 3	53.00 ± 2.75	51.10 ± 1.52	50.07 ± 2.64	50.49 ± 1.68
	p	> 0.05	> 0.05	> 0.05	> 0.05
Platelet count (G/L)	Group 1	544.80 ± 86.02	576.00 ± 86.31	525.80 ± 86.51	598.40 ± 82.90
	Group 2	533.60 ± 70.85	513.50 ± 99.48	609.30 ± 154.70	582.10 ± 157.70
	Group 3	553.30 ± 139.84	531.10 ± 156.84	605.00 ± 132.69	644.70 ± 168.17
	p	> 0.05	> 0.05	> 0.05	> 0.05

Table 3. Effect of of RA liquid extract on total WBC count and WBC differentials

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
Total WBC count (G/L)	Group 1	7.76 ± 1.53	7.86 ± 1.67	8.22 ± 1.73	7,54 ± 1,93
	Group 2	8.02 ± 1.88	8.35 ± 1.77	9.64 ± 1.91	9.20 ± 2.58
	Group 3	7.44 ± 2.12	8.92 ± 2.02	9.29 ± 2.14	8.86 ± 2.23
	p	> 0.05	> 0.05	> 0.05	> 0.05
Lymphocytes (%)	Group 1	75.98 ± 3.90	74.78 ± 7.07	71.95 ± 9.71	72.29 ± 9.09
	Group 2	72.79 ± 10.08	72.94 ± 8.98	73.24 ± 5.05	73.94 ± 6.45
	Group 3	74.92 ± 7.63	71.04 ± 6.36	71.27 ± 5.40	71.52 ± 6.00
	p	> 0.05	> 0.05	> 0.05	> 0.05

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
Neutrophils (%)	Group 1	6.72 ± 2.00	7.82 ± 1.92	9.74 ± 3.09	9.52 ± 2.74
	Group 2	7.31 ± 2.38	9.21 ± 2.18	9.49 ± 2.93	8.65 ± 2.61
	Group 3	7.88 ± 2.93	9.33 ± 2.88	10.02 ± 2.70	10.23 ± 3.24
	p	> 0.05	> 0.05	> 0.05	> 0.05

4. Effect on liver parameters

Table 4. Effect of RA liquid extract on liver parameters

Parameters	Group	Before treatment	30 days after treatment	60 days after treatment	90 days after treatment
AST level (UI/L)	Group 1	86.40 ± 23.75	87.20 ± 12.72	76.80 ± 18.39	87.40 ± 17.69
	Group 2	81.90 ± 22.27	95.10 ± 26.11	95.10 ± 25.92	77.50 ± 20.80
	Group 3	96.70 ± 21.40	97.00 ± 19.84	86.40 ± 14.66	88.70 ± 13.22
	p	> 0.05	> 0.05	> 0.05	> 0.05
ALT level (UI/L)	Group 1	35.70 ± 6.31	36.20 ± 3.22	38.20 ± 4.57	36.00 ± 6.82
	Group 2	38.20 ± 10.25	43.50 ± 12.29	44.00 ± 12.09	35.40 ± 9.31
	Group 3	40.20 ± 10.21	40.10 ± 10.92	39.10 ± 3.57	38.00 ± 4.83
	p	> 0.05	> 0.05	> 0.05	> 0.05
Total bilirubin (mmol/L)	Group 1	13.21 ± 0.92	13.22 ± 0.34	13.53 ± 0.37	13.48 ± 0.60
	Group 2	13.46 ± 0.37	13.45 ± 0.40	13.47 ± 0.37	13.28 ± 0.39
	Group 3	13.28 ± 0.30	13.38 ± 0.36	13.40 ± 0.33	13.54 ± 0.52
	p	> 0.05	> 0.05	> 0.05	> 0.05
Albumin concentration (g/dL)	Group 1	3.23 ± 0.89	3.44 ± 0.35	3.14 ± 0.48	3.32 ± 0.23
	Group 2	3.47 ± 0.43	3.28 ± 0.29	3.47 ± 0.39	3.18 ± 0.27
	Group 3	3.65 ± 0.85	3.15 ± 0.34	3.43 ± 0.29	3.42 ± 0.36
	p	> 0.05	> 0.05	> 0.05	> 0.05
Total cholesterol concentration (mmol/L)	Group 1	1.37 ± 0.29	1.40 ± 0.21	1.31 ± 0.32	1.58 ± 0.27
	Group 2	1.23 ± 0.28	1.44 ± 0.23	1.41 ± 0.22	1.40 ± 0.16
	Group 3	1.30 ± 0.26	1.42 ± 0.28	1.48 ± 0.22	1.49 ± 0.33
	p	> 0.05	> 0.05	> 0.05	> 0.05

There were no significant differencesin aspartate amino transferase (AST), alanine amino transferase (ALT) level, total bilirubin, albumin concentration and total cholesterol concentration between groups treated RA and the control group (p > 0.05) (Table 4).

5. Effect on kidney function

Table 5. Effect of RA on serum creatinine level

Days	Creatinine (mg/dl)			p (t - test Student)
	Group 1	Group 2	Group 3	
Before treatment	1.06 ± 0.10	1.07 ± 0.05	1.09 ± 0.14	> 0.05
After 30 days	1.03 ± 0.15	1.03 ± 0.13	1.03 ± 0.14	> 0.05
p (before – after)	> 0.05	> 0.05	> 0.05	
After 60 days	1.00 ± 0.11	1.08 ± 0.16	1.02 ± 0.21	> 0.05
p (before - after)	> 0.05	> 0.05	> 0.05	
After 90 days	1.01 ± 0.13	1.09 ± 0.19	1.08 ± 0.19	> 0.05
p (before – after)	> 0.05	> 0.05	> 0.05	

The Table 5 demonstrates that after treatment, RA caused no significant differences in serum creatinine level between the control group and groups treated RA (p > 0.05).

6. Histopathological examination

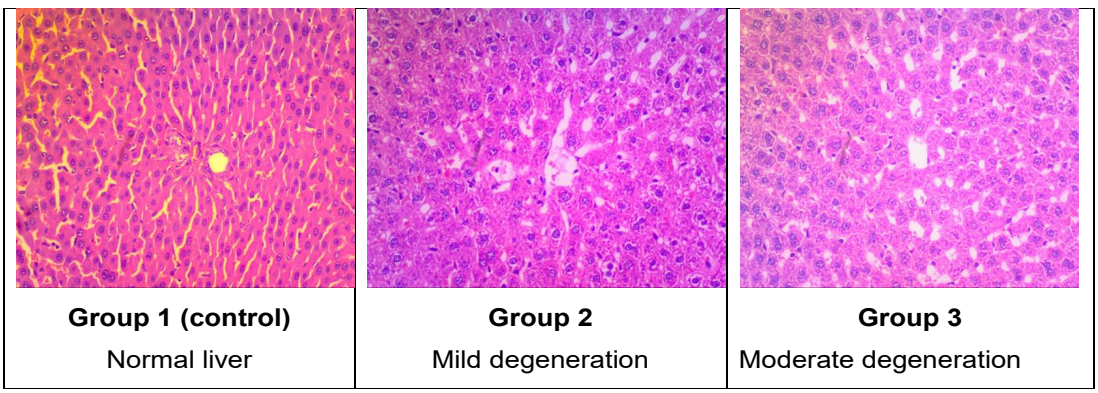

Figure 1. Histopathological images of liver (HE × 400)

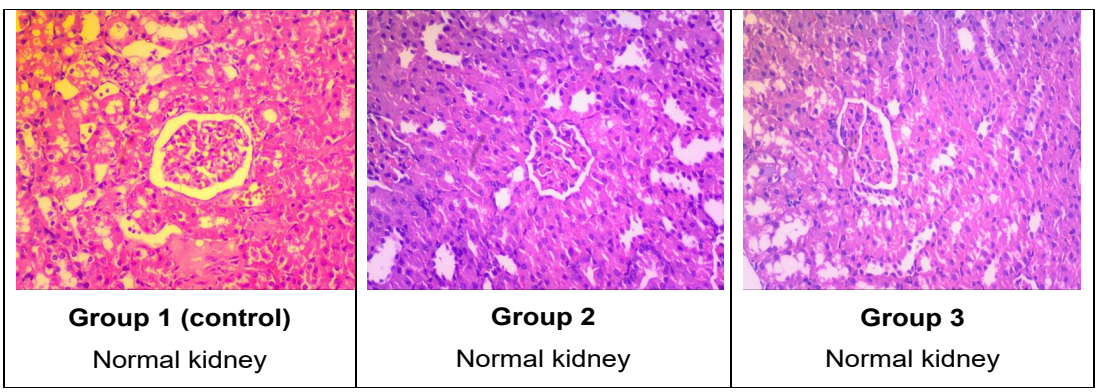

Figure 2. Histopathological images of kidney (HE × 400)

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

In group treated RA at the dose of 14 g/kg b.w/day, mild hepatic degeneration images were observed after 90 days of treatment. RA at the dose of 42 g/kg b.w/day caused the mild and moderate hepatic degeneration in experimental rats (Figure 1 and 2). In terms of micro - histopathological study of kidney, there was no significant difference between groups treated RA and control group after 90 days of treatment.

IV. DISCUSSION

Subchronic toxicity of RA liquid extract

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. To determine the safety of drugs and plant products for human use, toxicological evaluation is carried out 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.[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 of body weight 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 RA did not interfere with the normal metabolism of animals as corroborated by the nonsignificant difference from animals in the distilled water control group.

The blood circulatory system performs important functions, for example, delivering of 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 human and animals.[6,9] After 30 days, 60 days and 90 days of treatment, there were no significant changes in the number of blood cells and platelet; hematocrit, hemoglobin level and WBC differentials between groups treated RA and control group. So it can be concluded that the administration of RA liquid extract did not affect the hematological profile and blood formation process.

Analysis of 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 the increase in the serum.[8] Creatinine level can be used in describing the function of the kidneys.[9] Nonsignificant differences in the blood biochemistry level at control group and groups treated RA were presented between groups treated RA and control group ($p > 0.05$). Thus, RA did not affect the liver and kidney function in the rats.

In various organs, liver and kidney are strong for drug's affinity and conducive to the elimination of the drug, but also have a certain role in the accumulation. The histopathological

examination revealed the alteration in cell structure when viewed under the light microscope.[11] Our study showed that there was no alteration in cell structure of kidneys between group treated RA and the control group when viewed under the light microscope. Besides, RA caused the degeneration of liver cells and higher dose of RA (42 g/kg b.w/day) had more deleterious effect than the low dose of RA (14 g/kg b.w/day) on the histopathological images of liver.

V. CONCLUSION

In light of these findings, for continuous 90 days, RA liquid extract at doses 14 g/kg b.w/day and 42 g/kg b.w/day did not have deleterious effects on body weight, histopathological signs, hepato - renal function and micro - histopathological images of kidney. RA at both doses, however, have been shown to cause the liver degeneration through microscopic images and this is dose - dependent toxicity. Further studies to ascertain the mechanism of liver injury and the effects of RA 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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