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The Acute and Sub-Chronic Oral Toxicity Testing of *Cordyceps militaris* in Wistar Rats

Kittigan Suwannasaroj Passaraporn Srimangkornkaew Pakamon Yottharat and Aunchalee Sirimontaporn

National Laboratory Animal Center, Mahidol University, Nakhon Pathom 73170, Thailand

ABSTRACT *Cordyceps militaris* consists of Cordycepin, Adenosine, Polysaccharides and other compounds. For many countries including Thailand used *C. militaris* as an ingredient of a dietary supplement for many years. Whereas, toxicological testing data of *C. militaris* has less been to conduct. This study was conducted for the toxicity assessment of *C. militaris* by acute and sub-chronic oral toxicity testing in Wistar rats. The evaluation of *C. militaris* for acute oral toxicity in Wistar rats were performed at dose level 300 and 2,000 mg/kg body weight according to OECD Guidelines for the testing of chemicals 423, Acute Oral Toxicity –Acute Toxic Class Method. The results in all animals were not shown signs of toxic effects, moribund and mortality. Thus, *Cordyceps militaris* was classified in GHS (Globally Harmonised System for Classification and labeling of Chemicals) category 5 or unclassified, the LD_{50} cut off at 5,000 mg/kg body weight to infinity (∞). The sub-chronic oral toxicity of *C. militaris* was performed and the methodology was modified from OECD Guideline for testing of chemicals 408, Repeated Dose 90-Days Oral Toxicity Study in Rodent. The assessment and evaluation of toxic effects were investigated at the dose levels 5, 20 and 80 mg/kg body weight of *C. militaris* in Wistar rats and the result conclude that, the no observed adverse effect level (NOAEL) of *C. militaris* was considered to be 80 mg/kg body weight per day for Wistar rats.

Keywords: *Cordyceps militaris*, Acute oral toxicity, Sub-chronic oral toxicity, OECD Guidelines 423, OECD Guidelines 408

Corresponding author E-mail: kittigan.suw@mahidol.edu

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Introduction

The *Cordyceps militaris* has several bioactive compounds such as cordycepin, adenosine, polysaccharides, amino acid, sterols, cordycepic acid and multivitamins.[1, 2, 3, 4] These compounds exhibit various beneficial biological activities such as anticancer,[5] immunomodulatory, antioxidant,[6] antifibrotic,[7] renal protective,[8] anti-inflammatory,[9] antiangiogenetic[9, 10] and aniti-diabetic.[11] Thus, *C. militaris* is one of the most important fungi in traditional Chinese medicine that using for the treatment of asthma, bronchial and lung inflammation[12] including longevity promoting herbs.[13, 14] *C. militaris* has been sold as dietary supplements through online marketing and television in many countries including Thailand. Consequently, the safety assessment in animals should be evaluated before initial use in humans. However, the toxicity study of *C. militaris* has less been conducted.[4, 15, 16, 17] Therefore, the purpose of this study is to estimated acute oral toxicity of *C. militaris* in Wistar rats complied with OECD Guidelines for the testing of chemicals 423, Acute Oral Toxicity – Acute Toxic Class Method.[18] Then, sub-chronic oral toxicity of *C. militaris* in Wistar rats was performed to confirm the edible safety and evaluate the no-observed-adverse-effect level (NOAEL) for long term use. The methodology of this study modified form OECD Guideline for testing of chemicals 408, Repeated Dose 90-Days Oral Toxicity Study in Rodent.[19]

Materials and Methods

Cordyceps militaris powder

Cordyceps militaris powder has a bright orange color which storage at room temperature, away from heat, sunlight, oxygen and moisture. The constituent of *Cordyceps militaris* powder had cordycepin and adenosine for 0.289 and 0.037 % wt/wt, respectively and the stability was one year after manufacture date. The level of microbial contaminants monitored by laboratory of Quality Control Office, National Laboratory Animal Center, Mahidol University, Thailand for aerobic plate count and coliform were less than 300 cfu/g and 3 MPN/g, respectively.

Preparation of the Dose

For acute oral toxicity, the *C. militaris* powder were calculated at 300 and 2,000 mg/kg body weight and freshly mixed with distilled water as vehicle prior to administration. In the sub-chronic oral toxicity, the 3 dose levels (low, medium and high dose) of *C. militaris* were 5, 20 and 80 mg/kg body weight and freshly mixed with distilled water prior to administration. The constant volume was not exceeded 1 ml per 100 g of animal body weight.

Preparation of Animals:

The 50 males and 50 females of Wistar rats were used for sub-chronic oral toxicity but the acute oral toxicity used only 12 females of Wistar rats. The Wistar rats was *Rattus norvegicus*

species, Mlac: WR stain and body weight range 200 g ± 20 which were obtained from Office of Laboratory Animal Production, National Laboratory Animal Center, Mahidol University, Thailand.

Animals Husbandry Conditions

The animals were husbandry under standard conditions 12 hours light, 12 hours dark at temperature 22 ± 3°C and 30-70% relative humidity which were housed in stainless steel cages with food (082, Perfect Companions, Thailand) and 5 - 7 ppm chlorinated water ad libitum. All the animals were quarantined 1 day and acclimatized at least 5 days prior to the study. Guidelines of "Guide for the care and use of laboratory animals" were strictly followed throughout the study.[20] The study was approved by National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), Mahidol University; Thailand, code RA2017-37 and RA2017-38 for acute and sub-chronic oral toxicity testing, respectively.

Administration of Dose

The acute oral toxicity: The test substance at the dose level 300 and 2,000 mg/kg body weight was administered orally to the animal in a single dose by gavage using stainless steel stomach tube and all animals were kept for over-night (15 - 18 hours) fasting (feed but not water) prior to administration. The *Cordyceps militaris* was administered orally to 3 animal of group 1 at 300 mg/kg body weight, if no animals or one animal was shown evidence of toxicity, moribund state or mortalities, new three animals of group 2 were repeated at 300 mg/kg body weight. After that, three animals in group 3 were administrated with the next dose level (2,000 mg/kg body weight) if no animals or one animal was shown evidence of toxicity, moribund state or mortalities new 3 animals of group 2 were repeated again at 2,000 mg/kg body weight. The animal was observed after administration for toxic effect at first 30 minutes with special attention given during the first 4 hours, periodically during the first 24 hours. The time between treatment groups was determined until confident of previously dose animals, 24 hours for this study.

The sub-chronic oral toxicity: The 50 male and 50 female of Wistar rats were used and randomized into 5 groups (10 animal per sexes/20 animal per group). In group 1 - control group (vehicle control; distilled water), group 2 - low dose, group 3 - middle dose and group 4 - high dose (5, 20 and 80 mg/kg body weight of *Cordyceps militaris*, respectively) and group 5 - recovery group (once daily for 90 days at 80 mg/kg body weight of *Cordyceps militaris* and no administrated by gavage for the following 14 days). The dosage of administration to each animal was calculated based on the body weight of animal prior to administrate at a constant volume not exceed 1 ml per 100 g body weight.

Observation

The observations were included changes in skin and fur, eye and mucous membranes, respiratory, circulatory, autonomic and central nervous systems, somatomotor activity and be-

haviour pattern daily. Addition will be directed to observations of tremors, convulsions, gasping, cyanosis, vocalization, salivation, diarrhoea, lethargy, sleep and coma. The data of animal body weights, feed and drinking water consumption were measured and recorded weekly throughout the termination period.

Necropsy Examination, Hematological and Clinical Biochemistry Analysis

On the last day of each group for the study, the animals were kept overnight (15 – 18 hours) fasting and then euthanized using CO_2 inhalation.[21] Blood samples were collected via cardiac puncture and were separated 2 tubes which first tube for hematological (200 µl) and second tube has EDTA tube for clinical biochemistry analysis (800 µl). Hematological analysis was analyzed using automated analyzer (Procyte Dx™, IDEXX Laboratories, Westbrook, Maine, USA) following the parameters: Red blood cell count (RBC), Hemoglobin (HGB), Hematocrit (HCT), Mean corpuscular volume (MCV), Mean corpuscular hemoglobin (MCH), Mean corpuscular hemoglobin concentration (MCHC), Reticulocyte count (RET), Reticulocyte hemoglobin (RET – He), Platelet (PLT), Mean platelet volume (MPV), Plateletcrit (PCT), White blood cell count (WBC), Neutrophil (NEUT), Lymphocyte (LYMPH), Monocyte (MONO), Eosinophil (EO) and Basophil (BASO). For the clinical biochemistry analysis was measured by using an automated blood analyzer (Cobas®c311, Roche Diagnostics, Basel, Switzerland) that the parameters consisted of Sodium (Na), Potassium (K), Choride (Cl), Glucose (SGLU3), Cholesterol (CHO2L), Triglyceride (TRIGL), Uric acid (UA2), Blood urea nitrogen (U – BUN), Creatinine (CREA2), Total protein (TP2), Albumin (ALB2), Globulin (GLO), High – density lipoprotein (HDLC4), Low – density lipoprotein (LDLC3), Alanine amino transferase (ALTL), Aspartate amino transferase (ASTL) and Alkaline phosphatase (ALP2S).

Organ Weighting

After blood samples collection, all animals were sacrificed. The positions, shapes, sizes and colours of internal organs were evaluated. The organs was measured by using an electronic balance 4 digits (Mettler – Toledo, Columbus, Ohio, USA) and converted the weights to relative organ weights by calculating in the relative weights per 100 g animal body weight.

Analysis of Result:

Quantitative results were expressed as mean ± standard deviation. The Kolmogorov–Smirnov and Levene's test[22] were statistically analyzed the data for normality and homogeneity of variances. For parametric statistics, all data of vehicle control group and each treatment group were compared by 2–sided Dunnett or Dunnett's T3 test, using ANOVA analysis. On the other hand of non–parametric statistics, Mann Whitney U test was selected for comparison all data of the vehicle control group and each treatment group. A significantly differences were considered at 0.05 levels with SPSS® Statistic software version 18.0.0.

Results

The acute oral toxicity of *Cordyceps militaris* in Wistar rats

All tested animals were normally for consumed feed and drinking water including clinical observations and health examinations. Body weights were continued to gain throughout the study and the body weight change was shown as percentage which within range 11.49 - 20.01%. In the necropsy examinations result of all animals were not found any lesion.

The sub-chronic oral toxicity of *Cordyceps militaris* in Wistar rats

Feed, Drinking Water Consumptions and Body Weights

The feed and drinking water consumptions data as consecutive data were shown normal in all animals by means of the result were transient change and no effect on animal health, represented in Figure 1-4. The body weights of all animals were continued to gain throughout the study and the data are presented in Figure 5 and Figure 6 for male and female animals, respectively.

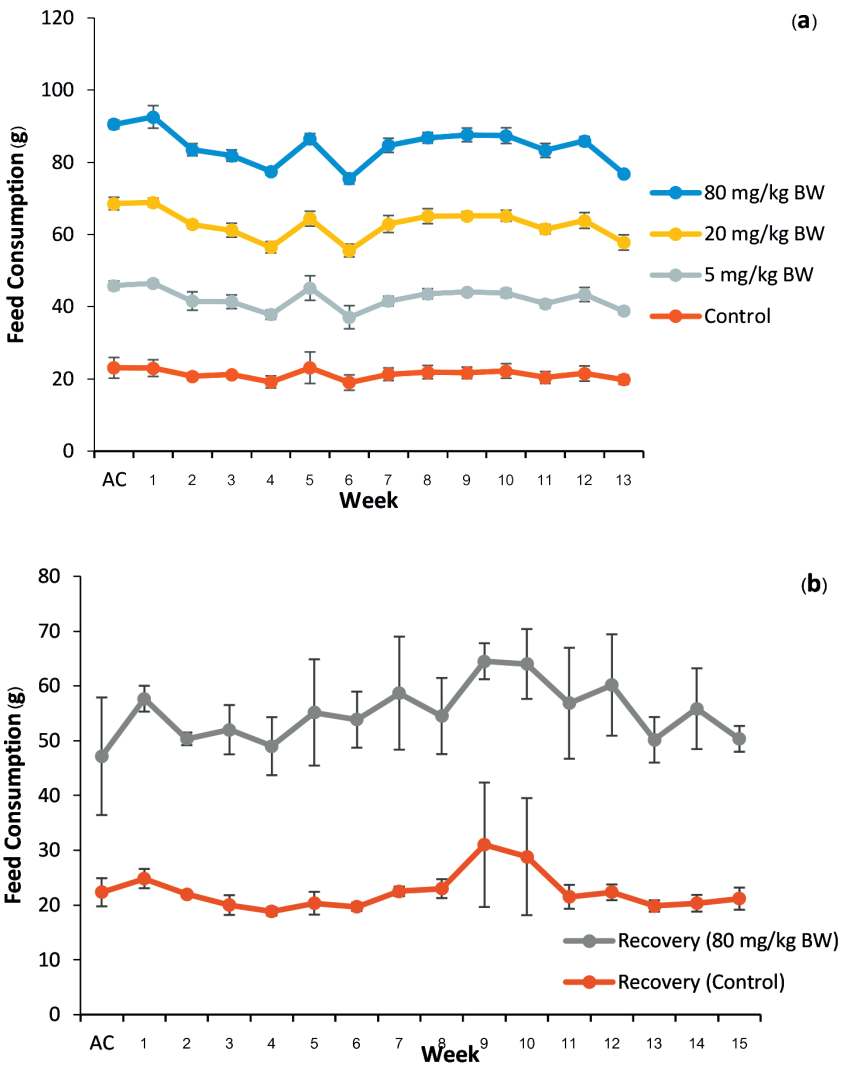

Figure 1 Effect of *Cordyceps militaris* on Feed consumption in male rats treated for 90 days. (a) Control group, Treatment group (5, 20 and 80 mg/kg BW) (b) Recovery group

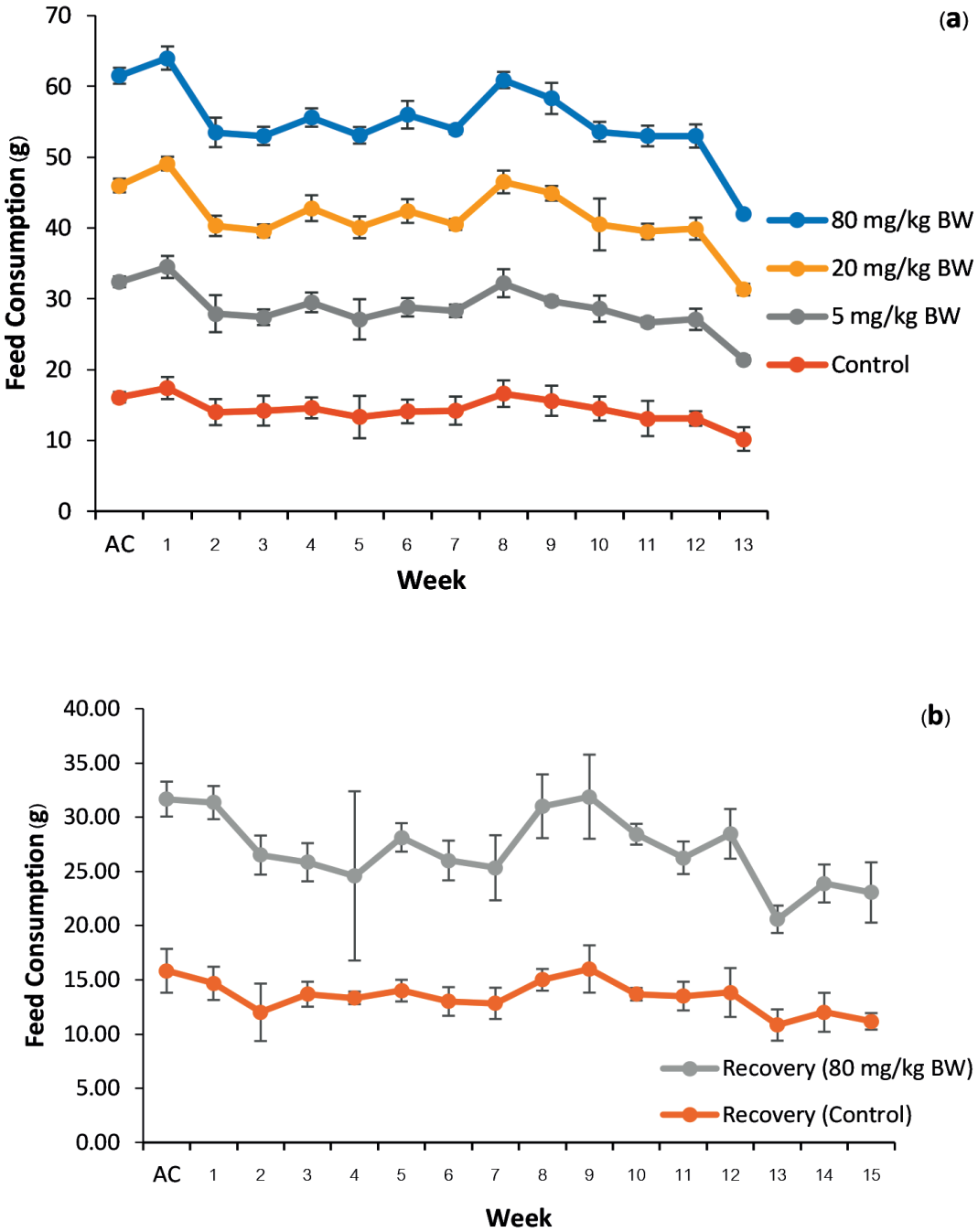

Figure 2 Effect of *Cordyceps militaris* on Feed consumption in female rats treated for 90 days. (a) Control group, Treatment group (5, 20 and 80 mg/kg BW) (b) Recovery group

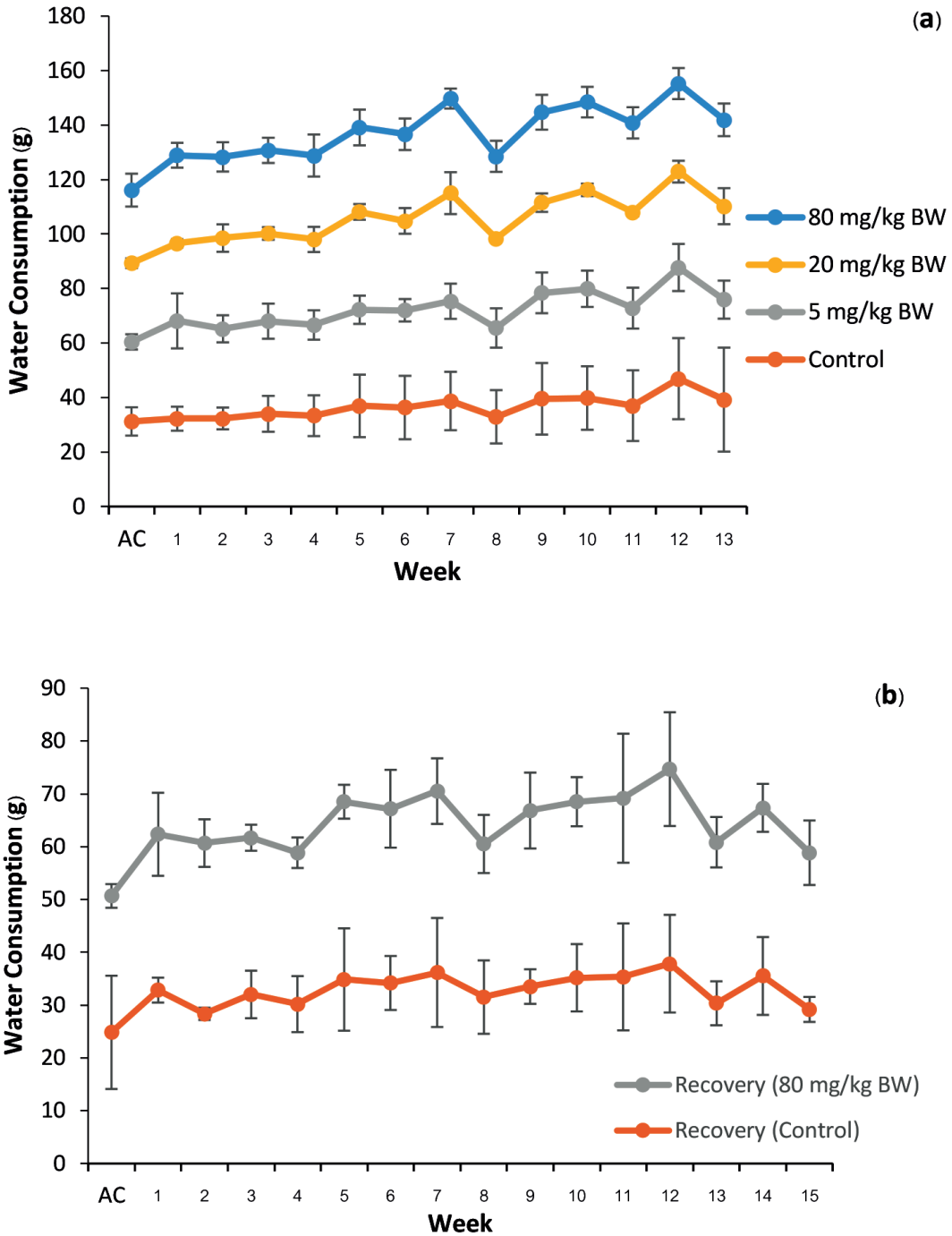

Figure 3 Effect of *Cordyceps militaris* on Water consumption in male rats treated for 90 days (a) Control group, Treatment group (5, 20 and 80 mg/kg BW) (b) Recovery group

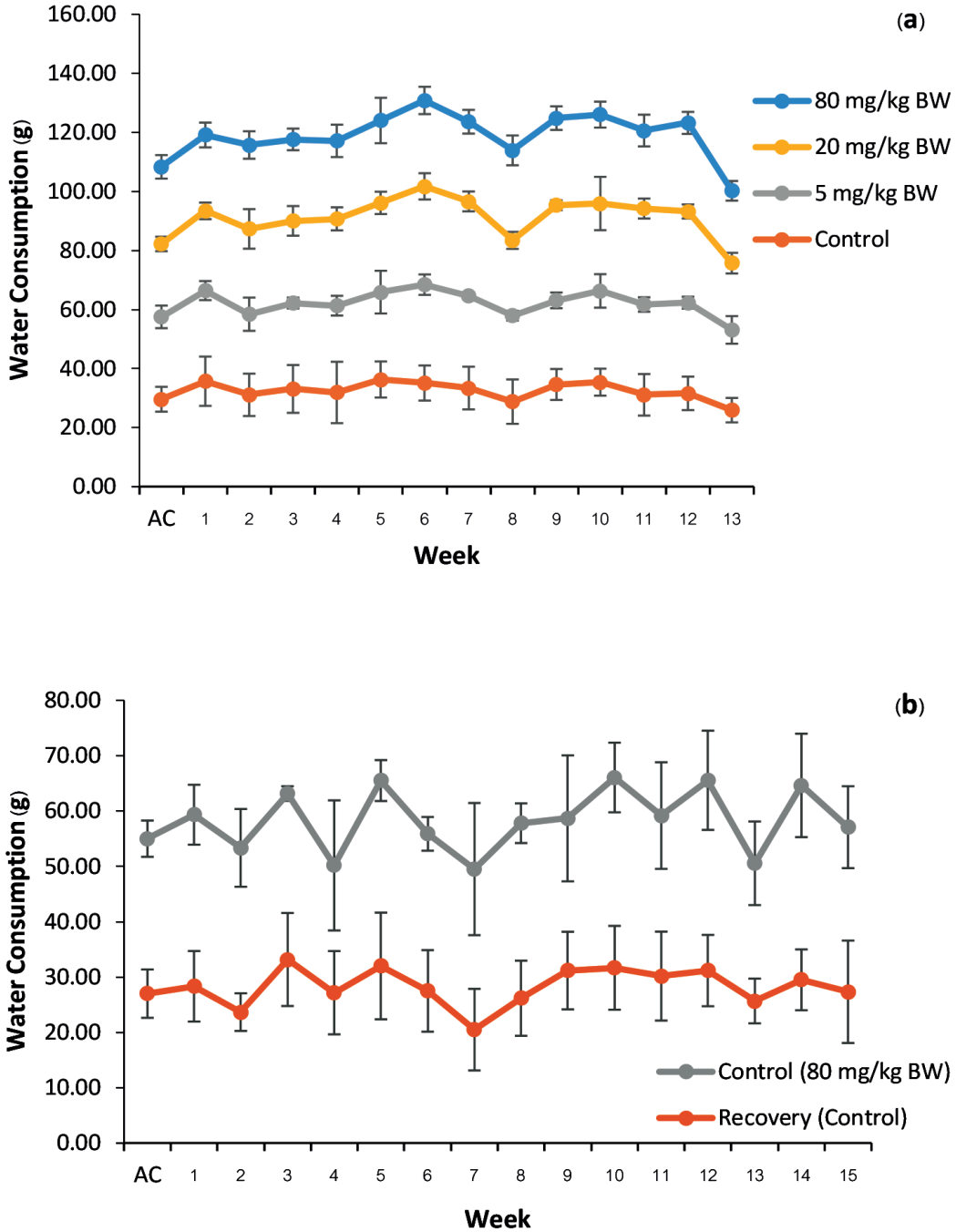

Figure 4 Effect of *Cordyceps militaris* on Water consumption in female rats treated for 90 days (a) Control group, Treatment group (5, 20 and 80 mg/kg BW) (b) Recovery group

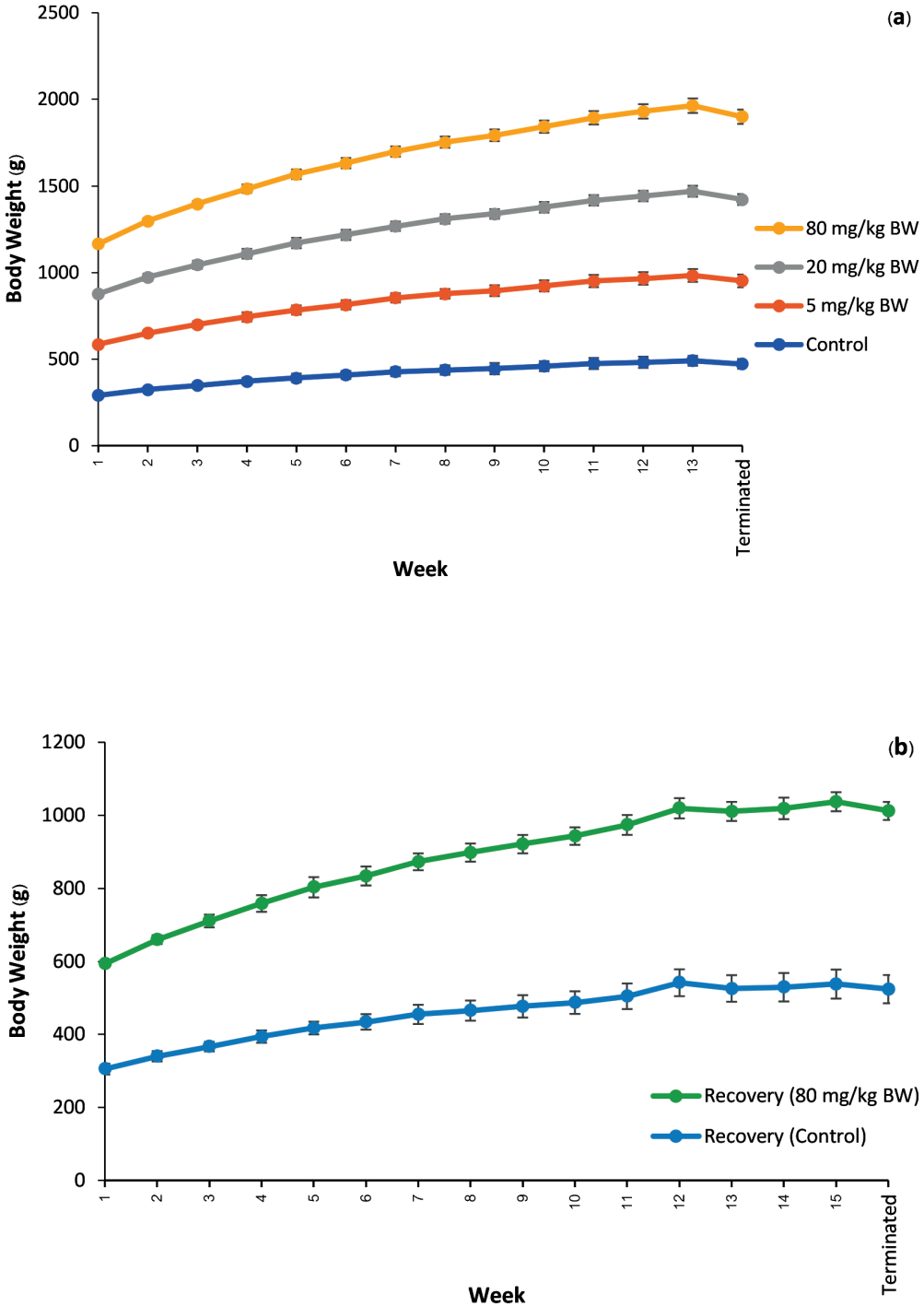

Figure 5 Effect of *Cordyceps militaris* on body weight in male rats treated for 90 days. (a) Control group, Treatment group (5, 20 and 80 mg/kg BW) (b) Recovery group

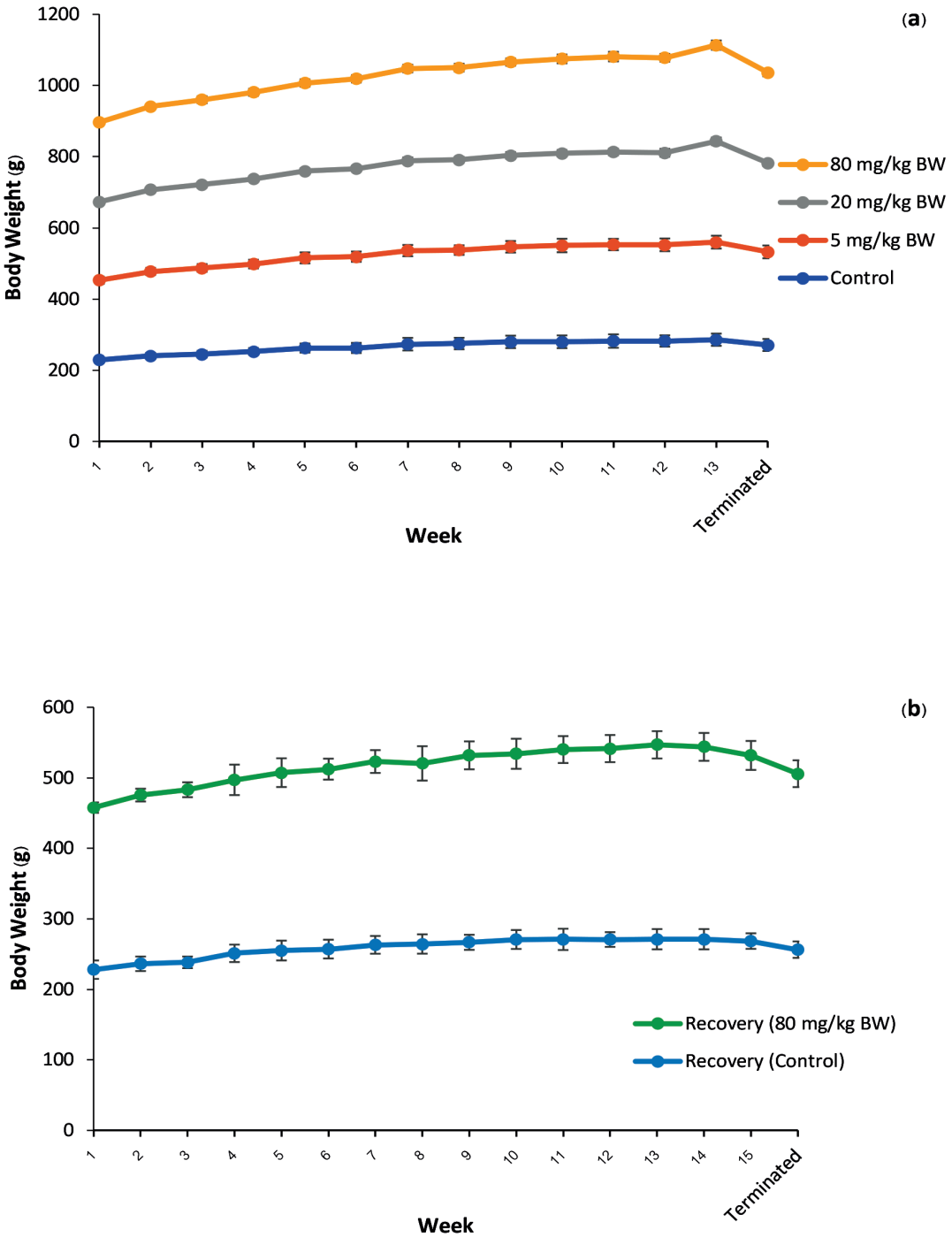

Figure 6 Effect of *Cordyceps militaris* on body weight in female rats treated for 90 days. (a) Control group, Treatment group (5, 20 and 80 mg/kg BW) (b) Recovery group

Observations

All animals were observed according to dosing period for 90 days and recovery period for 14 days which were no change in skin, fur/coat, eyes and mucous membrane, posture and response to handing. The health examinations such as respiratory pattern, social interaction, mobility and activity were normal. Moreover, all animal were not shown any neurological signs.

Necropsy Examination:

For macroscopic finding, petechial hemorrhage of the thymus was observed in control, all treatment group and control recovery group of male as well as in control and high dose group of female. Only one male in control group was observed with a subcutaneous mass on the ventral side of the neck. For abdominal cavity, in medium dose group, 1 of 10 male was observed with oval, yellow, firm, fat nodule (0.5 × 0.5 cm.) based on the right side and in low dose group, 1 of 10 female was observed with round, smooth, slightly firm, peritoneal fat mass (0.6x0.6 cm) adhesive with abdominal fat at right side based between right kidney and ovary. Besides, the accessory lobe of spleen was found in 2 of 10 females. For microscopic finding, there were no significant difference of severity grading mean between control group (Distilled water) and high dose group (80 mg/kg body weight). In addition, the other lesions which have been well known to occur spontaneously in Wistar rats of the same age were observed.

Hematological and Clinical Biochemistry Analysis

The all result of hematological and clinical biochemistry analysis that shown in Table 1 and 2, respectively. The summary result can be described following the group of dosing:

Low dose group (5 mg/kg BW); the means of WBC and LYMPH for male animal and the means of MPV and WBC for female animal were significantly different higher than control group ($p<0.05$) but the mean of NEUT and BASO for female animal were significantly different lower than control group ($p<0.05$). The mean of CREA2 in male animal was significantly different lower than control group ($p<0.05$).

Medium dose group (20 mg/kg BW); the means of HGB and PCT for male animal were significantly different lower than control group ($p<0.05$). For the female animal, the means of Na, CREA2, TP2, ALB2, GLO and ASTL were significantly different higher than control group ($p<0.05$) but the male animal has only the means of CREA2, UA2 and SGLU3 were significantly different higher than control group ($p<0.05$).

High dose group (80 mg/kg BW); the means of MPV and LYMPH were significantly different higher than control group ($p<0.05$) for both sexes animal and the mean of WBC that was significantly different higher than control group ($p<0.05$) only in male animal. Besides that, the male animal had significantly different lower than control group ($p<0.05$) for the means of NEUT and EO. For the female animal, the means of Na, U - BUN, CREA2 were significantly different

higher than control group ($p<0.05$) and the means of SGLU3 and LDLC3 were significantly different lower than control group ($p<0.05$).

Recovery group (control and 80 mg/kg BW); the means of PCT and NEUT were significantly different lower than control group ($p<0.05$) except the mean of RET – He was significantly different higher than control group ($p<0.05$) for male animal. In female animal, the means of MPV and EO were significantly different higher than control group ($p<0.05$). The mean of UA2 was significantly different lower than control group ($p<0.05$) for male animal but the mean of K in female animal was significantly different higher than control group ($p<0.05$).

Thus, all significantly different parameters of hematological and clinical biochemistry analysis were no treatment related effect except the mean of LDLC3 in high dose group (80 mg/kg body weight) of female animal was treatment related effect. Effects on LDL levels tended to significant decrease with the dose given, in the high dose group including HDL levels were not found to be significantly low. Therefore does not affect for the occurrence of Atherosclerosis which was according to the information from many journal that found of higher LDL and lower HDL levels are high risk of Atherosclerosis.[23, 24, 25]

Table 1 Hematological Analysis of control and *Cordyceps militaris* treated rat for 90 days

Sex	Parameters	Control	Mean of parameters ± standard deviation			Recovery	
			5 mg/kg BW	20 mg/kg BW	80 mg/kg BW	Control	80 mg/kg BW
Male	RBC (106/µl)	9.91±0.58	9.84±0.36	9.47±0.20	9.86±0.37	9.63±0.36	9.58±0.23
	HGB (g/dl)	17.3±0.68	17.2±0.35	16.7±0.48a	17.1±0.52	16.7±0.32	16.7±0.33
	HCT (%)	54.7±2.58	54.0±1.35	52.5±1.92	53.6±1.94	52.2±1.46	52.2±1.50
	MCV (fl)	55.2±1.20	54.9±1.63	55.4±1.33	54.5±1.15	54.2±1.01	54.5±1.32
	MCH (pg)	17.5±0.48	17.5±0.51	17.6±0.33	17.4±0.37	17.4±0.35	17.5±0.38
	MCHC (g/dl)	31.7±0.35	31.9±0.35	31.8±0.31	32.0±0.25	32.0±0.34	32.0±0.38
	RET (%)	2.78±0.50	2.66±0.37	2.85±0.31	2.51±0.24	2.23±0.44	2.07±0.20
	RET – He (pg)	19.4±0.39	19.4±0.51	19.5±0.25	19.2±0.40	19.3±0.46	19.4±0.23b
	PLT (103/µl)	916±123.75	910±52.83	820±76.25	908±102.71	855±48.20	883±75.43
	MPV (fl)	7.1±0.19	7.3±0.15	6.9±0.24	7.4±0.20a	6.9±0.23	7.1±0.23
	PCT (%)	0.65±0.08	0.66±0.04	0.57±0.05a	0.67±0.07	0.59±0.02	0.62±0.06b
	WBC (106/µl)	6.46±1.02	7.99±1.47a	6.40±1.20	8.18±1.28a	6.02±0.81	9.46±1.19
	NEUT (%)	13.3±2.69	9.0±2.17a	12.6±2.73	9.0±2.16a	14.7±3.51	9.6±1.24b
	LYMPH (%)	80.8±3.17	85.4±2.66a	80.8±2.60	85.7±2.35a	77.2±2.79	85.4±1.19
	MONO (%)	4.9±2.38	4.8±1.37	5.6±1.78	4.7±1.44	7.0±1.19	4.2±0.94
	EO (%)	0.8±0.28	0.7±0.25	0.9±0.30	0.5±0.19a	1.1±0.53	0.8±0.22
	BASO (%)	0.2±0.12	0.0±0.07a	0.1±0.09	0.1±0.07	0.0±0.09	0.0±0.05

Table 1 Hematological Analysis of control and *Cordyceps militaris* treated rat for 90 days (Continued)

Sex	Parameters	Control	Mean of parameters ± standard deviation			Recovery	
			5 mg/kg BW	20 mg/kg BW	80 mg/kg BW	Control	80 mg/kg BW
Female	RBC (106/µl)	9.19±0.30	9.38±0.50	9.20±0.62	9.65±0.65	8.96±0.47	9.36±0.71
	HGB (g/dl)	17.0±0.44	17.1±0.76	17.1±1.02	17.9±0.86	16.7±0.73	17.2±0.88
	HCT (%)	52.9±1.48	52.9±2.55	53.3±3.67	56.0±3.20	51.1±2.51	53.0±2.88
	MCV (fl)	57.6±1.25	56.4±1.05	57.9±1.08	58.1±2.14	57.1±0.90	56.8±1.97
	MCH (pg)	18.5±0.35	18.3±0.41	18.6±0.41	18.6±0.63	18.6±0.41	18.4±0.59
	MCHC (g/dl)	32.2±0.30	32.3±0.34	32.1±0.39	31.9±0.36	32.6±0.30	32.5±0.34
	RET (%)	3.25±0.42	3.30±0.65	3.41±0.42	3.41±0.94	2.40 ±0.17	1.77±0.57
	RET – He (pg)	20.3±0.44	20.2±0.58	20.6±0.61	20.5±0.60	20.1±0.49	20.4±0.57
	PLT (103/µl)	896±101.35	931±112.41	846±94.82	924±130.19	889±141.48	934±80.48
	MPV (fl)	6.9±0.20	7.3±0.28a	6.9±0.15	7.4±0.28a	6.8±0.11	7.1±0.43b
	PCT (%)	0.62±0.08	0.68±0.11	0.59±0.07	0.68±0.10	0.60±0.10	0.67±0.10
	WBC (106/µl)	5.60±0.75	7.28±1.52a	4.91±1.42	5.83±1.03	4.76±0.65	6.74±1.29
	NEUT (%)	8.2±2.29	6.2±1.21	9.1±2.91	6.0±1.60	10.2±0.91	8.6±2.16
	LYMPH (%)	87.1±2.07	87.9±3.00	85.9±2.96	88.7±1.88a	84.8±1.17	85.8±2.43
	MONO (%)	4.0±0.92	5.3±2.22	4.5±1.28	4.7±1.35	4.3±0.43	5.0±1.05
	EO (%)	0.6±0.26	0.5±0.31	0.5±0.33	0.5±0.33	0.6±0.17	1.7±2.62b
	BASO (%)	0.1±0.10	0.1±0.08	0.0±0.10	0.1±0.10	0.1±0.11	0.1±0.09

Note: [a]The mean difference is significant at the 0.05 levels of control group

[b]The mean difference is significant at the 0.05 levels of control recovery group

Table 2 Clinical Biochemistry Analysis of control and *Cordyceps militaris* treated rat for 90 days

Sex	Parameters	Control	Mean of parameters ± standard deviation			Recovery	
			5 mg/kg BW	20 mg/kg BW	80 mg/kg BW	Control	80 mg/kg BW
Male	TP2 (g/dl)	7.82±0.19	7.91±0.36	8.47±1.17	8.73±1.00	7.47±1.04	6.42±0.27
	CHO2L (mg/dl)	82.0±8.15	79.4±9.03	81.5±17.96	82.3±15.40	69.8±10.86	67.8±13.02
	TRIGL (mg/dl)	128.2±23.03	118.0±27.91	133.1±36.74	128.8±26.61	91.0±17.93	99.0±35.81
	ALTL (U/l)	94.0±62.01	69.3±20.38	96.3±40.29	86.7±26.02	76.5±17.96	70.0±24.39
	ASTL (U/l)	98.6±33.19	92.6±17.80	114.4±26.51	109.1±24.75	103.6±20.31	83.6±22.10
	ALP2S (U/l)	88±12.04	78±14.63	97±21.27	85±16.24	61±6.40	62±5.52
	ALB2 (g/dl)	5.41±0.10	5.39±0.12	5.95±0.71	5.96±0.65	4.54±0.32	4.27±0.11
	GLO (g/dl)	2.41±0.13	2.46±0.15	2.79±0.47	2.76±0.38	2.33±0.13	2.15±0.17
	CREA2 (mg/dl)	0.38±0.03	0.30±0.02a	0.43±0.05a	0.40±0.04	0.37±0.05	0.34±0.03

Table 2 Clinical Biochemistry Analysis of control and *Cordyceps militaris* treated rat for 90 days (Continued)

Sex	Parameters	Control	Mean of parameters ± standard deviation			Recovery	
			5 mg/kg BW	20 mg/kg BW	80 mg/kg BW	Control	80 mg/kg BW
	U - BUN (mg/dl)	23.0±1.57	25.2±4.20	23.9±2.88	22.6±2.12	18.9±1.68	19.8±3.62
	UA2 (mg/dl)	6.5±0.85	5.9±1.11	8.6±1.23a	8.0±2.08a	6.6±0.97	5.2±0.45b
	SGLU3 (mg/dl)	334.5±52.00	323.0±48.07	439.1±86.04a	336.1±60.02	326.5±29.91	289.5±37.63
	HDLC4 (mg/dl)	63.0±5.41	58.5±5.26	65.5±15.78	62.7±12.50	48.1±7.31	60.0±22.87
	LDLC3 (mg/dl)	6.7±1.76	6.6±1.55	8.2±1.98	5.1±1.86	7.2±2.08	7.0±2.31
	Na (10-3 mol/l)	153.35±1.45	155.45±4.38	171.63±20.34	165.92±8.79	137.52±7.54	132.06±5.42
	K (10-3 mol/l)	10.24±1.07	10.15±0.66	11.50±1.74	10.60±1.31	8.85±1.03	8.15±0.53
	Cl (10-3 mol/l)	101.68±1.59	102.19±3.43	116.83±15.44	109.31±6.17	89.84±5.27	85.68±4.43
Female	TP2 (g/dl)	7.88±0.20	8.09±0.44	8.83±0.53a	8.21±0.61	6.83±0.32	7.19±0.70
	CHO2L (mg/dl)	113.0±13.69	109.1±30.17	102.0±19.12	94.4±23.07	74.9±12.76	70.5±16.14
	TRIGL (mg/dl)	96.2±33.40	91.1±28.95	82.8±34.04	75.2±18.53	65.1±22.74	60.0±16.17
	ALTL (U/l)	46.8±6.19	48.4±12.14	51.7±8.49	48.5±10.51	53.4±15.88	53.6±13.67
	ASTL (U/l)	76.9±8.04	80.1±12.78	91.8±9.55a	86.8±12.17	88.0±15.29	86.0±12.15
	ALP2S (U/l)	41±6.77	46±9.08	44±7.57	40±6.60	35±5.86	35±7.73
	ALB2 (g/dl)	5.65±0.15	5.84±0.30	6.24±0.51a	5.85±0.41	4.74 ±0.20	4.98±0.43
	GLO (g/dl)	2.23±0.09	2.25±0.20	2.59±0.26a	2.36±0.23	2.09±0.15	2.21±0.28
	CREA2 (mg/dl)	0.42±0.03	0.40±0.03	0.48±0.04a	0.46±0.04a	0.42±0.03	0.43±0.05
	U - BUN (mg/dl)	20.9±2.64	23.2±2.61	21.5±2.12	24.1±2.33a	21.8±4.77	21.9±3.66
	UA2 (mg/dl)	4.3±0.93	4.8±0.99	4.3±0.65	4.2±1.39	3.4±0.48	3.4±0.59
	SGLU3 (mg/dl)	235.6±33.47	282.4±58.12	168.0±51.39	184.5±32.60a	197.5±46.66	153.8±63.27
	HDLC4 (mg/dl)	89.1±9.87	88.0±20.50	85.3±14.49	70.8±29.04	60.1±8.61	58.4±12.65
	LDLC3 (mg/dl)	9.2±3.06	8.4±7.78	6.0±1.94	4.3±1.85a	5.6±2.87	4.7±2.50
	Na (10-3 mol/l)	152.66±1.09	153.46±1.60	163.64±8.09a	158.69±4.34a	137.64±7.31	137.10±8.36
	K (10-3 mol/l)	9.74±1.10	9.50±1.44	10.92±1.26	10.13±1.12	8.82±0.55	9.19±1.66b
	Cl (10-3 mol/l)	104.02±1.36	101.96±2.55	112.29±6.56	106.59±2.31	93.24±5.27	93.08±6.34

Note: [a]The mean difference is significant at the 0.05 levels of control group

[b]The mean difference is significant at the 0.05 levels of control recovery group

Organ Weighting

For the female animal, the mean weight of right adrenal gland in medium dose group (20 mg/kg BW) was significantly different lower than control group ($p<0.05$) and the mean weight of heart and left adrenal gland in high dose group (80 mg/kg BW) were significantly different higher than control group ($p<0.05$). The organ's weight was calculated in the relative weights per 100 g animal body weight that showed the relative weight was not related with dose level what used in this study and the result of organ weight for male and female animals were represented in Table 3.

Table 3 Organ's Weight in relation to body weight of Wistar rats treated with Cordyceps militaris treated rat for 90 days

Sex	Parameters	Control	Mean of parameters ± standard deviation			Recovery	
			5 mg/kg BW	20 mg/kg BW	80 mg/kg BW	Control	80 mg/kg BW
Male	Liver	2.6552±0.15	2.6419±0.09	2.7351±0.14	2.5923±0.14	2.5526±0.06	2.4729±0.13b
	Kidney Rt.	0.2587±0.01	0.2598±0.01	0.2607±0.02	0.2540±0.02	0.2603±0.01	0.2423±0.03
	Kidney Lt.	0.2533±0.01	0.2570±0.01	0.2529±0.02	0.2458±0.02	0.2546±0.01	0.2391±0.02
	Heart	0.2839±0.02	0.2792±0.01	0.2560±0.07	0.2735±0.01	0.2838±0.01	0.2862±0.01
	Lung	0.3799±0.03	0.3634±0.04	0.3704±0.06	0.3369±0.04	0.3358±0.08	0.3907±0.05
	Spleen	0.1824±0.01	0.1766±0.02	0.1827±0.02	0.1700±0.02	0.1785±0.01	0.1746±0.03
	Brain	0.4744±0.03	0.4589±0.04	0.4705±0.02	0.4578±0.03	0.4394±0.02	0.4420±0.02
	Adrenal Rt.	0.0080±0.00	0.0083±0.00	0.0076±0.00	0.0075±0.00	0.0076±0.00	0.0083±0.00
	Adrenal Lt.	0.0088±0.00	0.0086±0.00	0.0085±0.00	0.0085±0.00	0.0089±0.00	0.0087±0.00
	Testis Rt.	0.3943±0.03	0.4009±0.04	0.3937±0.03	0.3762±0.03	0.3636±0.05	0.3905±0.04
	Testis Lt.	0.3895±0.02	0.4024±0.04	0.3985±0.03	0.3868±0.03	0.3701±0.04	0.3985±0.03
	Thymus	0.0714±0.02	0.0674±0.01	0.0564±0.02	0.0663±0.01	0.0578±0.01	0.0689±0.01
Female	Liver	2.5012±0.12	2.6613±0.14	2.6077±0.16	2.5907±0.18	2.5796±0.17	2.4362±0.21
	Kidney Rt.	0.2873±0.01	0.2948±0.02	0.2908±0.02	0.2824±0.02	0.2954±0.01	0.2783±0.01
	Kidney Lt.	0.2741±0.02	0.2875±0.02	0.2789±0.01	0.2750±0.02	0.2806±0.01	0.2746±0.01
	Heart	0.3272±0.02	0.3404±0.03	0.3334±0.02	0.3546±0.04a	0.3461±0.01	0.3397±0.02
	Lung	0.5054±0.07	0.5261±0.03	0.5386±0.10	0.5373±0.05	0.5073±0.09	0.5353±0.10
	Spleen	0.2403±0.03	0.2347±0.03	0.2400±0.02	0.2555±0.03	0.2535±0.03	0.2440±0.02
	Brain	0.7573±0.04	0.7735±0.06	0.8040±0.04	0.7969±0.04	0.8002±0.05	0.7758±0.04
	Uterus	0.1765±0.03	0.2319±0.09	0.2734±0.11	0.2378±0.08	0.1579±0.01	0.2169±0.07
	Adrenal Rt.	0.0169±0.00	0.0153±0.00	0.0142±0.00a	0.0168±0.00	0.0170±0.00	0.0168±0.00
	Adrenal Lt.	0.0157±0.00	0.0172±0.00	0.0145±0.01	0.0186±0.00a	0.0170±0.00	0.0174±0.00
	Ovary Rt.	0.0179±0.00	0.0173±0.00	0.0172±0.00	0.0166±0.00	0.0170±0.00	0.0181±0.00
	Ovary Lt.	0.0165±0.00	0.0184±0.00	0.0170±0.00	0.0168±0.00	0.0188±0.00	0.0184±0.00
	Thymus	0.0948±0.01	0.0887±0.02	0.0786±0.01	0.0929±0.02	0.0809±0.01	0.0943±0.01

Note: [a]The mean difference is significant at the 0.05 levels of control group

[b]The mean difference is significant at the 0.05 levels of control recovery group

Discussion

The *C. militaris* possesses extensive bioactive compounds which has been used as an ingredient in dietary supplement for many years but the safety data of *C. militaris* have not been conducted enough. In compliance with OECD Guidelines for the testing of chemicals 423, Acute Oral Toxicity – Acute Toxic Class Method of *C. militaris* at dose level 300 and 2,000 mg/kg body weight, all tested animals were not shown signs of toxic, moribund and mortality including the health observation, feed and drinking water consumptions was normal. For the body weights were continued to gain throughout the testing period.

In gross findings, there was petechial hemorrhage scattered in the thymus parenchyma that was no gross evidence of necrosis. Thus, this lesion in these animals likely resulted from necropsy technique. Accessory spleens are rare findings in rodents and may be congenital or a consequence of traumatic injury[26] caused by bisection of spleen. For the abdominal fat was considered to be non-adverse since it appears small in incidence and minimum in severity that can occasionally be seen spontaneously.

In our study, the analyzed parameters of the hematological analysis and clinical biochemistry analysis were mostly similar tendency to the histological control data of Wistar rat (Mlac:WR) including previous study that studies the toxicity study of *C. militaris*[6] except some parameters such as Neutrophils (NEUT), Lymphocytes (LYMPH), Red Blood Cells (RBC), Hemoglobin (HGB) for the hematological analysis and Alanine amino transferase (ALTL), Glucose (SGLU3) and Potassium (K) for the clinical biochemistry analysis. The fluctuation of data could be occurred due to age, gender, species, dose level of test item and other factors.

Thus, the toxicity level assessment of the *C. militaris* can be summarized no related effects at any dose level and no side effects or abnormalities after repeated oral administrations for 90 days. The sub chronic oral toxicity according to OECD Guidelines for the testing of chemicals 408, Repeated Dose 90 – Day Oral Toxicity Study in Rodents of *C. militaris* at dose level 5, 20, 80 mg/kg body weight. The all data result such as clinical signs of toxicity, body weight, food and water consumptions, necropsy examination, etc were not shown toxic of *C. militaris* in Wistar rats.

Conclusion

The acute oral toxicity of *C. militaris* in Wistar rats at 300 and 2,000 mg/kg body weight was not shown signs of toxic, moribund and mortalities in all animals. Thus, *C. militaris* was classified in GHS category 5 or unclassified, the LD_{50} cut off at 5000 mg/kg body weight to infinity (∞). For the sub-chronic oral toxicity result that the no observed adverse effect level (NOAEL) of *Cordyceps militaris* was considered to be 80 mg/kg body weight per day for Wistar rats. All result of this study were within only the test conditions which described earlier.

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References

1. Buenz EJ, Bauer BA, Osmundson TW, Motley TJ. The traditional Chinese medicine *Cordyceps sinensis* and its effects on apoptotic homeostasis. J Ethnopharmacol 2005; 96(1-2): 19-29.
2. Wen TC, Lei BX, Kang JC, Li GR, He J. Enhanced production of mycelial culture using additives and cordycepin by submerged in Cordyceps militaris. Food Ferment Ind 2009; 35(8): 49-53.
3. Li HC, Sun P, Feng CQ. The research of cordycepin as an active component in *Cordyceps*. J Jinggangshan Univ (Nat Sci) 2010; 31(2): 93-6.
4. Jhou BY, Fang WC, Chen YL, Chen CC. A 90 - day subchronic toxicity study of submerged mycelial culture of *Cordyceps militaris* in rats. Toxicol Res 2018; 7(5): 977-86.
5. Park C, Hong SH, Lee JY, Kim GY, Choi BT, Lee YT, et al. Growth inhibition of U937 leukemia cells by aqueous extract of Cordyceps *militaris* through induction of apoptosis. Oncol Rep 2005; 13(6): 1211-6.
6. Ng TB, Wang HX. Pharmacological actions of *Cordyceps*, a prized folk medicine. J Pharm Pharmacol 2005; 57(12): 1509-19.
7. Nan JX, Park EJ, Yang BK, Song CH, Ko G, Sohn DH. Antifibrotic effect of extracellular biopolymer from submerged mycelial cultures of *Cordyceps militaris* on liver fibrosis induced by bile duct ligation and scission in rats. Arch Pharm Res 2001; 24(4): 327-32.
8. Zhao-Long W, Xiao-Xia W, Wei-Ying C. Inhibitory effect of *Cordyceps sinensis* and *Cordyceps militaris* on human glomerular mesangial cell proliferation induced by native LDL. Cell Biochem Funct 2000; 18(2): 93-7.
9. Won SY, Park EH. Anti-inflammatory and related pharmacological activities of cultured mycelia and fruiting bodies of *Cordyceps militaris*. J Ethnopharmacol 2005; 96(3): 555-61.
10. Yoo HS, Shin JW, Cho JH, Son CG, Lee YW, Park SY, et al. Effects of *Cordyceps militaris* extract on angiogenesis and tumor growth. Acta Pharmacol Sin 2004; 25(5): 657-65.
11. Choi SB, Park CH, Choi MK, Jun DW, Park S. Improvement of insulin resistance and insulin secretion by water extracts of *Cordyceps militaris*, *Phellinus linteus* and P*aecilomyces tenuipes* in 90% pancreatectomized rats. Biosci Biotechnol Biochem 2004; 68(11): 2257-64.

12. Lin XX, Xie QM, Shen WH, Chen Y. Effects of fermented *Cordyceps* powder on pulmonary function in sensitized guinea pigs and airway inflammation in sensitized rats. China J Chin Mater Med 2001; 26(9): 622-5.
13. Panda AK, Swain KC. Traditional uses and medicinal potential of *Cordyceps sinensis* of Sikkim. J Ayurveda Integr Med 2011; 2(1): 9-13.
14. Kim YO, Kim HJ, Abu-Taweel GM, Oh J, Sung GH. Neuroprotective and therapeutic effect of *Cordyceps militaris* on ischemia-induced neuronal death and cognitive impairments. Saudi J Biol Sci 2019; 26(7): 1352-7.
15. Zhou X, Yao Y. Unexpected nephrotoxicity in male ablactated rats induced by *Cordyceps militaris*: the involvement of oxidative changes. Evid Based Complement Alternat Med 2013; 2013: 786528. (9 pages).
16. Zhu SJ, Pan J, Yang JJ, Zhou A. Immune activation and toxicity evaluation of fresh *Cordyceps militaris* extracts by high-pressure processing. Food Agr Immunol 2015; 26(5): 645-58.
17. Kwack SJ, Lee BM. Subacute oral toxicity study of a new type of Cordyceps, *Paecilomyces sinclairii*, in Sprague-Dawley rats. Toxicol Res 2009; 25(2): 101-6.
18. Organisation for Economic Cooperation and Development (OECD). Test no. 423: acute oral toxicity – acute toxic class method, OECD guidelines for the testing of chemicals. [online]. 2001; [cited 2021 Feb 16]; [14 screens]. Available from: URL: https://www.oecd-ilibrary.org/docserver/9789264071001-en.pdf?expires=1613451653&id=id&accname=guest&checksum=0023ABD857B31C6E4DF95A0FAA33E582.
19. Organisation for Economic Cooperation and Development (OECD). Test no. 408: repeated dose 90-days oral toxicity study in rodents, OECD guidelines for the testing of chemicals. [online]. 1998; [cited 2021 Feb 16]; [10 screens]. Available from: URL: https://www.oecd.org/env/ehs/testing/E408_1998.PDF
20. Institute for Laboratory Animal Research, Division on Earth and Life Studies, National Research Council. Guide for the care and use of laboratory animals. 8th ed. Washington, DC: The National Academies Press; 2011.
21. Office of Animal Care and Use. Guidelines for euthanasia of rodent fetuses and neonates. [online]. 2013; [cited 2019 Jun 18]; [3 screens]. Available from: URL: https://oacu.oir.nih.gov/system/files/media/file/2021-02/b4_rodent_euthanasia_pup.pdf.
22. Mooi E, Sarstedt M. Hypothesis testing & ANOVA. In: A concise guide to market research. 3rd ed. Berlin, Germany: Springer-Verlag GmbH; 2011. p. 113-160.
23. Ference BA, Ginsberg HN, Graham I, Ray KK, Packard CJ, Bruckert E, et al. Low - density lipoproteins cause atherosclerotic cardiovascular disease. 1. Evidence from genetic, epidemiologic, and clinical studies. A consensus statement from the European Atherosclerosis Society Consensus Panel. Eur Heart J 2017; 38(32): 2459-72.

24. Assmann G, Gotto AM. HDL cholesterol and protective factors in atherosclerosis. Circulation 2004; 109(23 Suppl 1): III8-14.
25. Fruchart JC, Nierman MC, Stroes ESG, Kastelein JJP, Duriez P. New risk factors for atherosclerosis and patient risk assessment. Circulation 2004; 109(23 Suppl 1): III15-9.
26. Hobbie K, Elmore SA, Kolenda-Roberts HM. NTP nonneoplastic lesion atlas: spleen - accessory spleen. [online]. [cited 2021 Jun 13]; [2 screens]. Available form: URL: https://ntp.niehs.nih.gov/nnl/immune/spleen/accessory/spleen-accessory-spleen-pdf_508.pdf.

การทดสอบความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรังทางปากของถั่งเช่าสีทอง *(Cordyceps militaris)* ในหนูแรทสายพันธุ์ Wistar

กฤติกาญจน์ สุวรรณสโรช ภัสสราภรณ์ ศรีมังกรแก้ว ภคมณ ยศทรารัตน์ และอัญชลี สิริมนตาภรณ์
ศูนย์สัตว์ทดลองแห่งชาติ มหาวิทยาลัยมหิดล จังหวัดนครปฐม 73170

บทคัดย่อ *Cordyceps militaris* ประกอบด้วย Cordycepin, Adenosine, Polysaccharides และสารประกอบอื่นๆ สำหรับหลายประเทศรวมทั้งประเทศไทยนำ *C. militaris* มาใช้เป็นส่วนผสมของผลิตภัณฑ์เสริมอาหารในช่วงหลายปีที่ผ่านมา ในขณะที่ยังไม่มีข้อมูลการทดสอบทางพิษวิทยาของ *C. militaris* อย่างเพียงพอ การศึกษาในครั้งนี้จัดทำขึ้นเพื่อประเมินความเป็นพิษของ *C. militaris* โดยการทดสอบความเป็นพิษทางปากแบบเฉียบพลันและแบบกึ่งเรื้อรังในหนูแรทสายพันธุ์ Wistar สำหรับการประเมินความเป็นพิษเฉียบพลันที่ขนาด 300 และ 2,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง ตามวิธีการมาตรฐาน OECD Guideline หมายเลข 423 ผลการทดสอบในสัตว์ทดลองทุกตัวพบว่า ไม่ก่อให้เกิดความเป็นพิษและการตายในสัตว์ทดลอง ดังนั้น *C. militaris* จัดอยู่ในระบบการจัดกลุ่มสารเคมี การติดฉลาก และการแสดงรายละเอียดบนเอกสารข้อมูลความปลอดภัยสากลหมวดที่ 5 หรือไม่ระบุประเภท มี LD_{50} ที่ 5,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลองขึ้นไป หลังจากนั้น ได้ทำการทดสอบความเป็นพิษของ *C. militaris* ทางปากแบบกึ่งเรื้อรังและวิธีการทดสอบได้มีการประยุกต์จากวิธีการมาตรฐาน OECD Guideline หมายเลข 408 การประเมินความเป็นพิษทำการศึกษาที่ขนาด 5, 20 และ 80 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง ผลการทดสอบสรุปได้ว่า ปริมาณที่มากที่สุดที่ได้รับต่อเนื่องเป็นเวลานานโดยไม่ก่อให้เกิดอันตรายใดๆ ต่อร่างกายต่อวันในสัตว์ทดลอง คือ 80 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง

คำสำคัญ: ถั่งเช่าสีทอง, การทดสอบความเป็นพิษเฉียบพลัน, การทดสอบความเป็นพิษกึ่งเรื้อรัง, OECD Guidelines หมายเลข 423, OECD Guidelines หมายเลข 408