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Acute and Sub-Chronic Oral Toxicity of Kratom leaves aqueous extract in Wistar rats

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ABSTRACT Kratom, or *Mitragyna speciosa* (Korth.) Havil. is widely used in pharmaceutical and medical applications, including food or dietary supplements. Toxicological testing data for Kratom has been less conducted in Thailand, although Kratom can be easily traded at present. This study aimed to assess the acute and sub-chronic oral toxicity of Kratom leaves aqueous extract in Wistar rats to obtain useful information for product registration, including confidence and product safety for consumers. The results showed the doses at 300 and 2,000 mg/kg body weight did not cause acute toxicity and mortalities in animals, thus Kratom leaves aqueous extract was classified in GHS (Globally Harmonised System for Classification and Labeling of Chemicals) category 5 or unclassified, with the LD_{50} cut off at 5,000-∞ mg/kg body weight of the animal. The sub-chronic oral toxicity testing was investigated at 250, 500, and 1,000 mg/kg body weight. It was concluded that the no observed adverse effect level (NOAEL) of Kratom leaves aqueous extract was 1,000 mg/kg body weight of the animal. From this study, the toxi city profile of Kratom leaves aqueous extract provided valuable data that was essential and could be supported for future studies, especially the toxicity study of Kratom product, which was produced and sold in Thailand. In the future, chronic toxicity studies will be emphasized to evaluate its safety and long-term effects.

Keywords: *Mitragyna speciosa* (Korth.) Havil., Kratom leaves aqueous extract, Acute and sub-chronic oral toxicity

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Introduction

Kratom (*Mitragyna speciosa* (Korth.) Havil.) is a plant belonging to the Rubiaceae family and native to Southeast Asia.[1,2] The botany of Kratom plants is perennial, with a height of around 15-30 meters.[3] The trunk is generally straight, and the outer bark is smooth and gray.[4] The leaves are dark green, glossy on their upper surfaces, ovate-acuminate in shape, and opposite in growth pattern.[5] The spherical inflorescences are grown in clusters of three at the ends of the branches and are deep yellow.[6] In Thai traditional medicine, Kratom leaves were used to suppress stomach pain, cure dysentery, cure diarrhea, relieve body aches and pains, and act as sedatives.[3] It produces and accumulates substances in various groups, including alkaloids, flavonoids, triterpenes, phenolic compounds, etc. The indole alkaloids are the largest group of compounds found in Kratom. The active substance is mitragynine, the main compound reported in Thai Kratom leaves as high as 66% by weight compared to the total alkaloids[3], while other alkaloids are reported in small amounts, which are not more than 2% of the total alkaloids, such as 7-hydroxymitragynine, speciogynine, and mitraciliatine.[7] Mitragynine is the main compound of alkaloids in Kratom leaves, and other alkaloids are derivatives or diastereomers of mitragynine (Figure 1).[8] The mitragynine substance was first isolated in 1907.[9] However, to synthesize mitragynine using organic synthesis, it was succeeded by Takayama H., et al.[10] and Ma J., et al.[11] However, the amount that could be synthesized was less than the natural extraction.

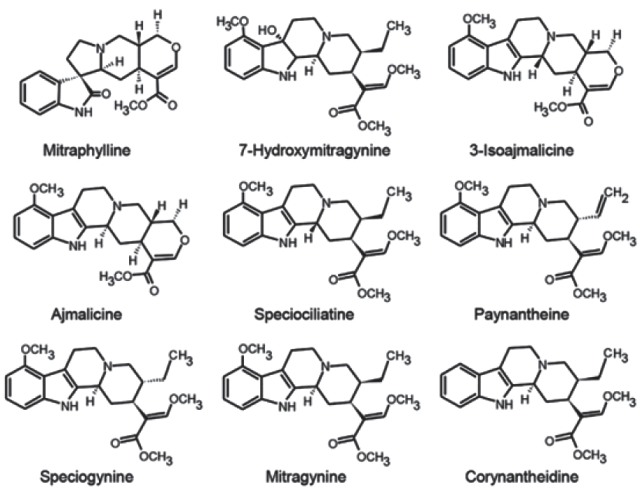

Figure 1 Chemical structures of alkaloids in *Mitragyna speciosa* (Korth.) Havil. Leaves [8]

The pharmacology of mitragynine affects the nervous system, digestive system, anti-inflammatory and antibacterial effects, and other systems such as high blood pressure, etc.[3] For the toxicology study of Kratom, there were some reports; for example, Sabetghadam A., et al.[12] indicated that the LD_{50} of mitragynine in mice with oral administration was 477.1 mg/kg

body weight, and kratom extract containing 20-22% mitragynine content had an LD_{50} of 591.6 mg/kg body weight, while the LD_{50} value of the mitragynine compound with oral administration was higher than the intravenous administration reported by Smith LC., et al.[13] and Harizal SN., et al.[14] The oral administration of the methanol extract from Kratom given to rats at doses of 100, 500 and 1,000 mg/kg body weight caused no deaths in 14 days. This substance did not affect feed and water consumption, including visceral weight and blood values, but was found to have an effect on the weight gain of animals, while at a dose of 100 mg/kg body weight, it inhibited weight gain. The results of the aforementioned were consistent with Ilmie MU., et al.[15] It was found that rats receiving methanol extract from Kratom for 14 days at doses of 100, 500 and 1,000 mg/kg body weight had significantly higher blood pressure than the control. At a dose of 1,000 mg/kg body weight, the liver tissue was damaged, but the lung and kidney tissues were not damaged, and no abnormality of the blood cell count was found. From Sabetghadam A., et al[16] it was reported that oral administration of mitragynine to rats for 28 days showed a decrease in red blood cell, white blood cell, and platelet counts, yet hemoglobin (HGB) and hematocrit (HCT) levels increased. Besides, those rats receiving the methanol extract from Kratom at doses of 100, 500 and 1,000 mg/kg body weight performed alanine aminotransferase (ALT) higher than the control group, which was taken morphine-treated patients at the toxic dose also had changes in albumin and triglyceride levels, which indicated abnormalities in liver function and hepatic tissue injury.[14]

The rational of the Kratom study was that, due to legal control in the past, Kratom was classified as a narcotic drug type 5, but this law had been abolished in the present which effective on 27th August 2022.[17] Thus, Kratom was popularly and widely used as medicine because it was believed to be safe with little or no side effects. Moreover, it was important to document toxicological data comprehensively for their safety reasons, resulting in more Kratom studies nowadays. Notwithstanding the widespread use of Kratom, there was still insufficient data about its toxicity. Therefore, this study was performed to evaluate acute and sub-chronic toxicity. The acute oral toxicity of Kratom leaves aqueous extract was studied for classification in the GHS (Globally Harmonised System for Classification and Labeling of Chemicals) category, which complied with OECD Guidelines for the Testing of Chemicals 423, Acute Oral Toxicity - Acute Toxic Class Method (Figure 2).[18] The methodology of sub-chronic oral toxicity modified from the OECD Guidelines for Testing of Chemicals 408, Repeated Dose 90-Days Oral Toxicity Study in Rodent[19] that was performed to confirm the edible safety and to evaluate the no-observed-adverse-effect level (NOAEL) for long-term use of Kratom leaves aqueous extract.

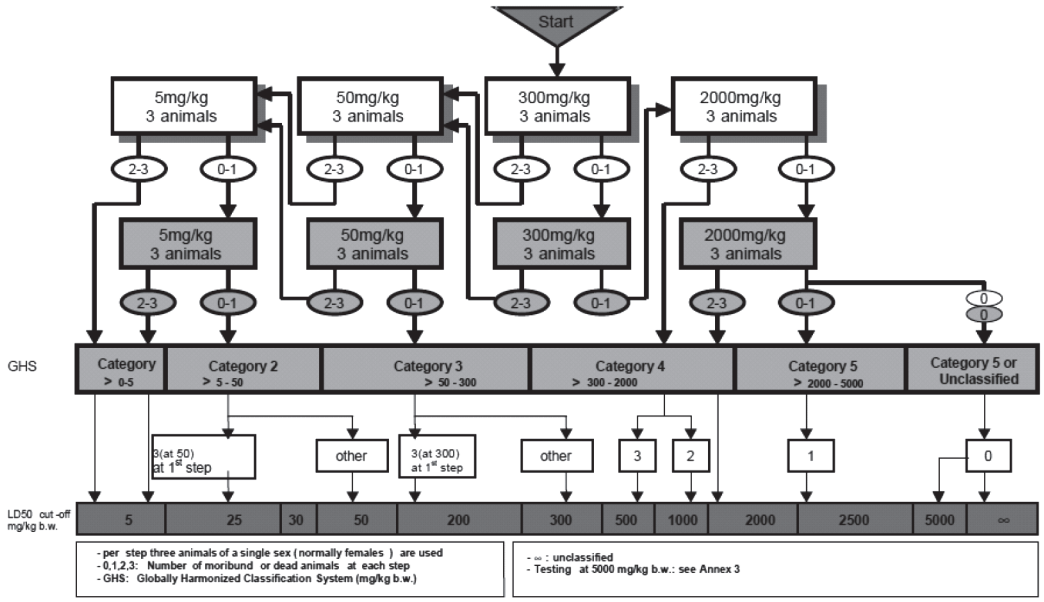

Figure 2 A test procedure for acute oral toxicity with a starting dose of 300 mg/kg body weight.[18]

Materials and Methods

Sample preparation

The Kratom leaves aqueous extract was provided by Ruay Anan 168 Co., Ltd. The quality control of samples was performed by Central Laboratory (Thailand) Co., Ltd. for heavy metal analysis such as arsenic, cadmium, etc. (Reference method: AOAC Official Method 2015.01; "Determination of Heavy Metals in Food by Inductively Coupled Plasma-Mass Spectrometry First Action" (2015)). The active ingredient (mitragynine), including microbiological contamination tests such as *Salmonella* spp., etc. (reference method: ISO 6579-1:2017, "Microbiology of the Food Chain – Horizontal Method for the Detection, Enumeration, and Serotyping of *Salmonella*-Part 1: Detection of *Salmonella* spp.) The mitragynine content in this lot that was used in both studies was reported at 1,822.84 mg/kg by high performance liquid chromatography (HPLC) with UV detector as the reference method.[20]

The leaves of Kratom were washed twice, drained, and finely chopped. After that, it was boiled and stirred with a boiling and stirring machine (SUS 304-4L, Khonthong 2520 LTD., Bangkok, Thailand). Then, it was extracted with a high-speed extractor using high-temperature and high-pressure water to flow through the sample, and then the water was evaporated and concentrated extracts were obtained. Then, it was filtered to remove residue, freeze dried at a temperature range of -80 to -60°C with a freeze dryer (GFD-200SM and GFD-400SM, Grisrianthong Co., Ltd., Ratchaburi, Thailand), and prepared into powder by blender with 50-250 kg/hr, 450 mesh (WFJ-450, Swentech Ltd., Bangkok, Thailand).

The Kratom leaves aqueous extract received from the method mentioned above was fine brown or brownish green powder. It was kept in a tightly-closed container, protected from moisture. The storage condition of this sample was kept at room temperature but not exceeding 30°C including sealed the container and stored away from direct sun light.

Preparation of the dose

For acute oral toxicity, Kratom leaves aqueous extract was being weighed and freshly mixed with sterile water as vehicle prior to administration, which was calculated at 300 and 2,000 mg/kg body weight. Kratom leaves aqueous extract for sub-chronic oral toxicity was prepared as same as the acute oral toxicity but using 3 dose levels (low, medium, and high dose), which were 250, 500, and 1,000 mg/kg body weight.

Preparation of animals

The 12 females of Wistar rats (9 weeks) and 50 males and 50 females of Wistar rats (6 weeks), whose their body weight ranged from 200 g ±20% were used for acute and sub-chronic oral toxicity, respectively. All animals were obtained from the Office of Laboratory Animal Production, National Laboratory Animal Center, Mahidol University, Thailand.

Animal husbandry conditions

The animals were housed in stainless steel cages with feed (082, Perfect Companions, Thailand) and 5–7 ppm chlorinated water *ad libitum* under standard conditions of 12 hours light, 12 hours dark, at 22±3°C, and 30–70% relative humidity. All the animals were acclimatized for 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.[21]

Acute oral toxicity test

For the administration of the dose for acute oral toxicity, the animal was fasted (feed but not water) overnight (15–18 hours) prior to administration. After that, a substance at dose levels of 300 and 2,000 mg/kg body weight was administered orally to the animal in a single dose and then feed to the animal after 3–4 hours that test substance had been administered.

Observational, the animal was monitored during the first 4 hours, with special attention during the first 30 minutes, and then periodically during the first 24 hours. A general observation of animals was observed individually for toxic effects such as changes in skin and fur, eye and mucous membranes, respiratory, circulatory, autonomic, and central nervous systems, somatomotor activity, and behaviour pattern daily. The addition would be directed to observations of tremors, convulsions, gasping, cyanosis, vocalization, salivation, diarrhea, lethargy, sleep, and coma. The body weight, feed, and water consumption of the survived animals were measured and recorded on days 1, 7, and 14 after each dose level administration.

Necropsy Examination, after 14 days, the survived animals were euthanized using CO_2 inhalation.[22] And all tested animals were sacrificed. The positions, shapes, sizes, and colours of internal organs were evaluated.

Sub-chronic oral toxicity test

Administration of doses for sub-chronic oral toxicity, 50 males and 50 females of Wistar rats were used and randomized into 5 groups (10 animals per sex/20 animals per group). Group 1 as the control group (vehicle control; sterile water), Group 2, 3, and 4 as the main groups at low dose, middle dose, and high dose (250, 500, and 1,000 mg/kg body weight of Kratom leaves aqueous extract, respectively), and Group 5 as the recovery group. The dosage

administration for each animal was calculated based on the body weight of the animal prior to administration at a constant volume of 1 ml per 500 g body weight. All animals were administered once daily for 90 days, and without dosing during the recovery period, 14 days for the recovery group.

Observational, all animals were monitored at least once a day for clinical signs at the similar time and were done outside the cage. After the last dose, animals in the recovery group were scheduled for follow-up observations to determine persistence and look at the exacerbation and/or reversibility of potential adverse effects for a total of 14 days. A general observation of animals was observed, as same as the acute oral toxicity test. In addition, the ophthalmological examination was performed before and after dosing, and the neurological examination was examined once a week, starting at week 11^{th}. The animal body weight, feed, and water consumption data were measured and recorded weekly, and the recovery group was continuously observed for another 14 days after 90 days of administration.

For necropsy examination, the animal was fasted (feed but not water) overnight (15–18 hours) on the last day of each group, and CO_2 inhalation[22] was used to euthanize. The cardiac puncture method was used for the blood sample collection, which was separated into 2 tubes for hematological (200 µl) as an EDTA tube and clinical biochemistry analysis (800 µl). The automated analyzer (Procyte Dx™, IDEXX Laboratories, Westbrook, Maine, USA) was used to perform the hematological analysis of 13 parameters: red blood cell count (RBC), hemoglobin (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet (PLT), white blood cell count (WBC), neutrophil (NEUT), lymphocyte (LYMPH), monocyte (MONO), eosinophil (EO), and basophil (BASO). The 17 parameters of clinical biochemistry analysis were measured using an automated blood analyzer (Cobas® c311, Roche Diagnostics, Basel, Switzerland), which consisted of sodium (Na), potassium (K), chloride (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). The necropsy was evaluated the positions, shapes, sizes, and colors of internal organs. The organs were trimmed and weighed (all the paired organs were weighed separately) to determine relative organ weights as liver, kidneys, lung, adrenals, heart, spleen, brain, thymus, testes, and epididymides for males, uterus, and ovaries for females. Paraffin section technique: the organs were trimmed and embedded for histopathology examinations, and then the liver, kidneys, lung, heart, and spleen were compared by a scoring system between the control and high-dose groups.

Statistical Analysis

The mean ± standard deviation was used to show the quantitative results. The Kolmogorov-Smirnov test and Levene's test[23]

were performed to statistically analyze the data for normality and homogeneity of variances. The data between the control group and each treatment group were compared by ANOVA and the Mann-Whitney U test. A significant difference using SPSS® Statistical software version 18.0.0 was consulted ($p \leq 0.05$).

Ethical approval

The study was approved by the National Laboratory Animal Center Animal Care and Use Committee (NLAC-ACUC), Mahidol University, Thailand, on May 25, 2022, coded RA2022-12 for acute oral toxicity and RA2022-13 for sub-chronic oral toxicity.

Results

For both toxicity test, it was shown that no animal mortalities occurred, and the clinical signs did not show any toxicity or changes in physical examination. The result of the ophthalmological and neurological examinations was normal.

The result of the animal motor activity assessment showed the average in the low dose (week 12), high dose (week 11), and recovery group (week 14) in male animals was statistically significant difference higher than the control group, but the recovery group of males (week 15) and females (week 14) was statistically significant difference lower than the control group ($p \leq 0.05$). Moreover, there was no statistically significant difference between animals for both sexes in fore-limb grip strength and hind-limb grip strength; only females of low dose (week 11) showed a statistically significant difference higher than when compared with the control group ($p \leq 0.05$). The individual body weights of the survived animals continued to gain throughout the testing period. There was a statistically significant difference for only male body weight in the main group, but in the recovery group for both sexes, there was no statistically significant difference when compared with the control group ($p \leq 0.05$). The data on acute oral toxicity and sub-chronic oral toxicity are presented (Table 1 and Figure 3), respectively. For oral toxicity tests, acute and sub-chronic, the feed and water consumption of animals were measured daily, and the data showed that all animal feed and water consumptions were regular. The result showed the feed and water consumption data for sub-chronic oral toxicity in animals (Figures 4 and 5).

Regarding relative organ weight, the following organs (Tables 2 and 3) were converted to relative organ weights (organ-to-body weight ratios). There was a statistically significant difference in organ weight when compared with the control group for both sexes ($p \leq 0.05$). The averages of left and right testis (low dose), right epididymides (low dose and middle dose), heart, and pituitary glands (high dose) for male animals, including the right and left kidney, right adrenal gland, and right ovaries, and oviduct (recovery) for female animals, were statistically significant differences lower than the control group, but only the left thyroid and parathyroid glands in low dose for female animals were statistically significant differences higher than the control group.

Macroscopic findings were observed as mild multifocal thymic hemorrhage (all group in male animals and low dose and high dose in female animals), clear fluid retention in

the uterus (all group in female animals), severe distension of the stomach (severe gastric dilation), and moderate reddening at the peripheral area of both caudal lung lobes in high dose male animals. For microscopic findings, according to the macroscopic lesions at necropsy findings, necrosis consisted of the minimal to moderate focal thymic hemorrhage observed in the thymus gland for all groups in male animals and low dose and high dose in female animals, the mild to moderate dilation of the uterine lumen (all groups in female animals), the endometrial and glandular epithelial degeneration, and necrosis (low dose in female animals). The kidneys revealed minimal focal hyaline cast (control and recovery group in male animals), which was characterized by homogeneous eosinophilic material (tubular proteinosis) filling the tubular lumen, minimal focal tubular mineralization in the kidneys (recovery group in female animals), and minimal focal mononuclear cell infiltration at periportal areas without associated alteration of adjacent hepatocytes in the liver (high dose, recovery group in male animals, and control group in female animals).

The clinical biochemistry analysis showed a statistically significant difference when compared with the control group for both sexes ($p \leq 0.05$). TP2 and ALB2 (low dose), U-BUN TP2 and ALB2 (middle dose), ASTL ALTL ALB2 (high dose), Na (recovery) of males and TP2 ALB2 (middle dose), and CHO2I TRIGL ALB2 HDLC4 (recovery) of females were statistically significant differences higher than the control group. The averages of K (middle dose), CREA2 (high dose) for males and CREA2 (middle dose) for females were statistically significant difference lower than the control group. In addition, the averages of Cl in low, moderate, and high dose groups for both sexes were statistically significant difference lower than the control group (Tables 4 and 5).

In the hematological analysis, the averages of RBC, HGB, and MONO of male animals in the moderate dose group were statistically significant difference lower than the control group ($p \leq 0.05$). The averages of RBC, HGB, and WBC of females in the moderate and high dose groups were statistically significant differences higher than the control group ($p \leq 0.05$), but only the WBC of females was related to the dose of the test item. For the recovery group, there was no statistically significant difference in hematological results when compared with the control group for both sexes ($p \leq 0.05$) (Tables 6 and 7).

Discussion

The toxicity study of Kratom leaves aqueous extract started with acute oral toxicity with a single dose of the test substance at 300 and 2,000 mg/kg body weight. All animals did not show signs of toxic effects, moribundity, or mortality, as in the studies of Harizal et al.[14] and Kamal MSA et al.[24] where the animals were orally administrated single doses of 100, 500 and 1,000 and 175, 500 and 2,000 mg/kg body weight, respectively. However, the other signs of animals were differently found in this study, such as a low level of activity (slow movement) and rapid breathing caused by an increase in blood pressure[14], fatigue and sleep sign[24], etc. Then, sub-chronic oral toxicity was performed by oral administration of the test substance for

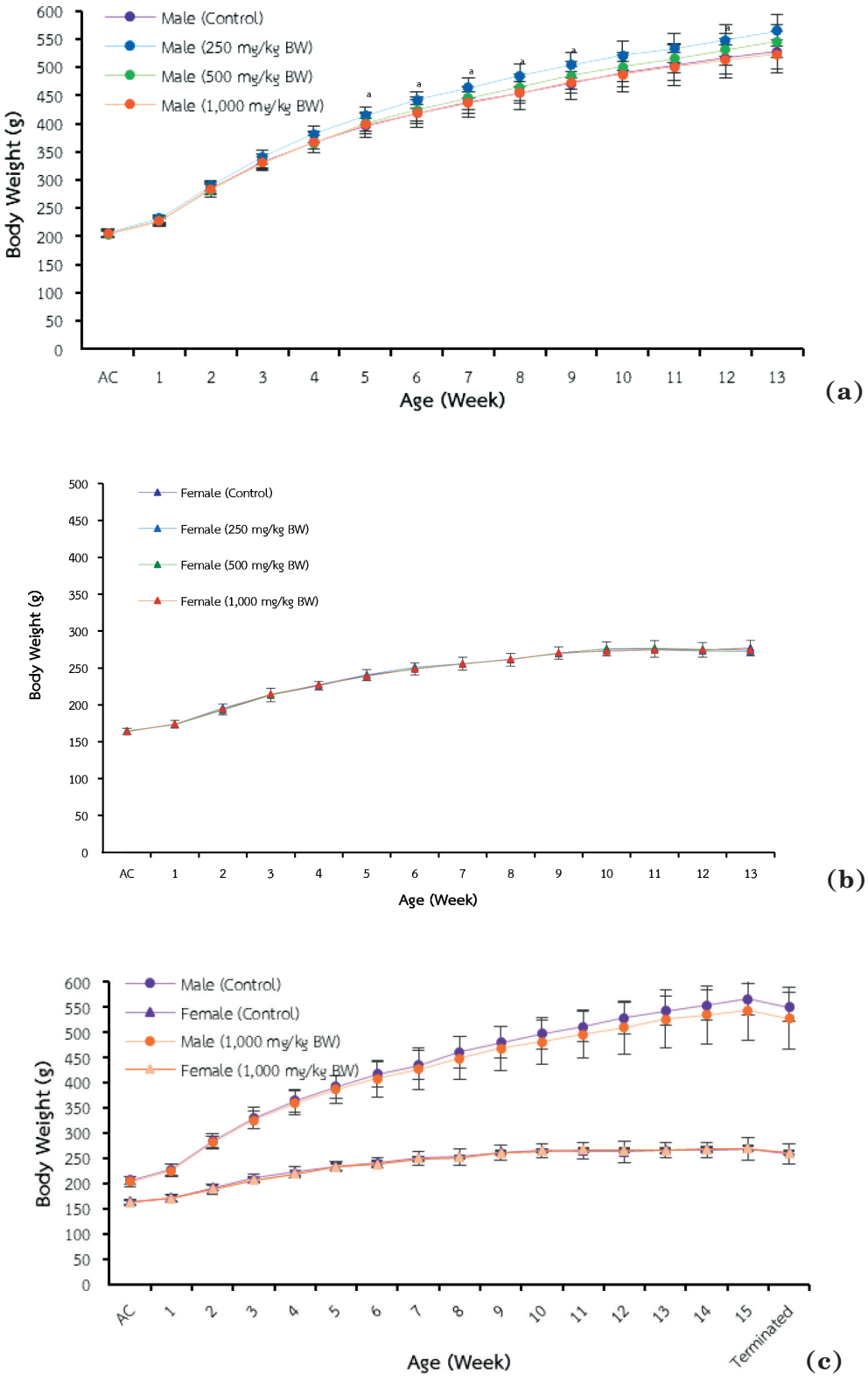

Figure 3 Effect of Kratom leaves aqueous extract on body weights (g) in animal treated for 90 days

(a) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in male animal (b) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in female animal and (c) Recovery group (n = 20) in male and female animal

Note: AC = Acclimatization

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

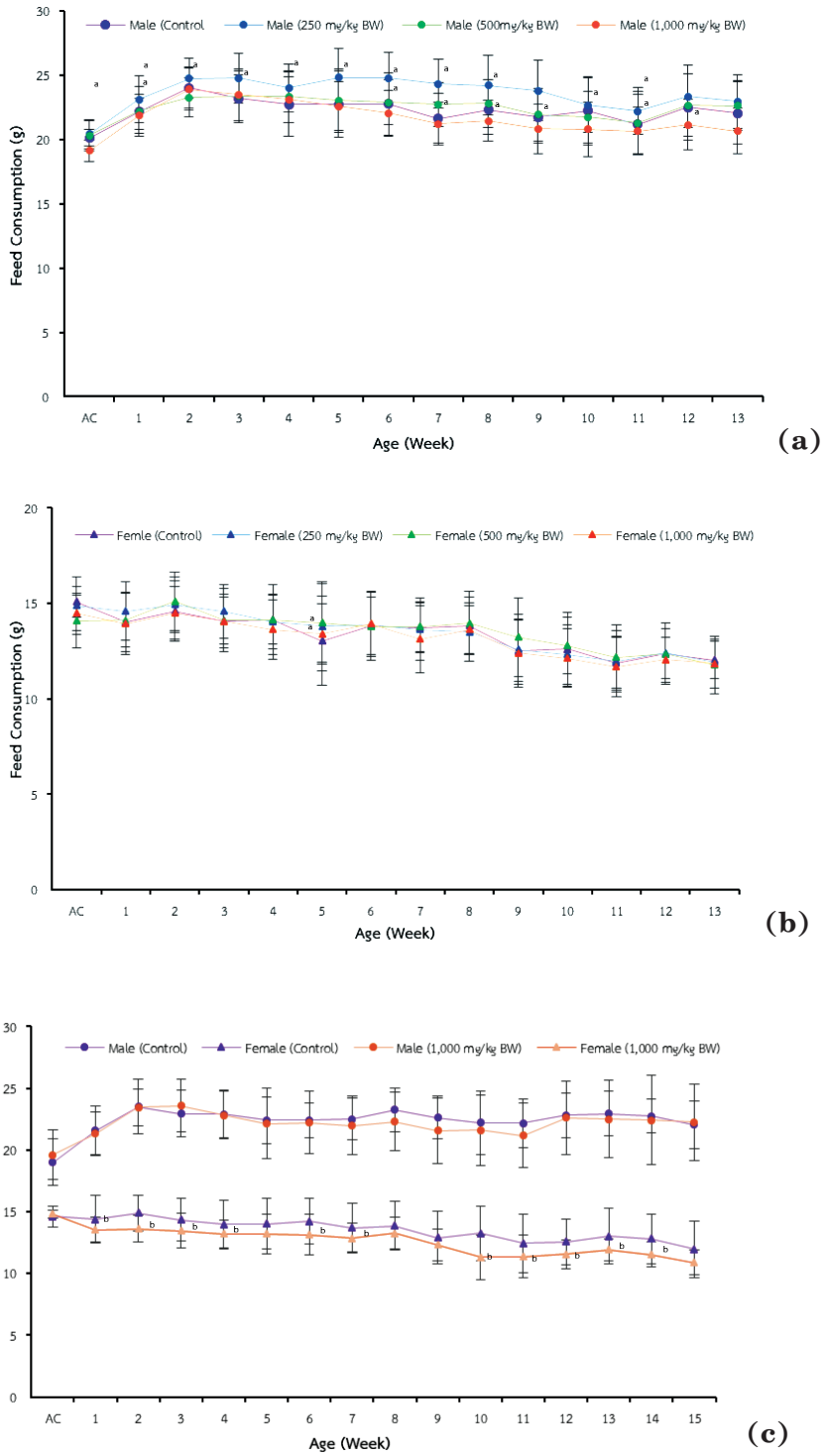

Figure 4 Effect of Kratom leaves aqueous extract on feed consumptions (g) in animal treated for 90 days

(a) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in male animal (b) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in female animal and (c) Recovery group (n = 20) in male and female animal

Note: AC = Acclimatization

[a] The average is statistically significant difference control group ($p \leq 0.05$)

[b] The average is statistically significant difference of control-recovery group ($p \leq 0.05$)

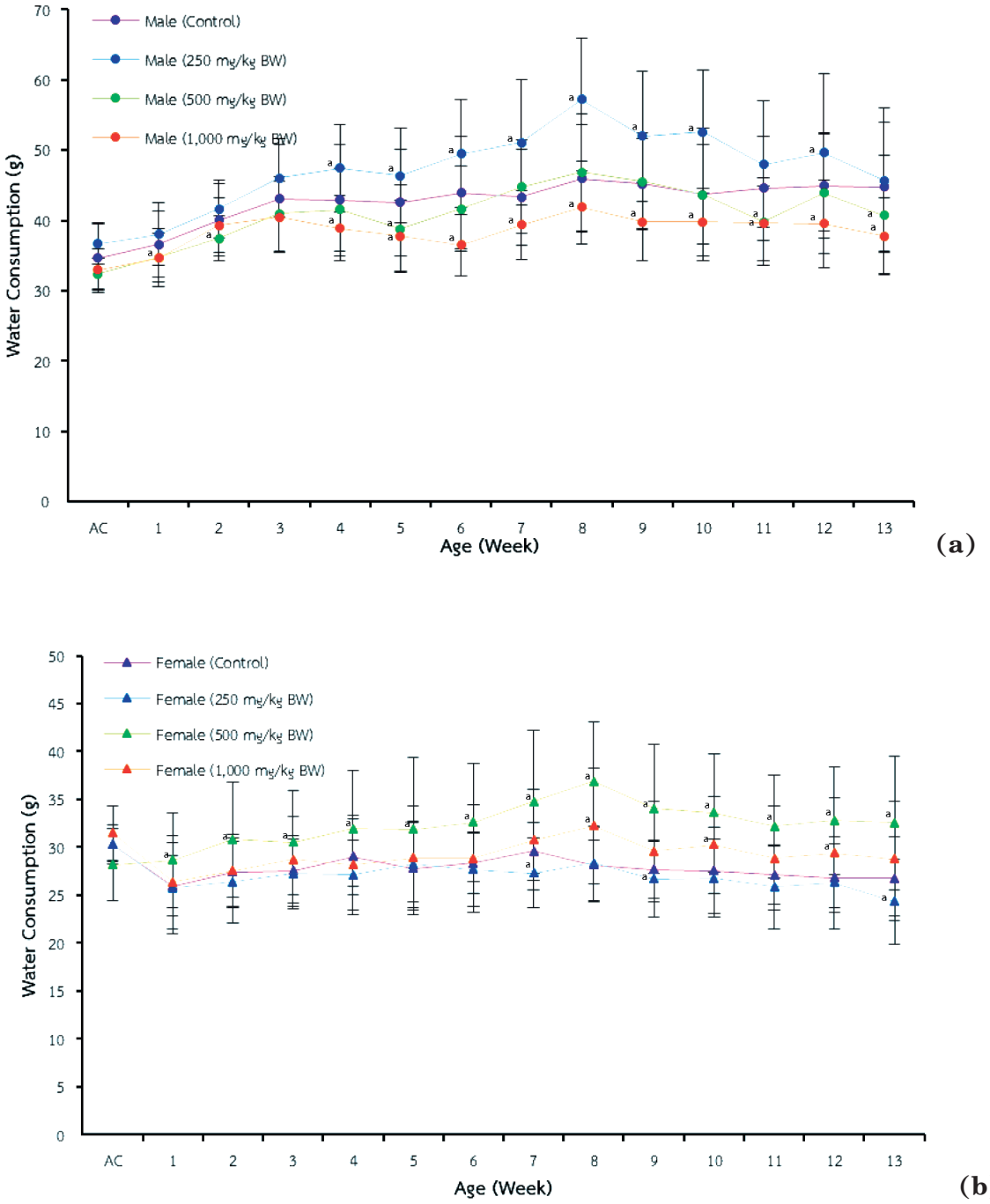

Figure 5 Effect of Kratom leaves aqueous extract on water consumptions (g) in male and female animal treated for 90 days

(a) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in male animal (b) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in female animal and (c) Recovery group (n = 20) in male and female animal

Note: AC = Acclimatization

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

[b] The average is statistically significant difference of control-recovery group ($p \leq 0.05$)

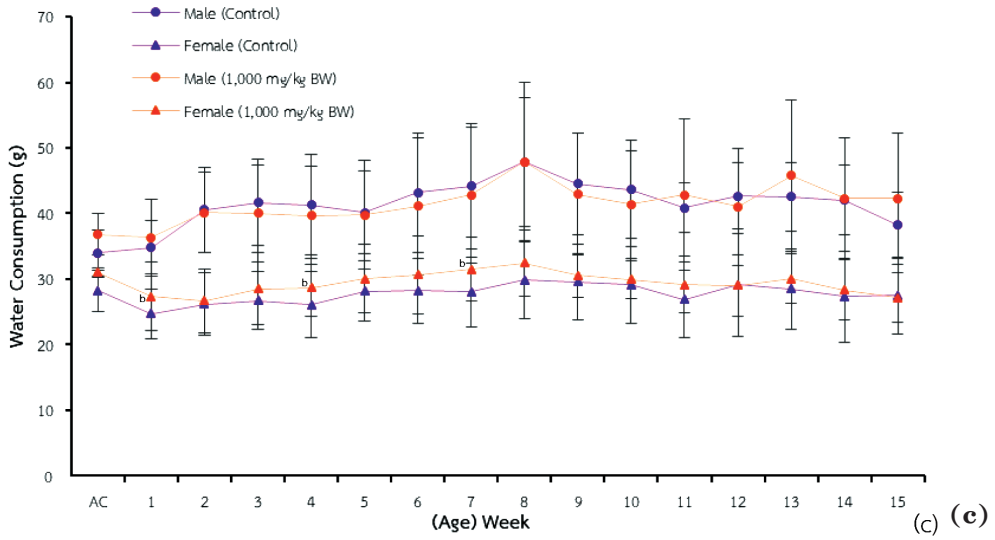

Figure 5 Effect of Kratom leaves aqueous extract on water consumptions (g) in male and female animal treated for 90 days

(a) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in male animal (b) Control group, Treatment group (250, 500 and 1,000 mg/kg BW) (n = 40) in female animal and (c) Recovery group (n = 20) in male and female animal

Note: AC = Acclimatization

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

[b] The average is statistically significant difference of control-recovery group ($p \leq 0.05$)

(continued)

Table 1 Effect of Kratom leaves aqueous extract on body weights (g) in animal for acute oral toxicity (n = 12)

Body Weight Change (%)	Animal No.	Body Weight (g)						Body Weight Change (%)
		Quarantine	Acclimatization	Day 0	Day 7	Day 14	Termination	
300	1	208	213	210	232	243	238.56	15.71
	2	207	208	204	224	232	230.35	13.73
	3	206	214	203	227	235	228.63	15.76
300	4	205	201	198	211	227	224.08	14.65
	5	201	201	198	221	223	224.69	12.63
	6	201	198	200	217	236	235.28	18.00
2,000	7	200	198	204	228	240	235.21	17.65
	8	200	200	204	221	230	229.07	12.75
	9	199	214	214	236	244	242.69	14.02
2,000	10	199	211	219	243	258	252.83	17.81
	11	197	203	205	224	240	234.22	17.07
	12	196	201	208	230	240	236.57	15.38

Table 2 Effect of Kratom leaves aqueous extract on relative organ weight in male animal treated for 90 days (n = 50)

Organ	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
Liver	2.6122±0.11	2.7419±0.23	2.7661±0.15	2.7118±0.09	2.5055±0.12	2.5039±0.11
Right Kidney	0.2582±0.03	0.2584±0.02	0.2550±0.02	0.2630±0.01	0.2476±0.02	0.2543±0.01
Left Kidney	0.2496±0.03	0.2523±0.02	0.2505±0.02	0.2463±0.01	0.2412±0.01	0.2520±0.01
Heart	0.3110±0.02	0.3023±0.01	0.2974±0.01	0.2921±0.02[a]	0.2926±0.01	0.3044±0.02
Spleen	0.1821±0.01	0.1774±0.01	0.1773±0.01	0.1699±0.01	0.1813±0.01	0.1678±0.03
Pituitary gland	0.0024±0.00	0.0022±0.00	0.0021±0.00	0.0020±0.00[a]	0.0020±0.00	0.0021±0.00
Brain	0.4271±0.03	0.3981±0.02	0.4141±0.02	0.4322±0.05	0.4100±0.02	0.4326±0.05
Right Thyroid, Para	0.0018±0.00	0.0019±0.00	0.0019±0.00	0.0018±0.00	0.0019±0.00	0.0018±0.00
Left Thyroid, Para	0.0019±0.00	0.0018±0.00	0.0018±0.00	0.0018±0.00	0.0016±0.00	0.0018±0.00
Right Adrenal	0.0073±0.00	0.0079±0.00	0.0075±0.00	0.0082±0.00	0.0071±0.00	0.0071±0.00
Left Adrenal	0.0092±0.00	0.0091±0.00	0.0086±0.00	0.0093±0.00	0.0083±0.00	0.0083±0.00
Right Testis	0.3973±0.03	0.3699±0.02[a]	0.3852±0.03	0.4037±0.02	0.3757±0.02	0.3875±0.06
Left Testis	0.3983±0.03	0.3699±0.02[a]	0.3910±0.03	0.4102±0.01	0.3865 ±0.02	0.3935±0.05
Right Epididymis	0.1282±0.01	0.1180±0.01[a]	0.1150±0.01[a]	0.1252±0.00	0.1168±0.01	0.1216±0.01
Left Epididymis	0.1238±0.01	0.1187±0.01	0.1157±0.01	0.1275±0.01	0.1195±0.01	0.1217±0.01
Prostate gland	0.1064±0.02	0.0885±0.02	0.1019±0.02	0.1007±0.01	0.0848±0.02	0.1042±0.01
Thymus	0.0636±0.01	0.0627±0.01	0.0694±0.01	0.0636±0.01	0.0510±0.01	0.0604±0.01

Note: Values are average ± standard deviation

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

Table 3 Effect of Kratom leaves aqueous extract on relative organ weight in female animal treated for 90 days (n = 50)

Organ	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
Liver	2.6361±0.20	2.5420±0.16	2.6335±0.22	2.5467±0.16	2.5177±0.04	2.4510±0.11
Right Kidney	0.2882±0.01	0.2912±0.01	0.2952±0.02	0.2895±0.01	0.3060±0.02	0.2830±0.01[b]
Left Kidney	0.2785±0.02	0.2819±0.01	0.2789±0.02	0.2766±0.01	0.2924±0.01	0.2720±0.01[b]
Heart	0.3565±0.02	0.3580±0.02	0.3516±0.01	0.3604±0.02	0.3538±0.02	0.3420±0.01
Spleen	0.2238±0.01	0.2343±0.02	0.2387±0.01	0.2308±0.02	0.2353±0.02	0.2220±0.01
Pituitary gland	0.0051±0.00	0.0052±0.00	0.0051±0.00	0.0049±0.00	0.0056±0.00	0.0048±0.00
Brain	0.7421±0.03	07748±0.05	0.7709±0.04	0.7701±0.05	0.7989±0.06	0.7684±0.02
Right Thyroid, Para	0.0029±0.00	0.0029±0.00	0.0030±0.00	0.0029±0.00	0.0031±0.00	0.0031±0.00

Table 3 Effect of Kratom leaves aqueous extract on relative organ weight in female animal treated for 90 days (n = 50) (continued)

Organ	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
Left Thyroid, Para	0.0025±0.00	0.0032±0.00[a]	0.0028±0.00	0.0029±0.00	0.0026±0.00	0.0030±0.00
Right Adrenal	0.0175±0.00	0.0187±0.00	0.0170±0.00	0.0155±0.00	0.0183±0.00	0.0154±0.00[b]
Left Adrenal	0.0190±0.00	0.0206±0.00	0.0198±0.00	0.0200±0.00	0.0196±0.00	0.0195±0.00
Right Ovary, oviduct	0.0267±0.00	0.0293±0.00	0.0284±0.00	0.0272±0.00	0.0288±0.00	0.0257±0.00[b]
Left Ovary, oviduct	0.0265±0.00	0.0271±0.00	0.0280±0.00	0.0279±0.01	0.0264 ±0.00	0.0276±0.00
Uterus	0.2035±0.04	0.1981±0.06	0.2335±0.07	0.2110±0.07	0.2450±0.09	0.2489±0.11
Thymus	0.1008±0.01	0.0984±0.02	0.0905±0.01	0.0865±0.02	0.0883±0.02	0.0868±0.02

Note: Values are average ± standard deviation

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

[b] The average is statistically significant difference of control-recovery group ($p \leq 0.05$)

Table 4 Effect of Kratom leaves aqueous extract on clinical biochemistry analysis result in male animal treated for 90 days (n = 50)

Parameter	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
SGLU3 (mg/dL)	415.48±74.33	396.76±41.87	410.54±71.77	416.44±33.33	375.1±42.99	331.5±63.93
U-BUN (mg/dL)	19.15±2.53	21.07±2.02	21.87±2.06[a]	19.60±1.72	19.2±1.18	19.0±2.34
CREA2 (mg/dL)	0.43±0.05	0.42±0.02	0.40±0.04	0.37±0.02[a]	0.44±0.02	0.42±0.03
UA2 (mg/dL)	7.04±0.91	6.91±0.81	7.18±0.71	7.58±0.63	6.6±0.75	6.5±0.76
CHO2I (mg/dL)	74.34±23.23	81.31±17.61	80.00±11.83	71.08±10.07	71.2±10.38	74.1±15.41
TRIGL (mg/dL)	96.50±32.29	109.83±39.54	100.09±22.12	101.54±21.43	87.5±20.15	79.3±14.84
HDLC4 (mg/dL)	54.82±15.75	64.79±13.77	63.37±9.46	56.26±6.91	53.3±6.93	55.8±12.37
LDLC3 (mg/dL)	12.97±8.80	10.13±5.14	10.89±2.59	8.98±3.21	8.3±2.68	10.4±3.96
ASTL (U/L)	82.29±10.28	90.71±14.48	84.36±20.22	103.23±25.58[a]	102.4±15.95	86.5±19.78
ALTL (U/L)	51.62±12.31	72.26±20.17	66.94±28.23	87.00±30.12[a]	81.2±27.40	60.5±22.02
ALP2S (U/L)	81.20±8.52	88.30±10.65	85.00±7.18	78.56±5.64	80±6.80	80±10.99
TP2 (g/dL)	7.01±0.20	7.28±0.21[a]	7.32±0.19[a]	7.20±0.17	7.15±0.08	6.97±0.28
ALB2 (g/dL)	4.91±0.13	5.10±0.11[a]	5.17±0.13[a]	5.06±0.11[a]	4.99±0.02	4.97±0.12
GLO (g/dL)	2.10±0.12	2.17±0.20	2.15±0.08	2.14±0.11	2.16±0.06	2.00±0.18
Na (mmol/L)	146.60±0.97	145.50±1.08	145.90±1.45	145.78±1.20	147±1.10	149±1.14[b]
K (mmol/L)	9.81±0.97	9.33±0.66	9.04±0.54[a]	9.55±1.00	9.71±0.43	9.74±0.58
Cl (mmol/L)	101.75±1.11	100.15±0.88[a]	99.34±0.78[a]	100.33±1.77[a]	101.8±0.55	101.9±1.36

Note: Values are average ± standard deviation

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

[b] The average is statistically significant difference of control-recovery group ($p \leq 0.05$)

Table 5 Effect of Kratom leaves aqueous extract on clinical biochemistry analysis result in female animal treated for 90 days (n = 50)

Parameter	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
SGLU3 (mg/dL)	250.73±71.62	285.52±57.62	274.10±96.51	255.42±64.73	189.78±18.52	175.44±20.08
U-BUN (mg/dL)	18.41±2.52	20.78±2.65	18.85±1.49	17.49±3.35	16.76±1.43	16.54±1.18
CREA2 (mg/dL)	0.47±0.04	0.43±0.04	0.42±0.03[a]	0.43±0.04	0.47±0.03	0.44±0.03
UA2 (mg/dL)	4.55±0.76	4.49±0.60	4.85±0.61	4.72±0.50	3.80±0.49	4.12±0.48
CHO2I (mg/dL)	80.03±21.29	83.24±11.53	88.22±12.26	81.26±9.02	75.42±9.37	90.12±10.21[b]
TRIGL (mg/dL)	66.79±15.43	58.99±13.74	63.78±21.69	63.27±16.82	46.58±6.19	66.80±14.71[b]
HDLC4 (mg/dL)	67.13±16.30	70.41±7.96	75.72±9.80	69.56±7.18	62.76±6.69	74.44±9.08[b]
LDLC3 (mg/dL)	8.27±4.60	7.71±2.28	8.16±1.68	7.51±1.59	6.36±2.25	8.44±0.61
ASTL (U/L)	76.19±6.56	76.65±6.21	83.91±25.81	83.08±7.23	87.04±13.65	87.22±7.71
ALTL (U/L)	41.41±9.22	39.82±7.43	40.20±12.62	42.24±4.06	45.60±10.76	50.08±8.26
ALP2S (U/L)	45.80±4.42	47.70±3.95	44.40±6.20	46.60±6.33	39.80±3.35	39.40±1.34
TP2 (g/dL)	6.89±0.14	7.09±0.20	7.20±0.23[a]	7.10±0.20	6.87±0.15	7.12±0.19
ALB2 (g/dL)	5.22±0.12	5.35±0.20	5.44±0.21[a]	5.40±0.24	5.23±0.11	5.47±0.18[b]
GLO (g/dL)	1.66±0.13	1.74±0.15	1.75±0.07	1.70±0.13	1.65±0.14	1.66±0.12
Na (mmol/L)	146.30±1.70	145.70±1.16	146.70±2.31	145.30±1.16	146.20±1.30	147.00±1.58
K (m mol/L)	10.27±1.02	9.78±0.61	10.31±1.62	10.69±0.93	10.26±0.64	10.75±0.83
Cl (mmol/L)	105.98±1.64	103.91±1.07[a]	103.38±1.28[a]	103.83±2.01[a]	105.50±0.93	105.32±1.32

Note: Values are average ± standard deviation

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

[b] The average is statistically significant difference of control-recovery group ($p \leq 0.05$)

Table 6 Effect of Kratom leaves aqueous extract on hematological analysis result in male animal treated for 90 days (n = 50)

Parameter	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
RBC (M/μL)	9.94±0.30	9.90±0.54	9.53±0.25[a]	9.98±0.65	9.64±0.23	9.84±0.47
HGB (g/dL)	17.61±0.42	17.38±0.93	17.00±0.29[a]	17.56±0.96	17.02±0.31	17.14±1.09
HCT (%)	55.43±1.54	54.54±2.98	53.79±1.00	55.43±3.37	53.34±0.91	53.50±3.44
MCV (fL)	55.75±0.78	55.10±1.31	56.50±1.27	55.57±1.31	55.34±1.27	54.36±2.48
MCH (pg)	17.72±0.32	17.57±0.39	17.85±0.39	17.62±0.34	17.66±0.35	17.44±0.82
MCHC (g/dL)	31.77±0.29	31.88±0.27	31.62±0.23	31.68±0.39	31.90±0.16	32.02±0.19
PLT (K/μL)	685.20±56.00	702.70±63.36	689.60±41.67	708.67±38.30	680.80±59.23	680.40±61.16
WBC (K/μL)	8.16±0.96	6.87±1.34	8.16±1.39	8.41±1.39	6.91±0.83	6.82±1.02
NEUT (%)	9.93±2.32	11.78±2.63	9.48±1.44	9.87±1.53	11.00±1.21	12.97±2.60
LYMPH (%)	84.07±2.32	82.58±3.25	85.93±2.00	84.99±1.57	83.50±1.32	81.74±2.80
MONO (%)	4.92±0.96	4.62±1.30	3.72±0.62[a]	4.09±0.76	4.20±0.64	4.16±0.79
EO (%)	0.89±0.38	0.86±0.10	0.70±0.26	0.83±0.37	1.18±0.69	0.82±0.36
BASO (%)	0.19±0.24	0.16±0.08	0.17±0.14	0.22±0.14	0.12±0.13	0.30±0.25

Note: Values are average ± standard deviation

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

Table 7 Effect of Kratom leaves aqueous extract on hematological analysis result in female animal treated for 90 days (n = 50)

Parameter	Main Group				Recovery Group	
	Control (Sterile water)	Low (250 mg/kg BW)	Medium (500 mg/kg BW)	High (1,000 mg/kg BW)	Control (Sterile water)	High (1,000 mg/kg BW)
RBC (M/μL)	9.00±0.48	9.37±0.30	9.48±0.34[a]	9.48±0.36[a]	9.46±0.47	9.80±0.59
HGB (g/dL)	16.81±0.88	17.31±0.32	17.85±0.56[a]	17.70±0.56[a]	17.64±1.01	18.34±1.11
HCT (%)	52.09±3.12	53.73±1.26	55.28±1.90	54.89±1.89	54.26±3.21	57.16±4.17
MCV (fL)	57.88±1.84	57.37±0.89	58.36±1.27	57.91±1.03	57.34±1.01	58.26±1.06
MCH (pg)	18.68±0.52	18.49±0.30	18.86±0.46	18.68±0.36	18.62±0.31	18.68±0.16
MCHC (g/dL)	32.28±0.37	32.23±0.24	32.29±0.24	32.24±0.24	32.52±0.11	32.12±0.43
PLT (K/μL)	690.60±56.84	733.00±40.16	710.40±58.58	653.90±88.84	701.60±45.93	691.00±93.69
WBC (K/μL)	4.31±0.62	5.11±0.99	5.85±0.60[a]	6.53±0.70[a]	4.31±0.80	4.34±1.03
NEUT (%)	8.59±2.18	9.89±2.26	8.12±2.42	8.14±1.81	8.90±1.44	8.70±2.29
LYMPH (%)	85.52±3.15	85.10±2.89	85.79±2.95	86.20±2.59	85.64±1.58	85.28±2.58
MONO (%)	4.81±0.87	4.19±0.80	5.26±1.22	4.93±1.07	4.44±0.47	5.30±1.41
EO (%)	0.78±0.29	0.58±0.18	0.54±0.16	0.49±0.22	0.68±0.24	0.62±0.08
BASO (%)	0.30±0.34	0.24±0.18	0.29±0.23	0.24±0.11	0.34±0.24	0.10±0.14

Note: Values are average ± standard deviation

[a] The average is statistically significant difference of control group ($p \leq 0.05$)

a total of 90 days, and the results showed that there was no mortality caused by the toxicity of the test substance up to the dose level of 1,000 mg/kg body weight. The body weights continued to gain throughout the study and were statistically significant differences but were not related to the dose of the test substance, including feed and water consumption. The feed and water consumption of animals is a normal, transient change that was not related to animal health.

The clinical biochemistry analysis results showed that U-BUN, CREA2, ASTL, ALTL, TP2, ALB2, K, and Cl in the main group and ALB2, HDLC4, TRIGL, CHO2I, and Na in the recovery group showed statistically significant differences. The results of the clinical analysis showed similarity with previous studies.[15,16,25,26] The hematological analysis result showed that RBC, HGB, WBC, and MONO showed statistically significant differences, but only the WBC of female Wistar rats was related to doses of the test substance. However, the increase in WBC levels in the moderate and high dose groups was still within the historical control range.[27,28]

Other studies showed that the hematological data, which contained RBC[16], HGB[16], and WBC[16,25] showed statistically significant differences, as in this study. But parameters NEU[16,25], HCT[16], PLT[25], and LYM[25] showed a statistically significant difference.

For pathological evaluation, according to the macroscopic lesions at necropsy findings, the minimal to moderate focal thymic hemorrhage might be attributed to necropsy technique and considered a dissection-induced artifact, and there was no evidence of a vascular lesion.[29] The mild to moderate dilation of the uterine lumen and the endometrial and

glandular epithelial degeneration and necrosis were physiological changes during the proestrus and estrus stage, respectively, of the estrus cycle.[30-32] The severe gastric dilation and red discoloration of the peripheral area of both caudal lung lobes showed no remarkable alteration on microscopic examination. The kidneys revealed a minimal focal hyaline cast, which was characterized by homogeneous eosinophilic material (tubular proteinosis) filling the tubular lumen and occurred incidentally at low severity and frequency.[33] In the liver, minimal focal mononuclear cell infiltration at periportal areas without associated alteration of adjacent hepatocytes was considered a background lesion and occurred spontaneously.[34] All findings were not considered to be treatment-related changes. Some studies found the abnormalities or changes in the internal organs of the animals, such as liver[14-16,24,26], kidney[14-16,25,26], heart[25], lung[15,25], and brain[16] In other studies for toxicity in accordance with Organization for Economic Co-operation and Development (OECD) guideline No. 407[35], it was reported that the Sprague-Dawley rats were orally administered test substances for 28 days with dose levels at 100, 200, and 500 mg/kg body weight[15], and 1, 10, and 100 mg/kg body weight[16], including 10, 50, and 150 mg/kg body weight.[36] The results for all studies mentioned above showed no deaths in rats during the 28-day period but showed some signs of toxicity, such as a toxic effect on the liver, kidney, and lung with abnormal values in biochemical analysis such as AST, creatinine, etc., as presented by Ilmie MU., et al[15] Sabetghadam A., et al[16] described that mitragynine was relatively safe at lower doses (1–10 mg/kg) and exhibited toxicity at the highest dose (100 mg/kg), which was confirmed by liver, kidney, and brain histopathological changed. In addition, Hassan Z., et al[36] explained evidence of toxicity from histological investigation of the kidney, liver, and brain tissues, including a low platelet count in hematological analysis and a high amount of uric acid, AST, ALT, and alkaline phosphatase in biochemical analysis. However, chronic preclinical toxicological investigations of mitragynine for a longer period are recommended to confirm this observation and also to provide adequate safety data for future clinical trials.

Conclusion

In conclusion, there were no related signs of the acute toxicity arising from the orally administration of Kratom leaves aqueous extract to Wistar rats. The result suggested that the test substance could be classified in GHS category 5 or unclassified, with the LD_{50} cutoff at 5,000–∞ mg/kg body weight. However, sub-chronic and chronic toxicity studies should be further carried out to assess the long-term safety of the test substance. The results of the sub-chronic oral toxicity study were shown that no mortality in the animals was caused by the toxicity of the test substance up to the dose level of 1,000 mg/kg body weight. In addition, the evidence from the test item-body weight, food and water consumption, hematological, clinical biochemistry, relative organ weight, and histopathological effects could confirm that

Kratom leaves aqueous extract did not show any signs of toxicity. Thus, the no observed adverse effect level (NOAEL) of Kratom leaves aqueous extract was considered to be 1,000 mg/kg body weight per day for Wistar rats under experimental conditions.

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Conflict of interest

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ความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรังทางปากของสารสกัดน้ำจากใบกระท่อม ในหนูแรทสายพันธุ์ Wistar

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บทคัดย่อ กระท่อมถูกใช้แพร่หลายในด้านเภสัชกรรมและการแพทย์ รวมถึงอาหารหรือผลิตภัณฑ์เสริมอาหาร ในขณะที่ข้อมูลการทดสอบทางพิษวิทยามีการดำเนินการในประเทศไทยไม่มากนัก อีกทั้งสามารถซื้อขายได้ง่ายในปัจจุบัน การศึกษานี้มีวัตถุประสงค์เพื่อประเมินความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรังทางปากของสารสกัดน้ำจากใบกระท่อมในหนูแรทสายพันธุ์ Wistar เพื่อให้ได้ข้อมูลที่เป็นประโยชน์ต่อการขึ้นทะเบียนผลิตภัณฑ์ รวมทั้งสร้างความมั่นใจและความปลอดภัยของผลิตภัณฑ์แก่ผู้บริโภค ผลการทดสอบพบว่าที่ขนาด 300 และ 2,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง ไม่ก่อให้เกิดความเป็นพิษเฉียบพลันและการตายในสัตว์ทดลอง แสดงให้เห็นว่าสารนี้จัดอยู่ในระบบการจัดกลุ่มสารเคมี การติดฉลาก และการแสดงรายละเอียดบนเอกสารข้อมูลความปลอดภัยสากลหมวดที่ 5 หรือไม่ระบุประเภท มี LD_{50} ที่ 5,000 มิลลิกรัมต่อกิโลกรัมขึ้นไปของน้ำหนักตัวสัตว์ทดลอง หลังจากนั้นได้ทำการทดสอบความเป็นพิษทางปากแบบกึ่งเรื้อรังที่ขนาด 250, 500 และ 1,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง สรุปได้ว่าปริมาณที่มากที่สุดที่ได้รับต่อเนื่องเป็นเวลานานโดยไม่ก่อให้เกิดอันตรายใด ๆ ต่อร่างกายต่อวันในสัตว์ทดลอง คือ 1,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัวสัตว์ทดลอง การศึกษานี้ทำให้ได้ข้อมูลความเป็นพิษของสารทดสอบดังกล่าวที่มีคุณค่า ซึ่งจำเป็นและสามารถสนับสนุนการศึกษาในอนาคต โดยเฉพาะการศึกษาความเป็นพิษของผลิตภัณฑ์กระท่อมที่ผลิตและจำหน่ายในประเทศไทย ในอนาคตการศึกษาเพิ่มเติมด้านความเป็นพิษเรื้อรังจะใช้ในการประเมินผลความปลอดภัยในการใช้ระยะยาว

คำสำคัญ: *Mitragyna speciosa* (Korth.) Havil., สารสกัดน้ำจากใบกระท่อม, การทดสอบความเป็นพิษเฉียบพลันและพิษกึ่งเรื้อรัง