

Acute and Sub-acute Toxicities of Thai Silkworm Powder (*Bombyx mori* Linn.) From Three Races in Male Wistar Rats and *In vitro* Antioxidant Activities

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ABSTRACT

Background: The silkworm powder from Japanese and Korean races has a high biological activity. However, there was less scientific evidence regarding bioactivities in Thai silkworm races. **Objective:** This research was interested in toxicity and antioxidant activities of Thai silkworm races. **Material and methods:** The 5th instar, 3rd day of growth stage of Thai traditional silkworm (Nanglai, Nangnoi and Samrong races) were lyophilized by freeze dryer and ground as silkworm powder. The acute and sub-acute toxicities studies were carried out in male Wistar rats. The antioxidant capacities of silkworm powder were investigated with DPPH, ABTS and FRAP methods. **Results:** The acute toxicity in rats by single dose oral administration show that the high dose at a concentration of 2,000 mg/kg b.w. did not have toxicity in rats. The sub-acute toxicity in rats by oral administration showed that the high dose concentration at 2,000 mg/kg body weight for 6 weeks had no toxicity in all group either. In addition, the hematological parameters and lipid profiles of treated group and control groups were not significantly different. The antioxidant study showed that silkworm powders were not significant difference in all groups of samples in three assays (DPPH, ABTS and FRAP). However, significant difference had been found in comparison to ascorbic acid ($p < 0.05$). **Conclusion:** Silkworm powder from three races of Thai silkworm did not produce any toxicity in male rats. In addition, the silkworm powder also exhibited antioxidant activities. **Keywords:** Silkworm, toxicities, hematological parameters, lipid profile, antioxidant activities.

INTRODUCTION

The biological activities of silkworm such as anti-hypertensive,¹ anticancer,² antibacterial,³ antiviral,⁴ antioxidant⁵ and hepatoprotective activities⁶ have been reported. Mulberry silkworm (*Bombyx mori* L.) has been reared in especially the northeastern part of Thailand for longtime.⁷ Silkworms are consumed mostly as functional food and pharmaceuticals for diabetes mellitus patient in Korea, China, and Japan.⁸ The major functional compound of silkworm powder was 1-deoxynojirimycin (DNJ) acting as alpha-glucosidase inhibitor.⁹ Therefore, the result from previous studies showed that silkworm powders were tended to be potential functional food. The safety of silkworm were still limited that only Korean silkworm races had been reported.⁸ However, Thai silkworm races had no scientific data regarding the safety for consumption. In order to make Thai silkworm powder widely used as food product, the safety should be investigated. This study was aimed to investigated the acute and sub-acute toxicities in male Wistar rat and *in vitro* antioxidant activities of three Thai silkworm races.

MATERIALS AND METHODS

Chemicals

Reagents of high quality were obtained from Sigma-Aldrich Chemical (St Louis, MO). Other chemical of analytical grade were purchased from Carlo Erba, Italy and Merck, Germany.

Preparation of silkworm powder

Three varieties of Thai mulberry silkworms including Nangnoi, Nanglai, and Samrong were reared at Silk Innovation Center, Maharakham University, Thailand. Identifications of silkworm races were performed with regard to the Queen Sirikit Department of Sericulture protocol. The 5th instar, 3rd day silk larvae were prepared according to the study of Ryu *et al.*¹⁰ The silkworms were then freeze-dried and ground in order to obtain fine powders. Silkworm powders were kept in freezer before use.

Animals

Male albino Wistar rats with weight of 150-200 g were purchased from the National Laboratory Animal Centre (NLAC), Mahidol University, Thailand. The rats were housed under standard environmental

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conditions (at $25 \pm 2^\circ\text{C}$, 40-60 % humidity with 12 h light/12 h dark cycle).¹¹ All animals were given a standard laboratory diet with access to water *ad libitum*. The experimental protocol and the experiments performed on the rats were approved by the Institutional Animal Care and Use Ethic Committee, Mahasarakham University, Thailand (License No. 0015/2011).

Acute toxicity test

The rats were divided into 10 groups, 6 rats per group, were fasted overnight (12 h) and weighed. The doses of silkworm powder from 3 races were calculated according to the body weight of fasted animals and administered at doses of 1000, 1500 and 2000 mg/kg body weight. The animals were regularly and individually observed for behavioral changes and general toxicity signs after dosing for the first 24 h, with special attention being given during the first 4 h. Thereafter, observation was continued daily for a total of 14 days. Mortality, body weights, bizarre behavior and abnormal sign including seizures, urination, vomiting, anorexia or depression were recorded.¹²

Sub-acute toxicity test

The rats were divided into 10 groups, 6 rats per group, were treated orally with silkworm powder (two days per dose) for 6 weeks. Silkworm powders were administered at 2000 mg/kg body weight in each silkworm races. Mortality, body weights and blood glucose level were recorded for every week. Hematological values and lipid profile were recorded at last week.¹²

Antioxidant activity

DPPH radical scavenging assay - The test was carried out using the 1,1-diphenyl-2-picryl hydrazyl (DPPH) free radical scavenging assay. The free radical scavenging activity of the silkworm powder and standard ascorbic acid solution in absolute ethanol were determined based on their ability to react with the DPPH free radical.¹³ A 750 μL aliquot of silkworm powder (10 - 1,000 $\mu\text{g}/\text{mL}$, dissolved in 10% DMSO and filtered) was added into 750 μL of DPPH in absolute ethanol (152 μM). After incubation at room temperature in the dark condition for 20 min, the absorbance of each solution was determined at 517 nm using 96-well plates with microtiter plate reader (BMG LABTECH GmbH, Ortenberg, Germany). The results were presented as a percentage of inhibition as follows:

$$\% \text{inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} was an absorbance of DPPH solution without sample. A_{sample} was an absorbance of DPPH solution with sample.

The inhibitory concentration of the sample requires scavenging DPPH radical by 50% (IC_{50} value) and obtaining a linear regression analysis of a calibration curve plotting, %inhibition versus concentration.

ABTS radical scavenging assay - 2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) diammonium salt radical cationic decolorization test was performed using spectrophotometric method from the previous studied¹⁴ with slight modification. The ABTS stock solution was prepared by mixing 88 μL of 140mM potassium persulfate ($\text{K}_2\text{S}_2\text{O}_8$) with 5 mL of 7mM of ABTS stock solution (pH 7.4). The working ABTS reagent was prepared by diluting the stock solution with ethanol to obtain absorbance of 0.7 ± 0.05 at 734 nm. The test concentrations of standard ascorbic acid, silkworm powders were prepared in the same manner as previously described in DPPH assay. The ABTS working solution (100 μL) with equal volume of ethanol served as a control. The reaction mixtures were incubated at room temperature for 30 min. The absorbance of each solution was determined at 734 nm using 96-well plates with microtiter plate reader (BMG LABTECH GmbH, Ortenberg, Germany). The results were expressed as percentage of inhibition as follows:

$$\% \text{inhibition} = [(A_{\text{control}} - A_{\text{sample}}) / A_{\text{control}}] \times 100$$

Where A_{control} was an absorbance of ABTS solution with ethanol. A_{sample} was an absorbance of ABTS solution with sample.

The effective concentration of sample requires to scavenge ABTS radical by 50% (EC_{50} value) and obtaining by a linear regression analysis of a calibration curve plotting % inhibition versus concentration.

FRAP assay (Ferric reducing antioxidant power) - FRAP assay was carried out with some modification from Benzie and Strain.¹⁵ The FRAP reagent was prepared from 300 mM acetate buffer (pH 3.6), 20 mM ferric chloride and 10 mM 2,4,6-Tris(2-pyridyl)-s-triazine (TPTZ) in 40 mM HCl. All 3 solutions were mixed together in the ratio of 10:1:1 (v/v/v), respectively. The FRAP reagent was freshly prepared daily prior to use. A total of 30 μL sample was added to 270 μL of the FRAP reagent and mixed well. After 30 min at room temperature incubation, the absorbances of final solutions were subsequently measured at 593 nm in 3 replicates using microplate reader. The antioxidant capacity of sample was calculated and compared with trolox calibration curve, and expressed as μmol trolox equivalent (TE) per gram of silkworm powders.

Statistical analysis

All data were expressed as mean \pm standard error of mean (SEM). The investigated parameters of each sample were evaluated in triplicate. The statistical analyses of the data were carried out by analysis of variance (ANOVA), using the statistical package SPSS 15 and the Duncan's multiple range test (DMRT) to indicate the statistical difference at P -value < 0.05 .

RESULTS

The acute and sub-acute toxicity

For acute toxicity study, the rats were single administered silkworm powder from 3 races at dose of 1,000, 1,500 and 2,000 mg/kg b.w. The mortality and behavioral sign and symptoms of toxicity were observed within 24 h and further for 14 days. During this experiment, rats body weights were measured as shown in Table 1. The result indicated that no dead animals and toxicity symptoms were found. In additions, the body weights of control and treated rats tended to increase week by week without significant difference among groups.

For sub-acute toxicity, the rats were administered every two days with silkworm powder from three races at a dose of 2,000 mg/kg b.w. During the experiment, body weight and blood glucose levels were measured weekly for 6 weeks (Table 2 and 3). At the end of the experiments, blood chemistry parameters and hematological values were analyzed (Table 4 and 5). The results showed that body weights of control and treated rats were not different but tend to be increased week by week. The body weights among control and silkworm powder treated rats were not significantly different.

On the other hands, blood glucose levels and blood chemistry parameters of the control and silkworm powder treated rats were not different. This finding suggested that silkworm powder did not alter organ system function in male rats and did not exhibit any toxicity in rats.

Antioxidant activities

The results of antioxidant activities of three races silkworm powders tested by DPPH, ABTS and FRAP methods were showed in Table 6. The antioxidant study indicated that the capacities of silkworm powders were not significant difference in all groups of samples in three assays (DPPH, ABTS and FRAP). However, significant difference has been found comparing with ascorbic acid ($p > 0.05$). It was found that Thai silkworm from three races have less antioxidant capacity than those of ascorbic acid.

Table 1: Body weight (g) of rats in the acute toxicity study (n=6)

Treatment	Week0	Week1	Week2
Nanglai-1000 mg	190.66 ± 6.00	200.00 ± 8.16	215.00 ± 7.63
Nanglai-1500 mg	181.66 ± 3.07	200.00 ± 5.77	210.00 ± 4.47
Nanglai-2000 mg	171.66 ± 6.45	180.00 ± 4.08	193.33 ± 4.65
Sumrong-1000 mg	188.33 ± 4.08	206.66 ± 4.47	230.00 ± 2.88
Sumrong-1500 mg	180.00 ± 4.65	183.33 ± 3.41	200.00 ± 3.65
Sumrong-2000 mg	185.00 ± 4.65	198.33 ± 5.00	216.66 ± 4.65
Nangnoi-1000 mg	181.66 ± 2.88	221.66 ± 4.08	251.66 ± 3.41
Nangnoi-1500 mg	188.33 ± 4.08	206.66 ± 4.47	228.33 ± 4.47
Nangnoi-2000 mg	191.66 ± 5.32	208.33 ± 3.41	221.66 ± 4.47
normal (0.5% Tween 80)	186.66 ± 3.41	201.66 ± 3.41	218.33 ± 3.41

Table 2: Body weight (g) of rats in sub-acute toxicity study (n= 6)

Treatment 2,000 mg/kg b.w.	week0	week1	week2	week3	week4	week5	week6
Nanglai-worm	180.66 ± 6.00	220.00 ± 8.16	300.00 ± 7.63	310.33 ± 7.14	320.66 ± 6.14	350.33 ± 5.57	360.00 ± 3.65
Sumrong-worm	181.66 ± 3.07	280.00 ± 5.77	310.00 ± 4.47	310.66 ± 3.07	340.66 ± 3.33	360.66 ± 3.07	380.33 ± 3.33
Nangnoi-worm	171.66 ± 6.45	260.00 ± 4.08	283.33 ± 4.65	303.33 ± 2.23	321.66 ± 3.41	370.33 ± 1.82	390.33 ± 2.88
normal (0.5% Tween 80)	198.33 ± 3.41	255.00 ± 4.65	321.66 ± 3.41	348.33 ± 4.65	383.33 ± 2.23	423.33 ± 2.23	435.00 ± 4.65

Table 3: Blood glucose level (mg/dL) of rats in sub-acute toxicity study (n = 6)

Treatment 2,000 mg/kg b.w.	week0	week1	week2	week3	week4	week5	week6
Nanglai-worm	104.50 ± 10.08	118.50 ± 7.25	98.50 ± 6.20	106.50 ± 9.03	79.50 ± 4.85	69.50 ± 4.34	81.50 ± 5.12
Sumrong-worm	101.16 ± 5.13	81.33 ± 4.07	79.33 ± 4.07	86.66 ± 4.40	65.50 ± 3.35	69.50 ± 3.49	89.66 ± 4.67
Nangnoi-worm	108.33 ± 12.49	115.66 ± 24.99	62.50 ± 4.96	102.66 ± 13.66	102.50 ± 8.12	101.33 ± 13.50	103.66 ± 8.14
normal (0.5% Tween 80)	79.16 ± 5.18	79.33 ± 5.33	93.50 ± 6.11	78.16 ± 5.18	98.50 ± 5.81	102.66 ± 6.75	92.00 ± 5.38

Table 4: Blood chemistry parameters of rats in sub-acute toxicity study (n=6) at 6th week

Treatment 2,000 mg/kg	BUN (mg/dL)	Creatinine (mg/dL)	Cholesterol (mg/dL)	Triglycerides (mg/dL)	HDL (mg/dL)	LDL (mg/dL)	ALP (mg/dL)
Nanglai-worm	40.73 ± 5.16	1.16 ± 0.13	128.00 ± 9.58	138.33 ± 33.89	26.00 ± 5.47	78.00 ± 12.09	287.00 ± 2.73
Sumrong-worm	39.70 ± 3.88	1.06 ± 0.05	148.00 ± 32.01	177.50 ± 24.64	29.33 ± 4.03	81.00 ± 3.57	329.66 ± 29.6
Nangnoi-worm	40.30 ± 5.91	1.00 ± 0.08	129.00 ± 13.88	118.33 ± 13.57	32.66 ± 4.92	76.33 ± 11.67	285.00 ± 16.23
normal (0.5% Tween 80)	34.56 ± 1.32	0.90 ± 0.08	119.00 ± 7.32	133.00 ± 5.16	30.00 ± 6.26	62.83 ± 7.27	273.00 ± 14.6

Table 5: Hematological parameters of rats in sub-acute toxicity study (n=6) at 6th week

Treatment 2,000 mg/kg	WBC (cells/mm ³)	RBC (10 ⁶ cell/mm ³)	Hb (g/dL)	%Hct	Platelet (cells/mm ³)
Nanglai-worm	3933.34 ± 361.40	8.20 ± 0.88	15.30 ± 6.85	45.66 ± 3.14	654000 ± 12521
Sumrong-worm	4333.00 ± 223.62	9.40 ± 0.88	17.96 ± 1.61	53.66 ± 4.50	670666 ± 58578
Nangnoi-worm	4332.34 ± 119.10	8.33 ± 0.62	17.73 ± 1.22	52.67 ± 3.61	679333 ± 84177
normal (0.5% Tween 80)	5166.00 ± 310.66	8.23 ± 0.41	16.50 ± 1.84	47.36 ± 9.68	764000 ± 51666

Table 6: Antioxidant activity of Thai silkworm powders from three races

Races	DPPH (IC ₅₀ ; mg/mL)	ABTS (IC ₅₀ ; mg/mL)	FRAP (Trolox equivalent; mmol/mL)
Nanglai	57.08 ± 5.32 ^b	123.08 ± 13.32 ^b	255.20 ± 36.98
Sumrong	39.54 ± 1.08 ^b	131.87 ± 11.00 ^b	248.83 ± 22.45
Nangnoi	42.41 ± 2.65 ^b	146.40 ± 12.12 ^b	243.44 ± 37.25
Ascorbic acid	5.268 ± 0.57 ^a	13.38 ± 0.33 ^a	-

The different letters (a, b) within a column show statistical differences (p<0.05)

DISCUSSION

The Acute and Sub-Acute Toxicity

For the acute toxicity study, rats were administered with single dose of 5th instar, 3rd day silkworm powder of 3 races at doses of 1,000, 1,500 and 2,000 mg/kg body weight. The behavior and body weight were recorded at 0, 7, and 14 days, respectively. For the sub-acute toxicity study, rats were administered with silkworm powders from three races at dose of 2,000 mg/kg body weight, every 2 days for 6 weeks. The result showed that no signs of toxicity or abnormalities in rats, such as seizures, vomiting, ataxia, lethargy, loss of appetite had been observed. This study is the first report on acute and sub-acute toxicities of Thai silkworms. In addition, there were only studies on the nutritional values of silkworm powder, reporting the main components of a protein about 40% in Indian silkworm.¹⁶ The protein components were not different from normal food resulting to low toxicity. Similar to the studies of Mondal *et al*,¹⁷ the substances in the silk powder were about 16-18 amino acids. The study of Ninpetch *et al*¹⁸ analyzed the protein and lipid contents of the fifth instar mulberry silkworm (*Bombyx mori* L.) for substitution of protein from meat. Three silkworm races (Jim thompson, Nanglay and Thai hybrid) showed a rich source of protein. Jim Thompson race had 68.97 % of protein, Nanglay race had 55.55 % of protein and Thai hybrid race had 54.19 % of protein.

The Antioxidant Activity

The antioxidant study indicated that silkworm powders were not significant difference in all groups of samples in three assays (DPPH, ABTS and FRAP). However, significant difference had been observed comparing with ascorbic acid. Antioxidant activities of mulberry silkworm may derive from some pigments in cocoon shell sericin. The coloring components are associated with phenolic compounds in the leaves of mulberry (*Morus alba* L.), the major feed for mulberry silkworm larvae. The two main pigments responsible for cocoon color are ether-soluble yellowish carotenoids and water-soluble green flavonoids.¹⁹⁻²⁰ The studies of Tamura *et al*¹⁹ isolated quercetin 5-glucoside, quercetin 5,7,4'-triglucoside and quercetin derivatives from the yellow-green cocoon shell of Multi-Bi race. The previous study showed that there are several native races of silkworm in Thailand with various color of cocoon shell. The most common native Thai silkworm is Nangnoi with intent yellow, while the color of cocoon shell from other races are varied from white to green yellow. Flavonoid compounds are also responsible for antioxidant properties of mulberry silk cocoon. The studies of Butkhup *et al*²¹ showed that the strong correlation between total active ingredients (phenolics and flavonoids) and antioxidant activities (DPPH, ABTS and FRAP assay) had been observed in Thai silk sericin.

CONCLUSION

Acute and sub-acute toxicities of silkworms on hematological parameters and lipid profiles had not been observed. Silkworms also present antioxidant capacities.

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CONFLICT OF INTEREST

Authors declare no conflict of interest.

ABBREVIATIONS USED

BUN: blood urea nitrogen content; **Creatinine:** creatinine phosphate content; **Cholesterol:** blood cholesterol content; **Triglycerides:** blood triglyceride content; **HDL:** high density lipoprotein cholesterol; **LDL:** low density lipoprotein cholesterol; **ALP:** alkaline phosphatase; **WBC:** white blood cell content; **RBC:** red blood cell content; **Hb:** hemoglobin; **%Hct:** %hematocrit; **Platelet:** platelet content.

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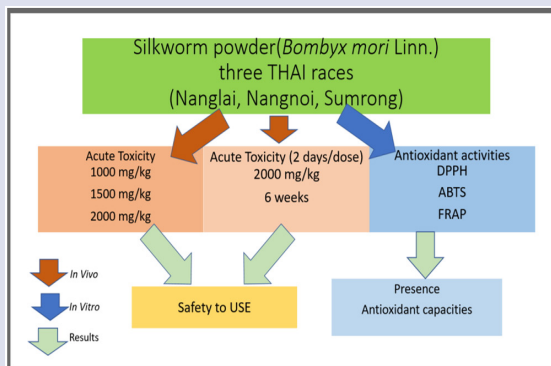
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GRAPHICAL ABSTRACT



HIGHLIGHTS OF PAPER

- The no acute toxicity in rats by single dose oral administration presented that the high dose at a concentration of 2,000 mg/kg body weight.
- The sub-acute toxicity in 2,000 mg/kg body weight for 6 consecutive weeks had no toxicity.
- The silkworm powders present antioxidant activity in three assays (DPPH, ABTS and FRAP).

ABOUT AUTHOR



Mr. Surapong Rattana: Obtained his M.Sc. degree in 2012 from Faculty of Pharmacy, Mahasarakham University, Thailand. Currently, He is Ph.D candidate in Biology major, Faculty of Science, Mahasarakham University, Thailand. Mr. Rattana is working on various aspect of medicinal plant related separation and isolation of both pure active compound and crude drug from herb for toxicity and biological activities test to finding therapeutic valuable of medicinal plant.

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