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EFFECTS OF PHYTOESTROGEN FROM WHITE KWAO-KRUA (*Peuraria mirifica*) ON THE DEVELOPMENTAL STAGES OF THAI MULTIVOTINE SILKWORM (*Bombyx mori*)

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ABSTRACT

This experiment was carried out to evaluate the effects of ethanolic extracts of phytoestrogen - rich plant, white Kwao-Krua (*Peuraria mirifica*) on toxicity, larval maturation and cocoon character of Thai Multivotine Silkworm, *Bombyx mori*. The toxicity (LC_{50}) at 96 hours was 9.51 % w/v (r^2= 0.94) after applying the extracts on mulberry leaves before feeding the 48 - hour old fifth in star silkworm. In addition, the crude extracts were administered topically on mulberry at 3 concentrations, 1%, 5%, and 10% w/v on the 48 -hour old fifth instar silkworm. The results showed decreased weight of larvae, pupae and at 5% and 10% w/v. However, the larval, cocoon and shell cocoon weights at 1% w/v treatment increased. Furthermore, the larval period was shortened up to 24 hours of all larval trialed. The results suggest that the extracts containing phytoestrogen may affect on growth and molting of silkworm. The physiology and biochemical mechanisms of phytoestrogen from this plant are discussed.

KEYWORDS: phytoestrogen, *Peuraria mirifica*, developmental stages**,** *Bombyx mori*

1. INTRODUCTION

In recent years, there are many reports demonstrated that vertebrate hormone in silkworm, *Bombyx mori*, enhanced silk production. The estrogen or estradiol-17β is one of the vertebrate hormones that had been reported that may act specifically in silk gland synthetic activity, silk protein synthesis and nuclear mediated way [1].

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Moreover, there was reported that it can effected in a significant enhancement of cocoon characters and qualities. Thus, the previous investigation offers a system for investigating the unique function of it in *B. mori* and offers potential for improvement of silk production [2].

However, exogenous estradiol-17β hasn't been used in Thailand's sericulture because it is expensive and it has to import from abroad. From this reason make ous considered some chemical instead of estrogen. Thus, in this study we have an idea to use phytoestrogen, in which it compounds are functional similar as animal estrogen, to study on silkworm. *Pueraria mirifica* (Leguminosae), a Thai herbal plant, containing a high amount of phytoestrogens was a choice of interest for this study. Also, Sompose *et al.* [3] reported *P. mirifica* powder could effect on growth and increase eggs laid of female moth when treated at the forth and fifth instar larvae of silkworm.

However, effects of *P. mirifica* crude extract have not been yet studied in *B. mori*. Thus, in this research we have studied the effect of ethanolic crude extraction of *P. mirifica* in the Thai Multivotine Silkworm 5th instar on toxicity, development and cocoon's character. The results from this study has been undertaken to understand the important role in physiological activities of silkworm larvae and its data could be applied for the benefit of sericulture.

2. MATERIALS AND METHODS

2.1 Experimental Animals

Disease-free eggs of the indigenous multivotine silkworm were used in the present investigation. Silkworms were reared under standard conditions at 26±2 °C, 70–85% RH and 12L: 12D photoperiod. The mulberry leaves harvested from the irrigated mulberry garden were used as food for silkworm: Silkworm were fed three times daily. After the third ecdysis, larvae were divided into 4 groups and reared under these same conditions.

2.2 Plant Materials and Preparation of Ethanolic Extract of the Plants

The tuberous roots of *Pueraria mirifica*, family Leguminosae were collected in the Kanjanabury tropical rainforest, 180 klilometers west of Bangkok. The collected plant materials were washed, sliced and completely dried in a hot-air oven at 70 °C. The dried materials was ground into small powder and subsequently extracted with ethanol at room temperature for 7 days. The ethanol solution was filtered and evaporated under vacuum to give ethanolic extract [4]. The weight/weight yields in terms of dry starting material were used in this experiment.

2.3 Experimental Procedures

This research used Completely Randomized Design with 3 replicated and 33 sampling unit each replicate. In the experiments, 3 concentration of crude were treated on the 48 hrs of fifth instar larvae. The experiment has been divided to 2 treatments, toxicity in term LC_{50} and development and cocoon character. In toxicity, crude was dissolved in distilled water and mulberry leaves were sprayed in the each concentration, 0, 0.5, 1, 5, 10, 15 and 20 % w/v and allowed a few minutes for water evaporation before being given to silkworms in second day (48 hrs) of the fifth instar larvae. While, in development and cocoon character, crude was dissolved in distilled water and mulberry leaves were sprayed in the each concentration, 0, 1, 5 and 10 % w/v and allowed a few minutes for water evaporation before being given to silkworms in second day (48 hrs) of the fifth instar larvae.

2.4 Silkworm Growth and Cocoon Characters of the Silkworm

Post-treatment, the larval and cocoon parameters were observed. The weights of larvae were determined in different days (3rd, 5th and 7th). Cocoon weights, cocoon shell weights, percentage spinning (larval duration) were determined between trial and control.

2.5 Data Analysis

CRD with five replicates is set for evaluation of data under laboratory. Statistical significance of the results was determined by analysis of variance (ANOVA) or Student's *t* test. For some experiments, ANOVA was followed by DMRT multiple comparisons test as described in Visetson [5].

3. RESULTS

3.1 Toxicity in Term of LC_{50} at 96 Hour and Behavior of the Fifth Instar Larval

The mortality percentage at 96 hours of fifth instar larval of *B. mori* after administrated by ethanolic crude extracts of *P. mirifica* 7 concentrations, 0 (distilled water: control), 0.5, 1, 5, 10, 15, 20 % w/v are 0±0, 0±0, 0±0, 23.67±4.04, 72.69±7.02, 82.33±5.50 and 92.67±5.033 respectively (Table 1 and Figure 1). The simple linear regration of this result is Y= 0.489 + 5.202 X (X means percent concentrations of *P. mirifica* extract and Y means mortality percentage of treated in fifth instar laval of *B. mori*). Therefore, LC_{50}-values at 96 hours of *B. mori* after treated with ethanolic extract are 9.51% w/v with correlation coefficient is 0.94. The mortality percentage values differ among groups and increase with differently significant at 0.05 level ($P<0.05$) when using Duncan's Multiple Rang Test.

Table 1 Percentage mortality of fifth instar larval against ethanolic crude extract of *P. mirifica* after 96 hour under the laboratory

Concentration (%w/v)	Total treated	No. replicate	% average mortality at 96 hr[(2)]
0[(1)]	100	3	0±0[a]
0.5	100	3	0±0[a]
1	100	3	0±0[a]
5	100	3	23.67±4.04[b]
10	100	3	72.69±7.02[c]
15	100	3	82.33±5.50[d]
20	100	3	92.67±5.03[e]

[(1)] Control = distilled water

[(2)] Means ± SD followed by different letters are significantly different ($p<0.05$) level by Duncan's Multiple Rang Test.

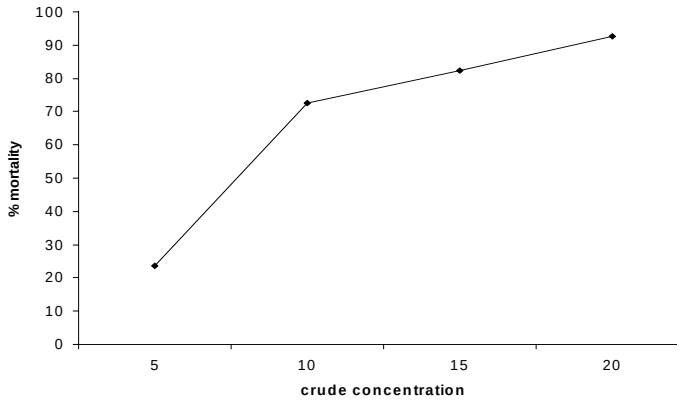

Figure 1 Mortality percentage of fifth instar larval against crude extract of ethanolic crude extraction of *P. mirifica* at the 96 hour under the laboratory condition

The behavior of larval after administration of crude extracts at 5%, 10%, 15% and 20% showed decrease obviously ingest mulberry leaves and slowly moved when compare to control and 0.5 , 1% of crude extract. All of mortal larval were diarrhea also.

3.2 The Effect of Ethanolic Extraction on the Silkworm Larval Weight (g). The larval weight of fifth instar larval of *B. mori* after administrated in 48 hour old fifth instar larval by ethanolic crude extracts of *P. mirifica* 4 concentrations, 0 (distilled water: control), 1, 5, 10, % w/v showed increased significantly as the days increased, in control and 1% w/v when compare with 5,10 % w/v (Table 2). However, there are a non significant different between the growth rate of control and 1 % w/v, 5 and 10 % w/v trialed (Table 2).

Table 2 The effect of ethanolic crude extracts of *P. mirifica* on larval weight

Concentration (% w/v)	Total treated	No. replicate	Weight of 5th instar larvae (g)			
			Before	3rd day	5th day	7th day
0(1)	100	3	0.303±0.19[a]	0.679±0.10[a]	1.091±0.15[a]	1.891±0.15[a]
1	100	3	0.300±0.19[a]	0.724±0.10[a]	1.061±0.12[a]	1.931±0.16[a]
5	100	3	0.314±0.12[a]	0.463±0.12[b]	0.756±0.25[b]	0.823±0.10[b]
10	100	3	0.303±0.14[a]	0.453±0.10[b]	0.755±0.16[b]	0.815±0.12[b]

(1)Control = distilled water

(2)Means ±SD followed by common letters in the same column are not significantly different ($p<0.05$) level by Duncan's Multiple Rang Test.

3.3 The Effect of Ethanolic Crude Extraction on the Cocoon Characters

The cocoon weight, cocoon shell weight and pupa weight in this study were divided by sex, female and male. The female cocoon weight and cocoon shell weight were decrease significantly in 5, 10% w/v when compared to control and 1% w/v but not significant different in male. All of treat groups are not significantly different in weight of pupae (Table 3).

Table 3 The effect of ethanolic crude extracts of *P. mirifica* on cocoon parameters

Character	Sex	Concentration (%w/v)			
		0(1)	1	5	10
Cocoon weight (g)	Female	0.699±0.02[a]	0.700±0.35[a]	0.588±0.33[b]	0.599±0.01[b]
	Male	0.509±0.01[a]	0.525±0.02[a]	0.518±0.04[a]	0.524±0.01[a]
Cocoon shell weight (g)	Female	0.07±0.01[a]	0.080±0.00[a]	0.059±0.00[b]	0.610±0.00[b]
	Male	0.064±0.01[a]	0.064±0.01[a]	0.057±0.00[a]	0.067±0.00[a]
Pupa weight (g)	Female	0.644±0.09[a]	0.601±0.01[a]	0.493±0.05[a]	0.538±0.01[a]
	Male	0.505±0.11[a]	0.456±0.02[a]	0.456±0.09[a]	0.456±0.01[a]

(1)Control = distilled water.

(2)Means±SD followed by common letters in the same column are not significantly different ($p<0.05$) level by Duncan's Multiple Rang Test.

3.4 The Effect of Ethanolic Crude Extraction on the Day of Spinning

The day of begin spinning was observed in control and trails. The spinning in 8th day fifth instar larval found in only trials and 1% w/v was increase significantly ($P < 0.05$) different between trials. The spinning of control began in the 9th fifth instar larval while trial spinning continued and completely cocooning in 10th day (Table 4).

Table 4 The effect of ethanolic crude extracts of *P. mirifica* on percentage of spinning

Day of begin spinning	Percentage of spinning (N=30, 3 replication) Concentration (%w/v)			
	0[(1)]	1	5	10
8th day	0.00±0.00[a]	43.33±5.77[b]	13.33±5.77[c]	3.33±5.77[a]
9th day	56.66±5.77[a]	53.33±5.77[a]	33.33±5.77[b]	56.66±5.77[a]
10th day	43.33±5.77[a]	3.33±5.77[b]	53.33±5.77[c]	40.00±0.00[a]

[(1)]Control = distilled water.
[(2)]Means±SD followed by common letters in the same column are not significantly different ($p<0.05$) level by Duncan's Multiple Rang Test.

4. DISCUSSION AND CONCLUSIONS

The previous studies reported that estradiol or estrogen hormone was found in ovary and haemolymph of silkworm larval [6-8]. Therefore the exogenous estradiol had been tested in silkworm and the results showed an appropriate dose and time of estradiol could positive effect on silk production and specific in silk gland response [9]. From the previous investigation offers a system for investigating the unique function of estradiol in *B. mori* and offers potential for improvement of silk production [2]. However, exogenous estradiol-17β hasn't been used in Thailand's sericulture because it is expensive and it has to imported from abroad. From this reason considered some chemical instead of estrogen.

Thus, in this study we have an idea to use phytoestrogen, in which it compounds are structurally similar to animal estrogen, to study on silkworm. Among the phytoestrogen-rich plants, *P. mirifica* with a local name of white Kwao Krua might be the most interesting one because it has been used beneficial for medicine, cosmetic and applied to improve production of animal husbandry [10]. The active in gradient to be found in tuberous root was belonging to the group of isoflavonoids which have an active similarity as estrogen [10-11].

Present study has been first reported LC_{50}-values at 96 hours of *B. mori* after spraying on the mulberry leaves with ethanolic crude extract of *P. mirifica*. The LC_{50} was 9.51% w/v with correlation coefficient is 0.94. The mortality percentage values differ among groups and the higher mortality percentage showed at 20 % w/v. From the toxicity test, some dose of crude extracts has been used on study development and cocoon character. The 48 hour old fifth instar larval were administrated by spray on the mulberry leaves. The result showed 5 and 10 % w/v of crude extracts could reduce the weight of larval, pupa, cocoon and shell cocoon in female while male was not different in all trial. As, Sompose reported that weight of larval was not different when tested with the of *P. mirifica* powder at 3 and 5% per weight larvae [3]. But the larvae weight at 10 % *P. mirifica* found decrease. That was the direct effect on decrease silk filament production when used high dose of *P. mirifica*. Furthermore, egg lying in silkworm female moth could be decreased. The behaviors of larval silkworms in this study were slowly move, non-appetite and diarrhea when treated with high dose of *P. mirifica*. Orajarusith reported the effect of *P. mirifica* extracted by 99% ethanol and distilled water on American cockroaches, *Periplaneta Americana*

[12]. The results showed morphological abnormalities such as body shining, body wall and muscle thickening, malformations of ejaculatory ducts and accessory glands in male and tumor ovaries in females [12]. Moreover, the results showed phytoestrogenic hormone behavior of both ethanolic and distilled water crude extracted on suppressed reproductive character when the concentration was consideration high 20% w/v of both extracts which was higher efficiency then the dried powder form. Similar report in present study that 20 % w/v of ethanolic extract is the higher dose caused higher percentage mortality of larvae.

Silkworm larvae have been found to be highly sensitive to exogenous hormones, and the effects of hormone applications are very much dependent on the age of the larvae and the particular dose of the hormone [2]. In the present study, the administration of ethanolic crude extract at 1% w/v to the 48 hour olds fifth instar larvae resulted increase non-significant ($P > 0.05$) cocoon, cocoon sell and pupa weight of female when compare to control group. Furthermore, the spinning in 8^{th} day fifth instar larval found in only trials and 1% w/v was increase significantly ($P < 0.05$) different between trials. The spinning of control began in the 9^{th} fifth instar larval while trials spinning continued and completely cocooning in 10^{th} day. Das demonstrated that estradiol-17β at dose of 2 µg/g when injected into two and five day-old fifth instar larvae, resulted in a shortening of larval duration without altering cocoon shell weight [6]. Kashan and Ray reported the increase in cocoon shell weight after the low dose of estradiol 17β was not due to the extention of feeding period as juvenile hormone treatment that induced a prolongation of the instar that caused increase in silk production [2]. Therefore, the increase of cocoon shell weight after estradiol treatment seemed to be the consequence of increased cellular activity of the silk gland [1-2]. Thus the results from this study will relative the silk gland of the silkworm

Major phytoestrogen such as puerarin daidzin, daidzein, genistin, genistein and coumestrol have been found in *P. mirifica* powder and extracts[10]. The phytoestrogens, are able to modulate growth, reproduction and molting by directly interacting with steroid hormone systems [8]. For example, the geneistein which is active ingredient of phytoestrogen showed a synergistic effect with ecdysteroid in the reduction of cell growth, while coumestrol was able to significantly inhibit edysone receptor- dependent gene transcription [9]. The shortening of larval duration in this study could effect from the phytoestrogen that contain in *P. mirifica*, however, the spinning of larval has not been synchronized and this was not economic character of silk production in silkworm. However, its precise mechanism of phytoestrogen on silkworm is not clear. The physiology and biochemical mechanism of phytoestrogen will be further studied.

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