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Total flavonoids, total phenolics, 1-deoxynojirimycin content and alpha-glucosidase inhibitory activity of Thai silkworm races (*Bombyx mori* Linn.)

Surapong Rattana[1], Teeraporn Katisart[1], Chirapha Butiman[2] and Bunleu Sungthong[3]*
[1]Department of Biology, Faculty of Sciences, Mahasarakham University, Kantharawichai, Maha Sarakham, Thailand
[2]Silk Innovation Center, Division of Research Facilitation and Dissemination, Mahasarakham University, Kantharawichai, Maha Sarakham Thailand
[3]Pharmaceutical Chemistry and Natural Products Research Unit, Faculty of Pharmacy, Mahasarakham University, Kantharawichai, Maha Sarakham, Thailand

Abstract: The silkworm powder (*Bombyx mori* Linn.) from Japanese and Korean races has well known used as a blood glucose lowering substance. However, the study of bioactive compounds and hypoglycemic effect on Thai silkworm races is still limited. This study was aimed to investigate the flavonoid, phenolic and 1-deoxynojirimycin (DNJ) contents, and α-glucosidase inhibitory activity of three Thai silkworm races (Nanglai, Nangnoi and Samrong) of the 5th instar, 2nd 4th day silkworm powders. The total flavonoid, phenolic contents and α-glucosidase inhibitory activity were performed using spectrophotometric methods. The DNJ content was determined using derivatization and further analyzed by HPLC. Nangnoi showed the highest flavonoid content with 12.32±0.41mg quercetin equivalent/g silkworm powder. Nanglai showed the highest phenolic content with 78.74±1.43mg gallic acid equivalent/g silkworm powder. Regarding DNJ content, Nangnoi expressed the highest at 3.11±0.01mg/g silkworm powder. According to the age of silkworm, powders of the instar 5th, day 3rd were provided the highest α-glucosidase inhibitory activity (p<0.05). This study clearly indicated that the 5th instar, 3rd day of Thai silkworms exhibited the highest flavonoid, phenolic, DNJ contents and α-glucosidase inhibitory activity. Further study on blood glucose lowering activity should be performed.

Keywords: Flavonoids, phenolic compounds, 1-deoxynojimycin (DNJ) content, α-glucosidase inhibitor, Thai silkworm (*Bombyx mori* Linn.)

INTRODUCTION

The silk products made from various parts of mulberry silk such as silk fiber, cocoon or pupae, which were recognized as high value products. In the past, silk was well known as the textiles. Later, silk products and silk proteins are increasingly used in medicines, according to their properties such as human tissue compatibility (Amol *et al.*, 2012) and high resistant to microbial degradation (Gomes *et al.*, 2011). Mulberry silkworm is commonly found as an edible insect out of 194 species previously reported in Thailand with respect to high quality protein as well as other nutritional benefits (Sirimungkararat *et al*., 2010). An approximately 48.7-58.0% protein and 30.1-35.0% fat found in silkworm pupae were demonstrated by Rumpold *et al*. (2013).

Besides food and nutritional aspects, the study on utilization of mulberry silkworm in other aspects was restricted in Japan or Korea. A previous study demonstrated that chemical compositions found in silkworm are 1-deoxynojorimycin and its derivatives (Nakagawa *et al*., 2010). Silkworm powder containing 1-deoxynojirimycin (DNJ) exhibits α-glucosidase inhibitory activity and is promising as a complementary and alternative medicinal (CAM) agent (Yatsunami *et al*., 2011). Han *et al*. (2007) reported that silkworm powder expressed inhibitory effects on glucose absorption in human intestinal epithelial cells by inhibiting α-glucosidase activation and glucose transporter (GLUT1) expression. Ryu *et al.* (1997) reported that Korean silkworms in different stages and in different preparation conditions such as a freeze dried silkworm powder of 5th instar, 3rd day exhibited a better glucose lowering activity at about 20% than heat dried mature silkworm powder.

Preliminary studies showed that the silkworm powder from Japanese and Korean races had a high therapeutic chemical compositions and biological activities. However, the bioactive compounds and hypoglycemic activity in Thai silkworm races are still unknown. This research aimed to determine total flavonoid, total phenolic, DNJ contents and alpha-glucosidase inhibitory activity of Thai silkworm races. The benefit from these studies will lead to a drug discovery, food supplementation and value-added of Thai traditional sericulture.

MATERIALS AND METHODS

Materials, chemicals and reagents

The silkworm races were identified according to The Queen Sirikit Department of Sericulture, Ministry of

**Corresponding author:* e-mail: bunleu.s@msu.ac.th

Agriculture, Thailand. Quercetin and gallic acid were obtained from Fluka (Buchs, Switzerland). 1-deoxynojirimycin, *α*-glucosidase enzyme, *p*-nitrophenyl glucopyranoside (*p*-NPG), 9-fluorenylmethyl chloroformate (FMOC), and glycine were purchased from Sigma-Aldrich (St. Louis, MO, USA). Acarbose was obtained from Wako Pure Chemical Industries (Osaka, Japan). Sodium nitrite, aluminium chloride, sodium carbonate, sodium chloride, methanol, sodium hydroxide, Folin-Ciocalteu phenol's reagent, hydrochloric acid, acetic acid and acetonitrile were purchased from Carlo Erba (Val-de-Reuil, France).

Sample preparation

Thai indigenous silkworms (Nanglai, Nangnoi and Samrong races) were reared by Silk Innovation Center, Mahasarakham University between May-June, 2012 and collected at 5^{th} instar, 2^{nd} - 4^{th} day of growth stage. The collected silkworms were then frozen at -40^{o}C and protected from light. Prior to experiment, the samples were lyophilized and ground into fine silkworm powder by blender, kept at 4^{o}C.

Determination of total flavonoid contents

Flavonoids contents were measured by the aluminium chloride colorimetric method as described by Ullah *et al*. (2017) with slight modification. Briefly, fifty milligrams of each silkworm powder was suspended in 10 ml of 80% methanol, sonicated for 15 minutes and filtered through Whatman filter paper No.42 (125 mm). In a test tube (10ml), 0.3 ml of filtrate, 3.4ml of 30% methanol, 0.15ml of 0.5 M $NaNO_2$ and 0.15 ml of 0.3 M $AlCl_3$-$6H_2O$ were mixed. Five minutes after the addition of 1ml of 1M NaOH, the absorbance was measured at 510 nm. A standard calibration curve was performed using known quercetin concentration against absorbance. Total flavonoid contents were determined on a basis of absorbance and calculated as mg quercetin equivalent per gram of silkworm powder (mg QE/g powder).

Determination of total phenolic contents

Phenolic determinations were performed with Folin-Ciocalteu method as previously described by Al-Dabbas (2017) with modification. The sample solution was prepared in the same manner as mentioned in "*Determination of total flavonoid contents*". Briefly, Folin-Ciocalteu reagent (400 µl) was mixed with 200µl of sample solution (1.0 mg/ml) in a volumetric flask. The solution was incubated at room temperature for 10 min. Then, 0.2ml of 7% Na_2CO_3 solution was added to stop the reaction. Finally, the mixture was made up to 10.0ml with deionized water. Before measuring the absorbance at 760 nm, the mixture was kept at room temperature for 2 hours. A standard gallic acid was plotted against absorbance. Total phenolic contents were then calculated according to the calibration curve of a standard gallic acid and expressed in term of mg gallic acid equivalent per gram of silkworm powder (mg GAE/g powder).

Determination of DNJ contents

DNJ content was determined according to the method by Kim *et al*., 2003 with modification. Briefly, an accurate weight of silkworm powder, approximately 30 mg, was mixed with 1 ml of 0.05 M HCl, followed by votexing for 1 minute and then ultra sonication for 40 minutes. After centrifugation at 12,000 rpm for 30 minutes, the supernatant was collected. The remaining silkworm powder was treated again by repeating the above steps. Then, the supernatants were combined and diluted to 2.0ml by adding distilled water. The obtained samples were used as crude extracts for DNJ derivatization.

For DNJ derivatization, the 35µl of larval powder crude extract was mixed with 169µl of 0.4 M potassium borate buffer (pH 8.5) in a 1.5-ml microtube. Twenty µL of 5mM FMOC dissolved in 50% (v/v) CH_3CN was added, and mixed thoroughly. The mixture was placed in 20°C water bath for 20 minutes. Subsequently, 25µL of 1 M glycine was added to quench the remaining FMOC. Finally, 66µL of 0.1% (v/v) acetic acid was added to stabilize the DNJ-FMOC complexation in the reaction tube, and distilled water was added to obtain the final volume of 1.0 ml. Each sample was filtered through a 0.2-µm nylon syringe filter before DNJ determination by HPLC.

A RP-HPLC was applied for DNJ content determination in silkworm powders. HPLC was procured from Shimadzu Corp. (Kyoto, Japan). HPLC system consisted of a Kromasil C18-5µm column (250 mm×4.60 mm i.d.), a Shimadzu SPD-10Avp UV-VIS detector at 254 nm and a LC-Solution working station used as data processing software. The column was eluted with a mobile phase of 0.1% (v/v) aqueous acetic acid in acetonitrile (42:58, v/v) at a flow rate of 1.0 ml/min. The sample was injected into HPLC system and calculated by using calibration curve of a standard DNJ.

In vitro α-Glucosidase inhibitory activity

The standard enzyme inhibition protocol was performed as described in a previous study with some modification (Moradi-Afrapoli *et al*., 2012). Enzymatic reaction was carried out in a 96-well microtiter plate and monitored the absorbance using microplate reader (BMG LABTECH GmbH, Ortenberg, Germany). Twenty microliters of *α*-glucosidase enzyme (0.04units/ml), 120µl of 100mM sodium phosphate buffer (pH 6.8) and 10µl of sample (0.39-5mg/ml in DMSO) were added and mixed thoroughly. After incubation at 37°C for 15 minutes, 20µl of 0.7mM *p*-nitrophenyl *α*-D-glucopyranoside (*p*-NPG) substrate was added into the sample reaction well and subsequently incubated for an additional 15 minutes. The reaction was stopped by adding 80 µl of 0.1 M Na_2CO_3. The absorbances of the reaction wells were measured at 405 nm as a *p*-nitrophenol produced from the hydrolysis of *p*-NPG by *α*-glucosidase. The reaction well without

sample added was used as a control for enzyme activity assay. Acarbose was also used as a positive control for the enzyme inhibitory assay. A blank solution was prepared for each sample concentration without adding enzyme. The different absorbance of each pair of reaction samples and blank solution was measured to subtract the color of sample. Percentage inhibition of enzyme activity by various samples was calculated according to the formula:

$$\%\,\text{Inhibition} = \left(\frac{(\text{ABS of Control} - \text{ABS of Sample})}{\text{ABS of Control}} \right) \times 100$$

Where *ABS of Control* was an absorbance at 405 nm of test condition without sample. *ABS of Sample* was an absorbance at 405 nm of test condition with sample.

STATISTICAL ANALYSIS

To analyze the total flavonoids, total phenolic compounds, 1-deoxynojirimycin contents and alpha-glucosidase inhibitory activity of silkworm powders, the assays were performed in triplicate and the results were expressed by mean ± SD (standard deviation). Experimental data were subjected to Analysis of Variance (ANOVA), using the statistical package SPSS 16 and the differences among means were evaluated by Tukey's test at $p<0.05$.

RESULTS

Total flavonoid and total phenolic contents

The highest amount of flavonoid contents was observed in Nangnoi race, followed by Samrong and Nanglai, respectively. The flavonoid contents of each race regarding the age at the 2nd - 4th day of the 5th instar was not statistically different ($p>0.05$). In case of total phenolic contents, the highest amount was observed at the 5th instar, 3rd day of Nanglai race, followed by Samrong and Nangnoi, respectively. However, no statistical difference had been found with respect to races and the ages ($p>0.05$). Total flavonoid contents of each sample were about 10-12mg QE/g silkworm powders. The silkworm powder of 5th instar, 3rdday of three races were the highest flavonoid content, However, there were not significant difference of flavonoid contents in all of samples groups ($p>0.05$).

Total phenolic contents of each sample were about 70-78 mg GAE/g silkworm powder. The silkworm powder of the 5th instar, 3rd day of three races were the highest phenolic contents. However, there were not significant difference in all of samples groups ($p>0.05$). The amounts of flavonoid and phenolic contents were showed in table 1.

HPLC analysis for 1-deoxynojirimycin contents

Quantitative analysis of DNJ was performed using FMOC derivatization and further determined by RP-HPLC. The peak of DNJ - FMOC in samples were identified with regard to corresponding retention time of a standard DNJ-FMOC. The calibration graph of DNJ (5 - 500 µg/ml) showed a good linearity, y=167912x+2648.1 (r^2= 0.9976). The DNJ content in silkworm powder from different ages and races were showed in table 2. The amounts were expressed in mg DNJ/g silkworm powders. The highest DNJ content was observed at the 3rd day of 5th instar for Nangnoi and Samrong races at 3.11±0.01 mg/g silkworm powder and 1.96±0.08 mg DNJ/g silkworm powder, respectively. In case of Nanglai race, the DNJ content was detected lower than 0.08mg/g silkworm powder.

The chromatograms of derivatized DNJ either in standard solution or silkworm powder were showed in fig. 1. The retention time of DNJ-FMOC, glycine-FMOC and FMOC-OH were approximately 4-5 minutes, 11-12 minutes and 14-15 minutes, respectively. The HPLC chromatogram of DNJ for a Thai silkworm sample was showed in fig. 1B. The DNJ contents of samples were calculated from linear equation of DNJ standard. The DNJ content determined by HPLC assay showed that silkworm powder in the 5th instar, 3rd day contained the highest DNJ contents. Based on silkworm races, the silkworm powder of the 5th instar, 3rd day of Nangnoi and Samrong races exhibited the highest DNJ contents. However, there were no significant differences in any of the samples ($p>0.05$).

In vitro α-glucosidase inhibitory effect

Inhibitory effects of acarbose (α-glucosidase inhibitor) and silkworm powders on *α*-glucosidase activity were determined by colorimetric method using *p*-NPG as a substrate. The product of enzymatic reaction was determined spectrophotometrically. The activities were further calculated and exhibited in IC_{50} as showed in fig. 2. The IC_{50} of silkworm races were expressed in terms of mg/g silkworm powder. The result shows that three silkworm races of 5th instar, 3rd day were the highest potency, which had similar potency comparing to acarbose ($p>0.05$).

DISCUSSION

Total flavonoid and total phenolic contents

The sources of flavonoids and phenolic compounds in Thai silkworms could be from the leaves of host plant, mulberry. Flavonoid components were also found in cocoon shell and Thai silk sericin extract (water and ethanol extracts) such as catechin, epicatechin, rutin, procyanidin (B1, B2), quercetin and luteolin (Butkhup *et al.*, 2012). Flavonoids have been found as a pigment in the cocoon shell of some silkworm races. Larvae of the silkworm sequester flavonoids into their cocoons that are possibly derived from the mulberry leaves. However, flavonol glycosides were not present in the mulberry leaves; these could be isolated from the cocoon (Tamura

et al., 2008). Therefore, it was inferred that flavonoids from their diet are modified within the insects by a glucosyl transferase that transfers a glucose residue to the C-5 hydroxyl position of quercetin, which increases fitness and helps to increase the antioxidative state of tissue (Hirayama *et al*., 2008). Besides antioxidant activity, flavonoids also exhibit α-amylase and α-glucosidase inhibitory activities (Asghari *et al*., 2015).

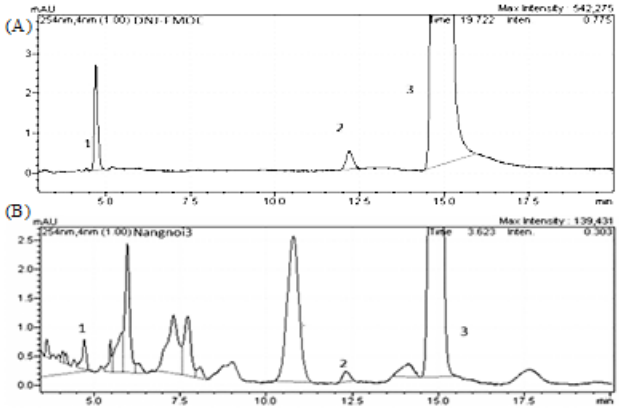

Fig. 1: HPLC chromatograms of (A) standard DNJ derivatization using FMOC, (B) derivatization of DNJ in Silkworm powder using FMOC (peak 1= DNJ - FMOC, peak 2 = glycine-FMOC, Peak 3 = FMOC-OH)

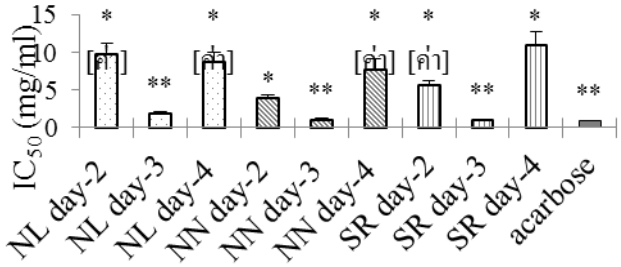

Fig. 2: α-glucosidase inhibitory effect of silkworm powders. The IC_{50} of activity was expressed in term of mg/ml silkworm powder. NL = Nanglai race, NN = Nangnoi race, SR = Samrong race.* Significant difference from acarbose group at $p<0.05$. **Non-significant difference from acarbose group at $p>0.05$

The different kinds of phenolic components in silkworms were also observed comparing with mulberry leaves. With the same explanation as mentioned in flavonoids, glycosylation of polyphenol in insect is catalyzed by UDP-glucosyl-transferase (UGT) (Tanaka *et al*., 2010). These phenolic components are the most commonly known for their antioxidant properties (Mhadhebi *et al*., 2014) and other diverse biological activities such as antimicrobial (Gomes *et al*., 2011) and antidiabetic activities including α-glucosidase inhibitory effect (Wong *et al*., 2014). Flavonoid and phenolic compounds had also protective effect in diabetes by decreasing oxidative stress and preserving pancreatic β-cell integrity (Coskun *et al*., 2005).

HPLC analysis for 1-deoxynojirimycin contents

Asano *et al*. (2001) reported that mulberry tree and silkworm consisted of polyhydroxy alkaloids more than 20 compounds. Yoshikuni *et al* (1988) also indicated that piperidine alkaloids, similar to DNJ expressed high capacity in α-glucosidase inhibitory activity and hypoglycemic activity. Besides mulberry leaves and silkworm, DNJ was isolated and detected in other plants or microorganisms (Asano *et al*., 1998; Asano *et al*., 2000). However, DNJ content in mulberry expressed the highest content comparing with other plants (Yatsunami *et al*., 2008). The presence of DNJ in silkworms might be from leaves of host mulberry plants, which contribute to the reduction of plasma glucose by inhibiting α-glucosidase. Ryu *et al*. (1997) firstly reported the diabetic patient treated by silkworm powder. These studies were carried out using freeze-dried of the 5th instar, 3rd day silkworm. Similar to the study of Yin *et al*. (2010), DNJ content of the 5th instar, 3rd day silkworm powder exhibited the highest amount at 0.3-0.4% w/w. The study also found that the DNJ content from male silkworm contained higher amount than the female. Interestingly, the highest DNJ content in silkworm was found in blood comparing to other organ. Bio-concentration is thought to occur when silkworms ingest the milky sap together with the mulberry leaves.

In vitro α-glucosidase inhibitory effect

Recently, the oral α-glucosidase inhibitor, acarbose has been prescribed to the type II diabetic patients, and clinical experiments are actively performed. Furthermore, this drug is known to mitigate levels of blood glucose, neutralize fats and cholesterol. Acting as α-glucosidase inhibitor, a higher DNJ content in silkworm powder showed more potent intestinal α-glucosidase inhibition than that with mulberry leaves (Yatsunami *et al*., 2011). Once silkworm powder containing DNJ reaches to the small intestine after ingestion. It inhibits the activity of an oligosaccharidase, α-glucosidase by competitive binding to the enzyme with other α-type disaccharides such as mannose, sucrose, *etc*. This binding prevents sudden hydrolysis of disaccharides. Therefore, the absorption of glucose into the blood vessel and blood glucose level are decreased (Bressler *et al*., 1992). Together with DNJ, rutin found in silkworm powder also inhibited α-glucosidase enzyme (Lee *et al*., 2015). Moreover, phenolic components also contributed to α-glucosidase inhibitory activity (Mai *et al*., 2007).

CONCLUSION

Biological evaluation of Thai silkworm powder on α-glucosidase inhibitor revealed that the silkworm powder expressed a promising activity comparable to a medication of acarbose especially from the 5th instar, 3rd day. This activity corresponded to the amounts of DNJ in silkworm powders. Further studies on isolation of

Table 1: Total flavonoid and total phenolic contents of three Thai silkworm races 5th instar, 2nd – 4th day (n=3)

Silkworm races(5thinstar)	Total flavonoids (mgQE/g silkworm powder)	Total phenolics (mgGAE/g silkworm powder)
Nanglai, 2nd day	10.53 ± 0.12	77.53 ± 0.95
Nanglai, 3rd day	11.62 ± 0.29	78.74 ± 1.43
Nanglai, 4th day	11.26 ± 0.32	75.28 ± 1.58
Nangnoi, 2nd day	10.36 ± 0.51	74.32 ± 0.71
Nangnoi, 3rd day	12.32 ± 0.41	75.97 ± 1.71
Nangnoi, 4th day	11.09 ± 0.44	74.07 ± 1.63
Samrong, 2nd day	11.96 ± 0.34	71.01 ± 2.09
Samrong, 3rd day	11.99 ± 0.32	77.91 ± 1.69
Samrong, 4th day	10.54 ± 0.42	70.54 ± 1.08

Table 2: DNJ content in three Thai silkworm races 5th instar, 2nd – 4th day by using HPLC (n=3)

Silkworm races (5th instar)	mg DNJ /g silkworm powder
Nanglai, 2nd day	< 0.08
Nanglai, 3rd day	< 0.08
Nanglai, 4th day	< 0.08
Nangnoi, 2nd day	1.38 ± 0.15
Nangnoi, 3rd day	3.11 ± 0.01
Nangnoi, 4th day	< 0.08
Samrong, 2nd day	< 0.08
Samrong, 3rd day	1.96 ± 0.08
Samrong, 4th day	0.45 ± 0.00

individual flavonoid and phenolic compounds in silkworm and the mechanism of such compounds in anti-diabetic study should be performed.

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