การพัฒนาการใช้หนอนไหมไทยเป็นแบบจำลองสำหรับทดสอบฤทธิ์เบื้องต้นในการลดระดับน้ำตาลในเลือด

นายอภิรุจ เชียงโสม

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรมหาบัณฑิต
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DEVELOPMENT OF THAI SILKWORM MODEL FOR SCREENING GLUCOSE LOWERING EFFECT

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science in Pharmacy Program in Pharmacology
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อ.ที่ปรึกษาวิทยานิพนธ์หลัก: อ.ภก.ดร.สันทัด จันทร์ประภาพ, 78 หน้า.

การศึกษานี้มีวัตถุประสงค์เพื่อพัฒนาการใช้หนอนไหมไทยเป็นแบบจำลองสำหรับทดสอบฤทธิ์เบื้องต้นในการลดระดับน้ำตาลในเลือด ศึกษาโดยใช้หนอนไหมไทยที่ถูกเหนี่ยวนำให้มีระดับน้ำตาลในเลือดสูงโดยการให้อาหารสังเคราะห์ที่ผสมกลูโคส 10% หรือซูโครส 10% และทดสอบฤทธิ์ในการลดระดับน้ำตาลในเลือดกับยาที่ใช้ในทางคลินิกสำหรับมนุษย์คือ อินซูลิน และยาลดระดับน้ำตาลในเลือดชนิดรับประทานคือ ไกลเบนคลาไมด์ เม็ทฟอร์มินและอะคาร์โบส และใช้แบบจำลองนี้ศึกษาฤทธิ์ดังกล่าวของสารสกัดมาตรฐานบัวบกอีซีเอ 233 (ECa 233) ผลการศึกษาพบว่าการให้อาหารสังเคราะห์ที่ผสมด้วยกลูโคส 10% หรือซูโครส 10% เป็นระยะเวลา 1 ชั่วโมงทำให้ระดับน้ำตาลในเลือดหนอนไหมไทยขึ้นสูงสุดโดยมีค่าเท่ากับ 7.37 ± 0.07 และ 7.70 ± 0.08 มก/มล ตามลำดับที่ระยะเวลา 5 และ 4 ชั่วโมงภายหลังการนำอาหารออก จากการศึกษาความเป็นพิษ พบว่าสารที่นำมาทำการศึกษานี้มีค่า LD_{50} ในหนอนไหมไทยดังนี้: ไกลเบนคลาไมด์ เม็ทฟอร์มิน อะคาร์โบส และ ECa 233 เท่ากับ 0.047, 0.041, > 25 และ 1.46 มก/ก ตามลำดับ อินซูลินของมนุษย์ ยาเม็ดรับประทานไกลเบนคลาไมด์ และอะคาร์โบส มีผลลดระดับน้ำตาลในเลือดหนอนไหมไทยได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ได้รับ 0.9% NaCl (P <0.01) และเป็นแบบขึ้นกับความเข้มข้น ในขณะที่เม็ทฟอร์มินไม่มีผลในการลดระดับน้ำตาลในเลือด และเมื่อทดสอบแบบจำลองนี้ด้วย ECa 233 พบว่า ECa 233 มีผลลดระดับน้ำตาลในเลือดหนอนไหมไทยได้อย่างมีนัยสำคัญทางสถิติเมื่อเปรียบเทียบกับกลุ่มควบคุมที่ได้รับ 0.9% NaCl (P <0.01) และเป็นแบบขึ้นกับความเข้มข้น จากผลที่ได้แสดงให้เห็นว่าแบบจำลองหนอนไหมไทยที่ถูกเหนี่ยวนำให้เกิดภาวะน้ำตาลในเลือดสูงสามารถนำมาทดสอบฤทธิ์เบื้องต้นในการลดระดับน้ำตาลในเลือดของสารทดสอบต่างๆ เช่น สารสกัดสมุนไพร สารสังเคราะห์ สารเคมีต่างๆ ที่มีฤทธิ์ในการลดระดับน้ำตาลในเลือดผ่านกลไกการออกฤทธิ์เช่นเดียวกับยามาตรฐานที่นำมาทดสอบดังกล่าวข้างต้น ยิ่งไปกว่านั้นแบบจำลองนี้ยังช่วยประหยัดเวลาและค่าใช้จ่ายในการวิจัยและพัฒนายาลงได้ในระดับหนึ่งอีกด้วย

ภาควิชา เภสัชวิทยาและสรีรวิทยา ลายมือชื่อนิสิต.....

สาขาวิชา เภสัชวิทยา ลายมือชื่ออ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

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KEYWORDS : SILKWORM/ HEMOLYMPH/ GLUCOSE LOWERING
ABHIRUJ CHIANGSOM: DEVELOPMENT OF THAI SILKWORM MODEL FOR SCREENING GLUCOSE LOWERING EFFECT. ADVISOR: SANTAD CHANPRAPAPH, Ph.D., 78 pp.

This study was aimed to develop the Thai silkworm model for screening glucose lowering effect. Thai silkworms were fed with 10% glucose or 10% sucrose diet to induce hyperglycemia. Human insulin and some oral hypoglycemic agents used in the clinical setting such as glibenclamide, metformin and acarbose were utilized to lower the glucose level in silkworm hemolymph. Later this hyperglycemic silkworm model was tested with ECa 233. The results showed that 10% glucose or 10% sucrose diet fed for 1 hr can increase sugar level in Thai silkworm hemolymph and sugar level reach the maximum concentrations of 7.37 ± 0.07 and 7.70 ± 0.08 mg/ml at 5 and 4 hr after the diet were taken out, respectively. For toxicity testing, LD_{50} of glibenclamide, metformin, acarbose and ECa 233 in Thai silkworm were 0.047, 0.041, > 25 and 1.46 mg/g respectively. Human insulin, glibenclamide and acarbose exhibited the hypoglycemic effect in Thai silkworm model with statistical significance comparing with 0.9% NaCl injected group (P< 0.01) and in concentration dependent manner, whereas metformin could not lower sugar level. Using this model to test ECa 233, the results showed that ECa 233 also exhibited hypoglycemic effect as shown by a statistical significance (P< 0.01) decrease of sugar level in Thai silkworm hemolymph in a concentration dependent manner as compared to the 0.9% NaCl injected group. These results suggested that Thai silkworm model can be proposed as an alternative for screening of glucose lowering effect in Thai herbal extract, chemical substances having mechanism of action similar to oral hypoglycemic drugs or insulin used in this study. Furthermore, the use of this hyperglycemic silkworm model can also save time and cost in some extent for drug research and development.

Department: Pharmacology and Physiology Student"s signature……
Field of Study: Pharmacology Advisor"s signature……
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LIST OF ABBREVIATIONS

%	= percent
^{o}C	= degree Celcius
µg	= microgram
µl	= microlitter
Akt	= the serine/threonine protein kinase Akt
AICAR	= 5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside
AMPK	= AMP-activated protein kinase
CHO	= carbohydrate
CMC	= carboxymethylcellulose
DNA	= deoxyribonucleic acid
DDP-IV	= dipeptidyl peptidase - iv
DMSO	= dimethylsulfoxide
ECa 233	= Standardized Extract of *Centella asiatica*, ECa 233
EC_{50}	= Median effective concentration
et al.	= et alii (and other)
g	= gram
gr	= group
g/group	= gram per group
g/kg	= gram per kilogram
GABA	= gamma butyric aicd
Hb A1C	= the glycated hemoglobin A1C
hr	= hour
hrs	= hours
IC_{50}	= Median inhibitory concentration
larva/gr	= larva per group
larvae/gr	= larvae per group
LD_{50}	= Median lethal dose
mg	= milligram
ml	= milliliter
mg/dL	= milligram per deciliter

mg/kg	= milligram per kilogram
mg/ml	= milligram per milliliter
min	= minute
MiRAU	= minor apbthous ulcer
mm	= millimeter
mmol/L	= millimolar per liter
MW	= molecular weight
Na	= sodium
NaCl	= sodium chloride
nm	= nanometer
PI3K	= phosphoinositide-3-kinase
PPARs	= peroxisome proliferator-activated receptors
RES	= reticuloendothelial system
rpm.	= revolutions per minute
S.E.M.	= standard error of mean
v/v	= volume by volume
vs.	= versus
w/w	= weight by weight
WHO	= World Health Organization

CHAPTER I

INTRODUCTION

Laboratory animals are a fundamental tool in biomedical research (Figure 1). Most scientists have been using animal models for their researches since the models can represent the life style and the human related diseases. In addition, laboratory animals are used to develop and test the possible outcome of therapies as a portion of the research process or to develop and test of diagnostic tools such as implants or scanner (heart pacemakers or artificial hip). Some animal models have physical characteristics like human such as anatomy, physiology including the responsible to the several pathogens invade. Moreover, in the recent time several of pathological or an ailments arisen in animals are often similar to those finding in human. Laboratory animals are able to set against the differences or similarities between human and animals and they can facilitate the forecast and extrapolate the results from one species to another. The great destination of the experimental research through using laboratory animals is to unravel the problems in clinical practice and to establish the new instrumentations and approaches to restore and relief of disease and competence (About animal model, 2011). Similarly, through of the new drug research and development in order to assure the invulnerable of the substances candidates for evaluating the therapeutic effects before bringing them to the clinical study in human, the preclinical data such as pharmacology, toxicology and pharmacodynamics *etc*. in laboratory animal are necessary. Human and animal have several of diseases in common and accordingly animals can be used as a model for the study of human illness. Thus, mammals have recently been used as animal models in the life science research because mammals or vertebrate models are accountable in several advanced pharmacology and illnesses research and the results are exceedingly powerful extrapolate. Vertebrate models include the use of small animal such as mice, rabbits (suffer from hardening arteries so favourable to study artherosclerosis) and rats *etc*. The large animal models such as dogs (favourable to suffer from diabetes, cancer, cataract, ulcer and bleeding disorder), cats (favourable to study some visual impairment as humans), monkeys, pigs *etc*. Vertebrate animal models are widely used for several advances in life sciences research (pharmaceutical research including the development of biologics, toxicological testing, cosmetic testing,

development and testing of new medical devices, surgical research and pathophysiological research *etc.*) (Chow *et al*., 2008). However, all models in the laboratory research often involve with several of limitations to be concerned. Recently, the use of vertebrate animal models in the life science research have shown the limitations that can be summarized as high cost of the maintenance of sufficient animal cultivation (amount 10s and 100s thousand of euro"s such as the cost of cage, feed, transportation fee and bedding) and the availability of qualified staff. This limitations also include the smallest group size selected of the laboratory animal are often without a powerful to analyze and the significant result can be made the unreliable expected.

The problems are exacerbated because the large information is often sought by studying manifold end points or time points exceptionably to adjust the statistics for the multiple testing (Hartung, 2008) and lastly the consequential respect is the ethical issue. The anguish issues are affected to the study process and postpone of the end point of the study. Therefore, in order to resolve this issue and overcome these problems, several research topics have been developed to use invertebrate animal as a drug screening models. By the time invertebrate animal models giving a screening of the effective compounds, it could bring to the further study in vertebrate animal models or in the clinical study. Establishment of the drug-screening system using invertebrate animal models is not only much lower cost than vertebrate animal model but also can avoid the association with the ethical issues occuring in the use of mammal or vertebrate models. Moreover, invertebrate have their small body weight and size result in the amounts of the compounds needed to be tested for the pharmacological effect generally smaller than in mammals or vertebrate model (Hamamoto *et al*., 2009).

Figure 1 Laboratory animal model

Invertebrate animal models such as pomace flies (*Drosophila melanogaster*) have been reported to use for *Escherichia coli* infected model (Bernal *et al*., 2000) and nematode (*Caenorabditis elegans*) have been used for *Pseudomonas aeruginosa* infected model (Lemaitre *et al*., 1996; Mahajan *et al*., 1999). However, these models have shown some limitations to be concerned including transferability and predictability since their body sizes are too small and can have a trouble for the pharmacodynamics studies. Recently, the researchers from the University of Tokyo, Japan have developed silkworm (*Bombyx mori*) as an animal model. Among the different species of invertebrate, silkworm have shown particular benefits as an animal model namely the silkworm body is a proper size enough to inject the sample solutions into hemolymph or mid-gut, silkworm hemolymph can be collected and preparations including silkworm organ can be isolated. These are necessary for the pharmacodynamic studies of the compounds in animal bodies. In addition, silkworms have a large number of larvae. They are easily to raise and rear at lower cost. Silkworms are also easily to handle and have less ethical issues. All of the reasons mentioned have made silkworm model a new invertebrate animal model.

Silkworms have been presented as an animal model for evaluating the therapeutic effect of drug candidates (Hamamoto *et al*., 2004), This model have been used for testing of bacteria that are pathogenic to humans and evaluating the therapeutic effects of clinically used antibiotics in humans through intra-hemolymph injection such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans.* The results have shown that clinically used antibiotics in humans were able to kill these pathogenic bacteria (Kaito *et al*., 2002, 2008). The further study have also shown that the oral administration of the clinically used antibiotics in human exhibited the consistent effective dose (ED_{50}) of antibiotics in the silkworm infection model with mammalian models (Hamamoto *et al*., 2004, 2005). In addition, use of silkworm infection model to evaluate the therapeutic effects of the antiviral from traditional medicines was reported (Orihara *et al*., 2008). Use of silkworm model for evaluating drug candidate toxicity and metabolism (Hamamoto *et al*., 2009) have shown that pharmacodynamics and pharmacokinetics of chemicals tested such as absorption, distribution, metabolism, and excretion in silkworms are resemble with those in mammals in greater extent. Furthermore, silkworms have been utilized as a model for screening the hypoglycemic effect of natural product by using human insulin and AICAR as a positive control. The results have shown that sugar levels in silkworm hemolymph can be increased by feeding with high glucose diet and human insulin and AICAR have also shown to decrease the hemolymph sugar levels of the hyperglycemic silkworm (Matsumoto *et al*., 2011).

This study was aimed to develop the Thai silkworm model for screening glucose lowering effect. Thai silkworms were fed with 10% glucose or 10% sucrose diet to induce hyperglycemia. Human insulin and some oral hypoglycemic agents used in the clinical setting such as glibenclamide, metformin and acarbose were utilized to lower the glucose level in silkworm hemolymph. Later this hyperglycemic silkworm model was tested with the Standardized extract of *Centella asiatica*, ECa 233.

Objective of the study

To develop the Thai silkworm as a model for screening glucose lowering effect

Hypothesis to be tested

Thai silkworm can be used as an animal model for screening glucose lowering effect.

Research Design

Experimental research

Scope of the study

Scope of the study was aimed to develop the Thai silkworm model for screening glucose lowering effect. Thai silkworms were fed with 10% glucose and 10% sucrose diet to induce hyperglycemia. Human insulin and some oral hypoglycemic agents used in the clinical setting such as glibenclamide, metformin and acarbose were utilized to lower the glucose level in silkworm hemolymph. Later this hyperglycemic silkworm model was tested with the Standardized extract of *Centella asiatica*, ECa 233.

Expected benefits and application

1. Obtain the data whether Thai silkworm model can be proposed as an alternative for screening glucose-lowering effect of substances.

2. Standardized extract of *Centella asiatica*, ECa 233 have hypoglycemic effect in Thai silkworm hemolymph or not and this information will be very useful to decide whether further step for drug development in vertebrate animal model such as mouse, rat or in clinical study is worth doing or not.

CHAPTER II
LITERATURE REVIEWS

Silkworm

Silkworm (*Bombyx mori* L.), the silk-producing larva of the domesticated silkmoth in a bombycidae family, is not only a worm but also known as a caterpillar. They consume mulberry leaves as their food. They are of economically great importance as the silk producer. When the silkworms have become the putates, they will secrete a single protein strand as the silk from two gland on the top of the head making the cocoon that is cropped for silk. Silkworm genome has full published in 2008 by the International Silkworm Genome consortium. Its genome size is about 432 Mb (The International Silkworm Genome Consortium, 2008). Recently silkworm has become a laboratory animal for the study of lepidopteran and arthropod biology, this research has found the genetic information of silkworm and the possibility of genetic engineering and several hundreds of strains have also been described. In addition, silkworm brain structures, physiology and chemical substances such as pheromones and hormones that produced in silkworm brain were also found in the study.

Life cycle of the silkworm goes through 5 stages. The first stage silkworm starts the life with a little egg laid by female moth. The small black eggs take about fourteen days to hatch and grow as the larvae, the larva stage are growing through 5 steps (1-5 instar larva) which continuously eat the mulberry leaves. For the 5 stages of life cycle, silkworms have change their bodies size through molting of their skin and go into the resting period by fasting for 24 hr every stage and total of 4 times. They continue to grow rapidly, not only molt their skin several times but also change their body‟s size. This stage has a long period about 20-24 days. After silkworms have molted for four time (the 5^{th} instar larva) their bodies have turned slightly white to gray-white and their skins become tighter. In this instar larva, body weights of silkworm are in the range of 0.9-1.7 grams, length about 8 centimeters and width about 1.5 centimeters. When the molting in larva stage reaches to 4 times, the caterpillar begins to putate. They do spinning a cocoon around themselves. This step often takes more than 3 days. The complicated silk cocoon will envelop the hard brown pupa, this stage remains around three weeks except the cocoon is taken to harvest for the silk. The last stage starts after three weeks in the pupa stage and the adult silkworm so called silkmoth appear from the cocoon, this stage the

life span of the silkmoth is about four to six days. When the silkmoth appears from the cocoon, firstly the male moth hatches out and moves rapidly around to search for the female moth. The female moth hatches 2 days later. Female moth releases the pheromone to attract male moth for mating and stands mating for about one day. After mating, female moth will separate and lay the yellow eggs. Female moth will urinate on the top of them and die later. While the male moth looks for another female to do mating and will die later on (Pigdon, 2004) (Figure 2).

Figure 2 The life cycle of silkworm (*Bombyx mori*)
(http://www.silkcollections.co.za/bombyxmorisilkfarm)

Silkworm is an invertebrate by nature. They can develop and change the body size throughout their life cycle. Silkworm, therefore, is favorable to serve as an animal model for studying the life cycle such as metamorphosis or the changes of body size. Furthermore, silkworm is relatively small size, is easy to handle, is easy to raise and it has organs involving drug metabolism which can be isolated. Since silkworm is an invertebrate animal, it has less ethical issues. In the mean time several research topics at the University of Tokyo, Japan have been using silkworms as animal models in the life sciences research. For instance the silkworm model were used for bacterial infection, these bacteria are pathogenic to humans such as *Staphylococcus aureus, Pseudomonas aeruginosa* and *Candida albicans*. Then the therapeutic effects of antibiotics used in clinical for humans were determined in silkworm through intra-hemolymph injection. The results have shown that these pathogenic bacteria have lethal effects in silkworm model when injected into the hemolymph and the clinical used antibiotics exhibited therapeutic effects against such pathogenic bacteria in silkworm. These results were also consistent with the silkworm and mammal (Kaito *et al*., 2002, 2007). In addition, the effective doses (ED_{50}) of antibiotics when given through the oral administration in this silkworm model are consistent with those in mammalian models (Hamamoto *et al*., 2004). Silkworm model also was used to study the pathogenic bacterial toxins. The stationary phase culture-supernatants of *Staphylococcus aureus* and *Pseudomonas aeruginosa* were injected into the hemolymph of silkworm larvae. The results have shown that slikworms were killed whereas a culture-supernatant of a non-pathogenic strain of *Escherichia coli* could not kill the silkworm. A culture-supernatant of a mutant of *agr*, a global virulence to handle of *S. aureus* that needed for the production of exotoxin, was less toxic to silkworm larvae. The culture-supernatant of a disruption mutant of the *S. aureus* beta-toxin gene did not kill larvae, whereas one of a deletion mutant of alpha-toxin, gamma-toxin, or aureolysin causes their death. These results suggested that beta-toxin gene is required for staphylococcal supernatant–mediated killing of silkworm larvae. As purified toxin killed the larvae, silkworm larvae could be used as a model to study the actions of pathogenic bacterial toxins in animal bodies (Hossain *et al*., 2006). The study to assess the antiviral effects of the Kampo medicines (Japanese traditional medicines) by using silkworm infection model with baculovirus has been conducted. The results have shown that antiviral drugs used in clinical for humans

(such as ganciclovir, foscarnet, vidarabine and ribavirin) showed therapeutic effects in silkworm-baculovirus infection model. The antiviral activity of the Kampo medicines Mao-to containing cinnamon bark, a component of the traditional Japanese medicine have shown a therapeutic effect in silkworm-baculovirus infection model whereas cinnzeylanine, purified compound existing in cinnamon bark, also inhibits the proliferation of herpes simplex virus type I in vero cells. These results suggested that the silkworm–baculovirus infection model is useful for screening antiviral agents capable of treating humans infected with DNA viruses (Orihara *et al*., 2008). The activation of the silkworm cytokine by bacterial and fungal cell wall components via a reactive oxygen species-triggered mechanism has been studied. The results have demonstrated that silkworm muscle contraction can be induced by paralytic peptide (PP), PP was stimulated by peptidoglycan and glucan, the bacterial and fungal cell wall components in silkworm hemolymph. In addition, muscle contraction induced by PP and peptidoglycan could be suppressed by Anti-PP antibody and also could be inhibited by free radical scavengers and serine protease inhibitors. When peptidoglycan or glucan generated the active form of PP was injected into the live silkworm, it generated the active form of PP which suppressed by free radical scavengers and serine protease inhibitors. This results have revealed that stimulating of the immunologic could induce reactive oxygen species production from the larval hemocytes and the activation of serine protease are pursued then mediates the PP processing reaction conduct to defensive reactions (Ishii *et al*.,2008). The silkworm model has been used to evaluate drug candidate toxicity and metabolism. It has been found that lethal dose of the cytotoxic substances (7-ethoxycoumarin and 4-methyl umbelliferone) were consistent between silkworm and mammals and the metabolized of these substances are also through a pathway common in both mammals and silkworms (reaction with enzyme cytochrome P450, conjugation with hydroxylated compounds and excretion) unless in silkworm the substances was conjugated with glucose whereas it is conjugated with glucuronate or sulfate in mammals. These results suggested that silkworms are useful as a model for evaluating the toxicity and metabolism of therapeutic drug candidate (Hamamoto *et al*., 2009).

Silkworm larvae as in the most insect use glucose and trehalose as a major source of energy. However, the main sugar in silkworm hemolymph is not glucose but trehalose (glucose is a minor component of insect hemolymph sugar, less than 4% of the total

sugar in silkworm larvae). Trehalose or its chemical name α-D-glucopyranosyl-(1-1)-α-D-glucopyranoside ($C_{12}H_{22}O_{11}.2H_2O$) is a naturally disaccharide that found in insect, plants, fungi and bacteria. Trehalose is a non-reducing sugar that consisting of two glucose units linked by a 1-1 alpha bond. It‟s resemble the functionality to sucrose unless it have the greater stability and less sweetness. Trehalose have been produced from the fat body. Fat body is a fuzz white organ that develops from the mesoderm tissue and distributes through the silkworm bodies. This organ is responsible for storage the nutrition such as protein, glycogen and the other substances that associated with the metabolism process. Trehalose is obtained from diet that silkworm have taking or mainly produced from the gluconeogensis in the fat body. In addition, this sugar also obtained from the glycogenolysis process and then metabolized as glucose-1-phosphate and glucose-6-phosphate both sugars are led into the glycolysis process to promote the energy reserves for silkworm (Thompson, 2003). Through the changes of the instar of silkworm, they were molted and in a starved period for 24 hr in this period trehalose levels in silkworm hemolymph were increased slightly during the first 6 hour of the starvation and decreased thereafter. This effect results from the glycolysis in the fat body, whereas glucose concentration decreased rapidly immediately after diet was deprivation (Satake *et al*., 2000).

The brain anatomical and physiology of silkworm and the several of chemical substances that silkworm produced have been found such as pheromones, hormone and also bombyxin. Bombyxin is an insulin-related peptide that synthesized in silkworm‟s brain. The immunohistochemical study have disclosed that this peptide is produced by four pairs of large medial neurosecretory cells (NSCs) in the brain and is axonally transported to the corpora allata (CA). The results suggested that this peptide is released into hemolymph from these organs and functions as a hormone. The effects of this substance to lower the concentration of trehalose, glucose and the others sugar in silkworm hemolymph. When bombyxin was injected into the neck-ligated silkworm larvae, the result showed a dose-dependent manner to decrease the major sugars concentration in their hemolymph. Bombyxin markedly lowers the glycogen content in the fat body of silkworm (Claeys *et al*., 2002). The effect of sugar on the release of bombyxin from the brain also have been examined. Glucose and trehalose were injected to 6 hr starved female day-1 5^{th} instar larva silkworm, the results have shown that

bombyxin level in silkworm brain reduced in a dose-dependent manner. Thus, bombyxin is involved to modulate carbohydrate metabolism in silkworm (Masumura *et al*., 2000).

The structures of bombyxin have been identified and found. In the mean time this peptide exists in a highly heterogeneous molecular forms namely bombyxin I, II, III, IV and V. However, the primary structures that have been completely determined are bombyxin II and IV and a partially determined for bombyxin I, III and V (Iwami, 2000). The inspection of solution structure of bombyxin by using the nuclear magnetic resonance have exhibited that the overall main-chain fold of bombyxin was resemble to those of insulin solution and insulin in the crystalline T-state form (Nagata *et al*., 1995). The hybrid molecule comprising the A chain of bombyxin and B chain of human insulin motivates 2-deoxyglucose uptake and DNA synthesis in CHO cells (Nagasawa et al., 1986). The study done by Bayazit (2009) have reported the heterodimer of insulin-like A and B chains whose amino acid sequences showing about 50% and 40% identity to the A and B chains of human insulin (Figure 3, 4). Based on these structures and characteristics, bombyxin was regarded to be a member of the insulin superfamily. Bombyxin A and B chains are also connected by two inter-chains and one intra-chain disulfide bonds the same as insulin and can be. The sequences of bombyxin genes is with human insulin families are display to be alike 41% to 56% and 28% to 35% when identity with human preproinsulin and also preprobombyxin have at least 73% identical amino acid sequences genes resembling compared to the variation structures of vertebrate insulin (Steiner *et al*., 1985). Thus, bombyxin are resemble to insulin not only the biological effects but also in a primary structures (Nagata *et al*., 1995).

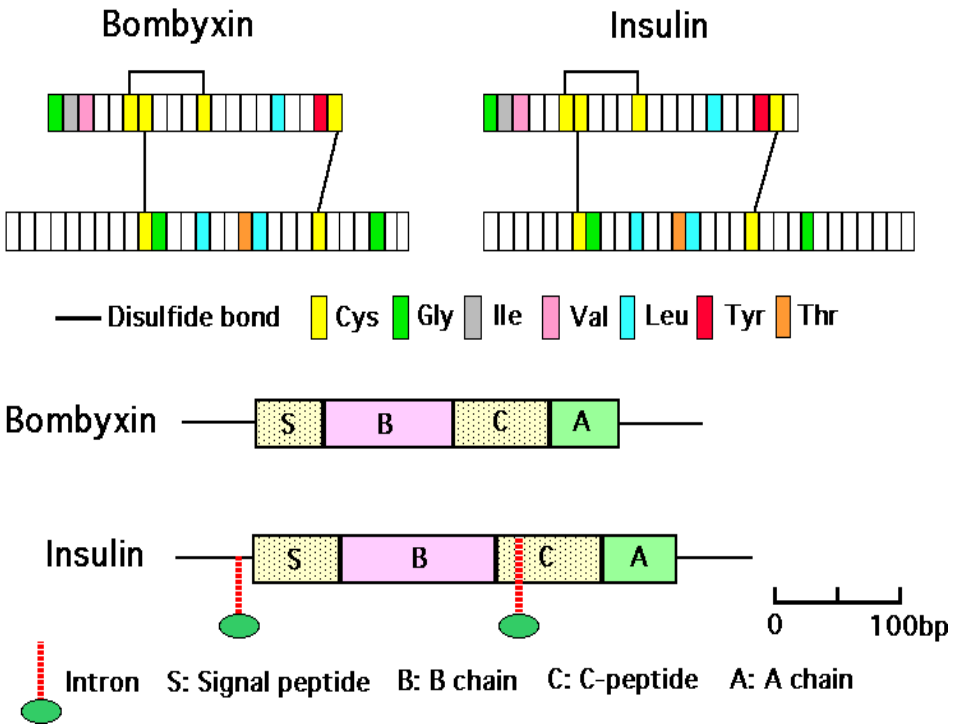


Figure 3 Amino acid sequences of Bombyxin (Left) and Insulin (Right) (Biyazit, 2009)

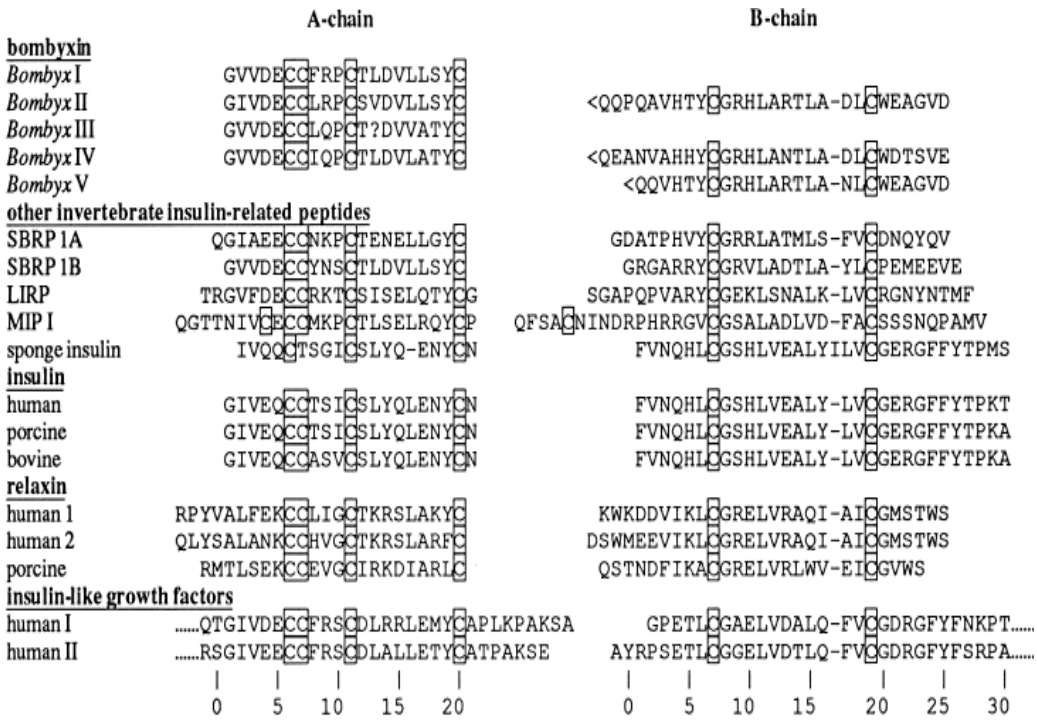


Figure 4 Amino acid sequences of insulin super-family peptides. Compared between bombyxins and insulin (Nagata *et al*., 1995)

Receptors of bombyxin were found and they distribute in several organs of silkworm such as ovaries, brain, fat body and prothoracic glands *etc.* The subunit structures of the putative bombyxin receptor have shown to be similar to the mammalian insulin receptor (Fullbright *et al*., 1997). When silkworm is taking the diet, bombyxin concentration in silkworm hemolymph is also increasing. This is to maintain the energy homeostasis since food intake apparently results in Akt phosphorylation. Silkworm Akt (BomAkt) has been identified and characterized on the pleckstrin homology, kinase domain and a dual phosphorylation site essential for kinase activation. It is composed of 443 amino acid and more than 80% identities with mouse and human Akts. Additionally, phosphorylation of BomAkt was demonstrated in the isolated fat body after exposure to bombyxin, in endogenous insulin like peptide and in the fat body of silkworm after feeding one time after starvation. These results indicating that food intake stimulates insulin signaling pathway including Akt phosphorylation in silkworm similar to diet taking stimulate the activation of insulin signaling in mammals by starting with insulin secretion and resulting in the regulation of carbohydrate and lipid metabolism (Nagata *et al*., 2008).

Insects including silkworm are quite similar in mammals in some aspects, food intake can induce the secretion of insulin-like peptides followed by activation of the insulin signaling pathway and phosphorylation of Akt. The signal is transferred in cascade manner and lastly giving a biological effect by taking up sugars into the cells. These processes cause the decrease of sugar levels in the bloodstream (Nagata *et al*., 2008) (Figure 5). Upon injection of bovine insulin to silkworm, the results have shown that insulin stimulated ecdysteroidogenesis during a long-term incubation period and in a dose-dependent manner. In addition, bovine insulin stimulated insulin receptor and also stimulated DNA synthesis. Viability of prothoracic glands and phosphorylation of Akt, stimulation of Akt phosphorylation appeared to be PI3K-dependent and these regulate the development of many organs (Gu *et al*., 2009).

Furthermore, several studies on the biological activities of bombyxin have been reported. This substance functions as a peptide of insulin superfamily as insulin functions in mammals. In a starvation period, bombyxin titer in silkworm hemolymph was decreased whereas its titer level was increased in the brain. After re-feeding the starved larvae, this resulted in an increasing of bombyxin levels in silkworm hemolymph while the level of this peptide in the brain of silkworm start decreasing rapidly. When glucose was injected into starved larvae, the result in a same manner of re-feeding could be observed. This indicated that glucose is working as a signal to bring over the release of mammalian and insect insulin (Masumura *et al*., 2000). Similar to insulin, the release of bombyxin was stimulated by the sugars such as glucose or trehalose. It is highly credible that bombyxin also functions as a regulator of carbohydrate metabolism. This peptide facilitates glucose uptake and glycogen synthesis. On the other hand, this peptide has shown to inhibit the breakdown of glycogen and the gluconeogenesis process (Newsholme *et al*., 1992).

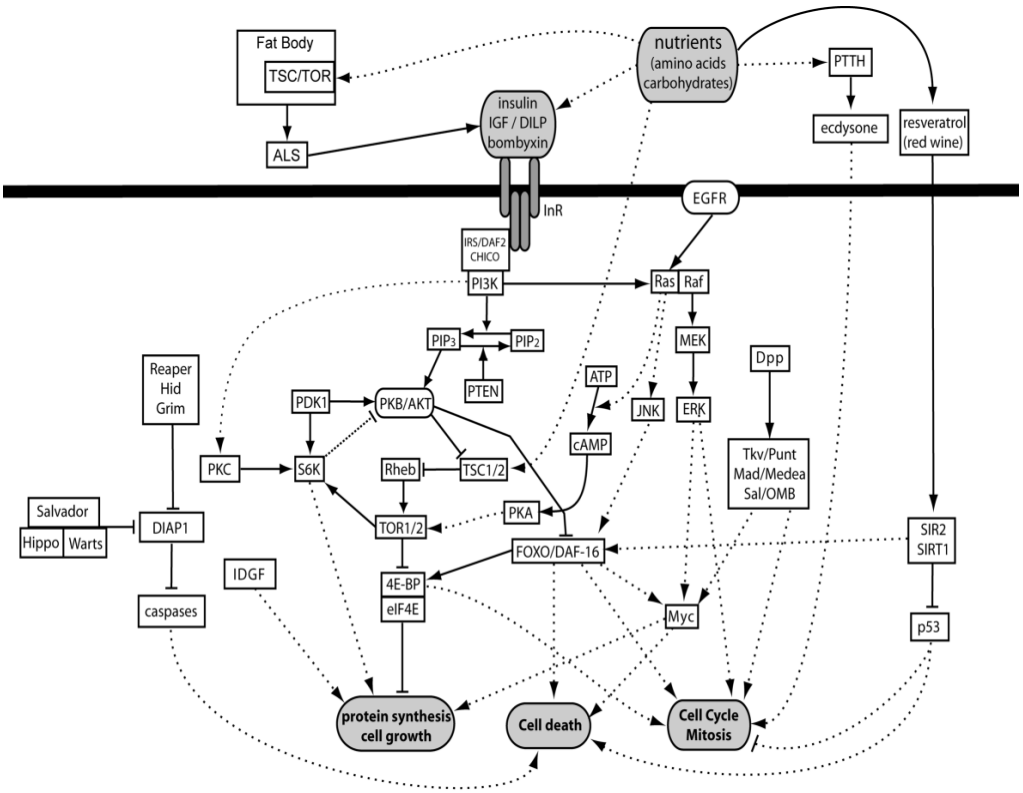


Figure 5 Bombyxin and insulin activate Akt phosphorylation (Nijhout, 2003)

The mid-gut enzymes of silkworm functioning to break disaccharides into monosaccharide before absorption have been reported for 3 isoforms as sucrose 1, sucrase 2 and sucrase 3. The activities of the three isoforms are first increased during the 5^{th} instar larva and then reach the maximum effects in the middle stage of the 5^{th} instar larva. Finally their activities are decreased towards the last stage of the 5^{th} instar larva. Three isoforms of these enzymes have been isolated and purified in order to do the characterization and homogeneity. The results have shown that sucrase 1 and sucrase 2 were beta-fructofuranosidase enzyme whereas sucrase 3 was alpha-glucosidase enzyme respectively (Toshihiro *et al*., 2002).

Silkworm has been established as a hyperglycemic model for the identification of anti-diabetic drugs and such model was also used for screening anti-diabetic effect of the natural products as well (Matsumoto *et al*., 2011). The study was done by using hyperglycemic silkworm induced by feeding high glucose diet. The hemolymph sugar levels were measured by phenol-sulfuric acid method. The results have shown that a high glucose diet group (10% glucose diet) fed for 1 day has high sugar level in silkworm hemolymph more than 4 folds comparing to normal diet group (no glucose diet) and the sugar levels in silkworm hemolymph in the fasted group was less than half that of the silkworm fed with normal diet. The time course of the increase in total sugar in silkworms hemolymph after feeding with a high glucose diet have also been examined. The results have shown that sugar levels in silkworm hemolymph fed high glucose diet was 2 folds, 4 folds and 6 folds at 30, 60 and 180 minutes after feeding respectively comparing with sugar level at 0 minute. Whereas in the fasted group and normal diet feeding group, sugar levels in silkworms hemolymph did not increase for up to 180 minutes. Glucose levels in silkworm hemolymph were measured and confirmed by using glucose oxidase method. Glucose levels in silkworms hemolymph fed high glucose diet were increased rapidly while in normal diet and the fasted group glucose levels cannot be detected. In general, the major sugar in insect hemolymph is trehalose (a dimer of two glucose units linked by 1-1 alpha bond) whereas glucose is not detected. Thus, high sugar levels in silkworm hemolymph detected were coming from the high glucose diet given. Hyperglycemic silkworm model was tested with human insulin and AICAR (5-aminoimidazole-4-carboxamide-1-β-D-ribofuranoside, an AMP-activated protein kinase (AMPK) activator). The results have shown that both of substances can decreased the

sugar levels in hyperglycemic silkworm hemolymph. This effect results from the increase of Akt kinase phosphorylation in the fat body of silkworm larvae and facilitates glucose uptake and glycogen synthesis. This has been re-investigated by isolation of the fat body of silkworm and treating with human insulin in an *in vitro* culture system. After adding glucose to the medium, total sugar in the fat body was increased and Akt phosphorylation was stimulated. These results were inhibited by added wortmannin (an inhibitor of phosphoinositide 3 kinase) into system. The results were similar to the effect of insulin that induces glucose uptake through the activation of phosphoinositide 3 kinase in mammalian adipose tissue. The activation of AMPK by AICAR was shown to lower sugar levels in silkworm hemolymph. When AICAR was added to an *in vitro* culture medium of silkworm fat bodies, it stimulated AMPK phosphorylation in silkworm fat body too. This result was consistent with the activation of AMPK signaling pathway resulting in decrease the blood sugar levels in mammals. Furthermore, the hyperglycemic silkworm model was used to test the hypoglycemic effect of the Chinese herbal (Jiou). The result was also shown that Jiou can decrease the sugar levels in hyperglycemic silkworm hemolymph. This study was the first report to demonstrate the possibility of using silkworm model for screening glucose lowering effect of substances.

Diabetes Mellitus

Diabetes mellitus is a group of chronic metabolic diseases in which a person has a high sugar level in the bloodstream (Hyperglycemia), either because the conditions in which the pancreas does not produce enough insulin, or because tissues do not respond and/or fail to properly use of insulin that is produced. The classical symptoms of diabetes in patients are polyuria (frequent urination), polyphagia (increased hunger) and polydipsia (increased thirst) *etc*. There are four main types of diabetes as followings (American Diabetes Association, 2004).

Type 1 diabetes: results from the destruction of beta-cell of pancreas and usually leading to absolute insulin deficiency

Type 2 diabetes: predominantly insulin resistance with relative insulin deficiency or predominantly an insulin secretory defect with or without insulin resistance

Gestational diabetes: meet in pregnant women in case who have never had diabetes before, have a high sugar level in the bloodstream during the period of pregnancy and this type may precede development of type 2 diabetes

Other specific types: these types group associated with the several causes and pathology such as genetic defects of beta-cell function of pancreas, genetic defects in insulin action, disease of the exocrine pancreas, endocrinopathies, infections of pancreas, drug or chemicals induced diabetes, uncommon but specifics form of immune-mediated diabetes mellitus and the other genetic syndromes sometimes may involved with diabetes.

Diabetes mellitus have characterized by the criteria for abnormal glucose level recurrent or persistent of hyperglycemia and is diagnosed by any one of the several methods such as Fasting blood sugar > 126 mg/dL (≥ 7.0 mmol/L). Glucose loading test plasma glucose > 200 mg/dL (≥ 11.0 mmol/L) after 2 hr 75 g oral glucose load test, Patients who have symptoms of hyperglycemia and causal plasma glucose > 200 mg/dL (≥ 11.0 mmol/L), the glycated hemoglobin A1C (Hb A1C) > 6.5% and WHO diabetes criteria 2006 recognized criteria to diagnosed diabetes shown in table 1

Condition	**2 hrs glucose tolerance test** mg/dL, (mmol/L)	**Fasting glucose** mg/dL, (mmol/L)
Normal	<140 (<7.8)	<110 (<6.1)
Impaired fasting glycaemia	<140 (<7.8)	≥110 (≥6.1) and < 126 (< 7.0)
Impaired glucose tolerance	≥140 (≥7.8)	< 126 (< 7.0)
Diabetes mellitus	≥200 (≥11.0)	≥ 126 (≥ 7.0)

Table 1 WHO diabetes diagnostic criteria 2006

Chronic of high blood sugar level or hyperglycemia that may cause to damage the microvascular and produces many complications to the major organs especially cardiovascular system, eyes, renal etc. (Unite for diabetes, 2006). The prevalence of diabetes is rapid increasing worldwide in 1985 WHO have reported the numerical estimates was to 30 million adult people of diabetes and rise to 135 million people in

1995. While at least 170 million people worldwide in 2000 and will rise to 300 million people in the year 2025 and the number of diabetes people increase by 122 % (King *et al*., 1998). Recently, the prevalence of diabetes worldwide all group of diabetes in 2000 has estimated to be 2.8% and to rise to 4.4% by the year 2030. The total diabetic people is appeared to increase from 171 million in 2000 to 366 million by the year 2030 and the higher prevalence was seen in men than women but diabetic women have seen to more in men (Wild *et al*., 2004). The prevalence of diabetes was seen a higher in developed countries more than in developing countries, In developing countries diabetes people are in the age range 45-64 years, while in developed countries the majority of people with diabetes are aged ≥ 65 years. In Thai populations the number of people with diabetes was 2.6-6% and mostly prevalence in aging rise to 13-15.3%, estimates Thai people with diabetes 3.7% in the year 2025 (Lohsunthorn V. and Jiamjarusrangsi V., 2008).

Diabetes mellitus is a chronic disease in which difficult to treat and care. However, in the recent time the management of diabetes is intent to keep blood sugar levels as close to normal as possible without serious effects to the patients (AACE Diabetes Mellitus Clinical Practice Guidelines Task Force, 2007). First always advise on life style modification with close dietary management, exercise, and use of appropriate oral hypoglycemic agents. Several types of oral hypoglycemic agents have been used for a good control of blood sugar levels (AACE Diabetes Mellitus Clinical Practice Guidelines Task Force, 2007) (Figure 6). For instance sulfonylureas group, these agents action through stimulation of insulin release from β-cell of pancreas. As a result, insulin levels and thus utilization of glucose and other metabolic fuels are increased (Evans *et al*., 2009, Inzucchi, 2002). Hypoglycemia is also more likely to occur when the caloric intake is inadequate or after strenuous and prolonged exercise. Similar to insulin, sulfonylureas can induce weight gain. Biguanides, the therapeutic uses are derived from their tendency to reduce gluconeogenesis in the liver and resulting in the reduction of glucose level in the bloodstream. Most common side effects are diarrhea, metallic taste and the most important and seriously side effect is lactic acidosis. Therefore, metformin possess the contraindication in the renal insufficient patients (Moon et al., 2007, Evans *et al*., 2009, Inzucchi, 2002). Alpha-glucosidase inhibitor, this group acts as competitive inhibitor of enzyme needed to break carbohydrate namely alpha-glucosidase enzymes in the brush border of the small intestines, resulting in less glucose absorption. Since alpha-

glucosidase inhibitors prevent the degradation of complex carbohydrates into glucose, the carbohydrates will remain in the intestine. In colon, bacteria will digest the carbohydrates complex, thereby causing gastrointestinal side effects such as flatulence and diarrhea (Moon *et al*., 2007, Evans *et al*., 2009, Inzucchi, 2002). Thiazolidinedione, this group actions through binding with PPARs, a group of receptor molecules inside the cell nucleus and thus activating of PPARs specific to $PPAR_{\gamma}$ resulting in decreasing in insulin resistance, adipocyte differentiation, also increase of the synthesis of certain proteins involved fat and glucose metabolism. In addition, thiazolidinediones stimulate the uptake of fatty acids by adipocytes and the production of triglycerides result in gaining of the body weight (Moon *et al*., 2007, Evans *et al*., 2009, Inzucchi, 2002)**.** Non –sulfonylurea insulin secretagogues such as repaglinide, these groups lower blood glucose by stimulating the release of insulin from β-cell of pancreas. However, weight gain, diarrhea and joint pain may occur (Inzucchi, 2002, Campbell, 2007). Incretin based therapy, in the recent time clinician has turned to attend to the new option of hypoglycemic agents using the endogenous gastrointestinal hormone such as the glucagon-like peptide-1 or incretin hormone for diabetes patients. The release of incretin into bloodstream after food intake affects blood sugar by decreasing glucagon release, delaying gastric emptying time, promoting a felling of fullness after meal and increasing insulin produced from the pancreas. Sitagliptin (DDP-IV inhibitor) is an example for these agents (Knop *et al*., 2009, Ceriello *et al*., 2008). In addition, insulin have been given to diabetes patients for a good of glycaemic control (Singh *et al*., 2006, Ceriello *et al*., 2008) either alone or concomitant for diabetes management. However, several hypoglycemic agents always carry some adverse effects. Recently, many research topics, therefore, have been looking for the natural substances as candidates for the anti-hypoglycemic drugs instead in order to reduce some adverse effects existing in the recent clinical used antidiabetic drugs and to be able to lower the expense of diabetes managements too.

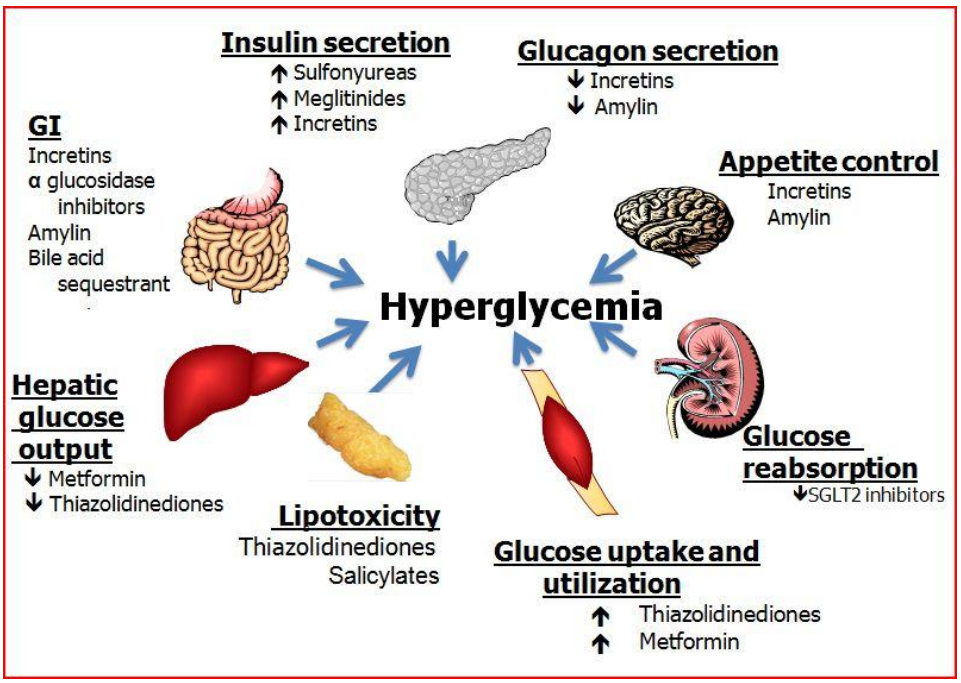


Figure 6 Pharmacotherapy of Diabetes mellitus (Evan and Rushakoff, 2002)

Several Thai herbal extracts have been studied and reported about their hypoglycemic effects for instance *Tinospora crispa* (Boraped). Sriyapai and *et al*. (2009) administered 250 mg/capsule of *T. crispa* dried powder 1 capsule twice daily 30 min before meal to diabetic out-patient for 2 months. The results have shown that it has significantly decreased in fasting blood sugar levels. *Pandanus odorus* (Toei-Hom) also have been studied and reported. The water soluble root extract of this plant 5 mg/kg was shown to decrease plasma glucose levels in diabetic rats. Additionally, the compound has shown to increase serum insulin levels and liver glycogen content in normal rats (Peungvicha *et al*., 1998). Other several of Thai herbals also have been used in traditional medicine to reduce blood sugar such as *Arcangelisia flva*, *Albizia myliophylla, Xylinbalia minutiflor* and *Holarrhena pubescens* etc. (Pannangpetch *et al*., 2006) including the study of the glucose lowering effect of *Centella asiatica*.

Centella asiatica (L) is small herbaceous-branched prostate plant. This plant is a weekly aromatic smelling herb of the family Umbelliferare that consisting of several active principles such as triterpenoid glycosides (asiaticoside, madecassoside, brahmoside, brahminoside, centelloside, *etc*.), aglycones (asiatic acid, madasiatic acid, madecassic acid, thankunic acid, isothankunic acid, volatile oil *etc*.) and the other amino

acids, flavonoids, alkaloids *etc.* (Brinkhaus *et al*., 2000) (Figure 7). This plant has been used wildly as a traditional medicine and a medicinal herb for centuries.

Indian Ayurveda medicine have used *Centella asiatica* to support the faster healing of a small wound, burning treatment, treatment of the leprosy, eczema, psoriasis and the others skin disease *etc*. In Thailand, this plant is also well known. This herb has been used in the folk medicine due to its ability to relieve and recover for pain, fever and sickness. Recently, several biological effects of this herb have been studied and reported. For instances, wound healing effect. This effect has shown to facilitate the formation of tissues at the wound site and promote the tensile strength of the newly skin made. And also this includes the enhancement of the quantity of fibronectin and mitotic activity of the germ layer together with the ability to uplift the keratohyaline granules in tissues at the wound site too (Bonte *et al*., 1994).

For anti-ulcer and the ulcer protective effect, several concentration of the extracts of *C. asiatica* (0.05, 0.1. 0.25 g/kg) and asiaticoside (1, 5, 10 mg/kg) were administered to peptic ulcer rats induced by acetic acid. The results have shown that these extracts can reduce the ulcer size and increase the microvascular supply in tissues at the wound site (Cheng *et al*., 2004; 2006). Neuroprotective effect of the extracts also have been reported for 3 derivatives of asiatic acid using primary cultures of rat cortical neurons insulted with glutamate the neurotoxin in *in vitro* screening system. The results have shown that the 3 derivatives significantly mitigated the neurotoxicity from the over production of nitric oxide induced by glutamate in this screening system (Lee *et al*., 2000). In addition, psycho-neurological effect of alcohol extracts of *C. asiatica* has been studied by administration the alcohol extracts to rats. The results have shown that the alcoholic extracts of *C. asiatica* can increase GABA levels, the mainly inhibitory neurotransmitter in central nervous system, in a concentration dependent manner (Brinkhaus *et al*., 2000). Immunomodulatory effects have been reported both for water and alcohol extracts of *C. asiatica*. It has been shown that both water and alcohol extracts of *C. asiatica* can activate the reticuloendothelial system (RES) both the classical and alternative pathway of complement system (Jayathiratha and Mishra, 2004). Furthermore, anti-bacterial and anti-fungal effects have also been reported. The chloroform extract of *C. asiatica* showed to have the highest activity against the growth of *Staphylococcus aureus and Vibrio Parahemolyticus* through the inhibition zone (16 mm) and these extracted have also

shown good activities against the growth of *Samonella typhi* (inhibition zone 15 mm), *Echerichia coli* (inhibition zone 15 mm), *Pseudomonas aeruginosa* (inhibition zone 14 mm) and *Shigella boydii* (inhibition zone 14 mm). For anti-fungal effects, the chloroform extract strongly inhibited *Candida albicans* (inhibition zone 15 mm) whereas n-hexane extract inhibited *Aspergillus niger* (inhibition zone 14mm) (Ullah *et al*., 2009)**.**

For antioxidant effects, 0.2% asiaticoside were applied to the excision wound in mice 2 times/day for 7 days. The results have shown that both enzymes and non-enzymatic antioxidants increased in the newly skin made at the wound site (Shukla *et al*., 1999). Moreover, cognitive and memory enhancing effects have also demonstrated by administrating *C. asiatica* extract 200 mg/kg/day for 14 days to impaired cognition mice from oxidative stress induced by streptozotocin. The results have shown that *C. asiatica* could enhance learning and memory ability of these mice. When the dose was increased to 300 mg/kg/day, the levels of glutathione and enzyme catalase also increased (Veerendra and Gupta, 2002). Hepatoprotective effect has been reported when glucosides from *C. asiatica* was given for 6 weeks to liver fibrosis rat induced by dimethylnitrosamine. The results have shown that glucosides could decrease the levels of the liver enzymes AST and ALT and also the levels of hyaluronic acid comparing to colchicine-treated group. These results may contribute to the anti-liver fibrosis effect of glucosides (Ming *et al*., 2004).

In addition, the hypoglycemic effect of *C. asiatica* has been reported by Yutaka *et al*., 2007. The *C. asiatica* concentration extract and asiaticoside were given to mice orally. Then fasting blood sugar and glucose load test were performed. The results have shown that *C. asiatica* concentration extract and asiaticoside could lower fasting blood sugar level significantly after 14 days of administration. For glucose load test, asiaticoside could lower glucose level significantly when measured. Moreover, asiaticoside also showed the significant sugar absorption inhibitory effect too. When the ethanolic and methanolic extract of *C. asiatica* leaves were given to alloxan-induced diabetic rats (Chauhan *et al*., 2010). The results have shown that both ethanolic and methanolic extract of *C. asiatica* leaves could lower the blood glucose level to normal range and the maximum reduction of blood glucose levels was observed after 3 hr at a dose of 250 mg/kg of body weight. When glucose loading test was performed in the male wistar rat by administration of glucose 4.5 g/kg followed by the treatments as followings:

1% Na CMC, glibenclamide 0.225 mg/kg and *Centella asiatica* in dose range 0.01, 0.5 and 1 g/kg (Efek Antihiperglikemik Ekstrak Etanol Herba Pegagan (Centella asiatica (L) Urb.) Pada Tikus Jantan (Rattus norvegicus, L) Galur Wistar DenGan UJi Toleransi Glukosa Oral, 2009). The results have shown that *C. asiatica* could decrease the blood glucose levels for 16.05%, 32.35%, and 45.81% respectively whereas glibenclamide with dose 0.225 mg/kg could decrease blood glucose levels for 78.28%.

Figure 7 *Centella asiatica* (L.) (http://centella-asiatica.101herbs.com/)

However, all the extracts of this plant from the previous studies were prepared from several solvents. This resulted in an uncontrollable concentration of the extracts prepared together with the chemical constituents. Thus, these can affect the study and cause the uncertainty and inconsistence of the results.

Standardized extract of *Centella asiatica*, ECa 233

At the present, researchers from the faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand have prepared the Standardized Extract of *Centella asiatica*, ECa 233. This extract is a white to off-white powder, and the triterpenoid glycoside in the extract is well controlled by fixing the ratio of madecassoside/asiaticoside at 1.5 ± 0.5 and both of glycoside constituents in this extract

not less than 80% , the shelf-life is more than 2 years (เอกรินทร์ สายฟ้า และคณะ, 2549) (Figure 8).

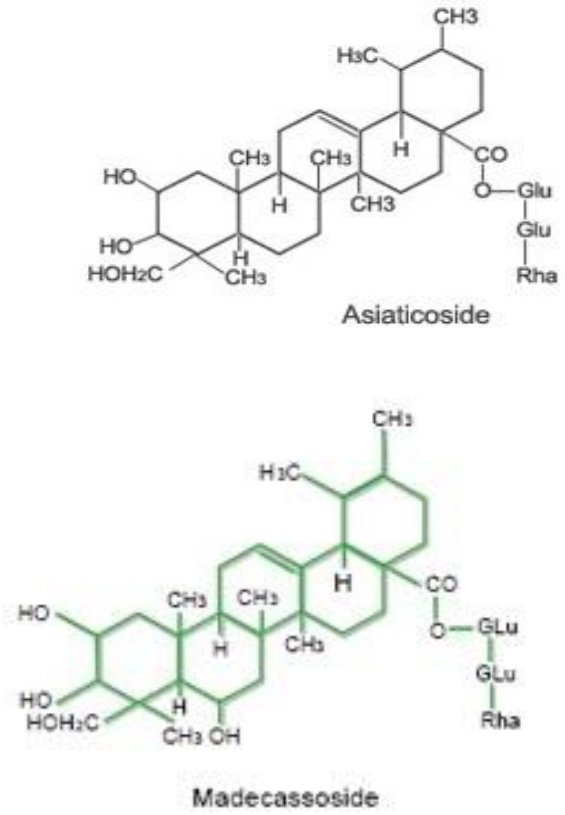


Figure 8 Structure of asiaticoside (upper) and madecassoside (lower) (http://asiaticoside.com/)

ECa 233 has used to study on the pharmacological effect in several topics such as wound healing effect. Tanintaraard *et al.*(2009) have reported that after 3 day of application of gel containing 0.05% of ECa 233 once daily to the incision wound in rat the tensile strength and epidermal thickness in the wound site were significantly higher comparing to gel base given group and untreated group. Furthermore, after application of ECa 233 oral paste in various concentration (0.05, 0.10, 0.20%) to minor apbthous ulcer (MiRAU) in 24 eligible subjects, the results showed the significant reduction of the ulcer size and also pain score (Ruengprasertkit *et al*., 2010). In addition, when applied gel containing 0.05% ECa 233 to second degree wound burn once a day in wistar rat, the results have shown that gel containing 0.05% ECa 233 have the healing effect better than the gel base treated group. This also included the rate of wound healing in the ECa 233

treated group which was significantly higher than gel base treated group in day 7 post-burning and also cutaneous blood flow was increased on day 7 only in 0.05% ECa 233 gel treated group (Wannarat *et al*., 2009).

The improvement of learning and enhancing of memory have been reported after administration of 10 mg/kg of ECa 233 twice daily to mice induced by bilateral common carotid arteries occlusion (Kam-eg *et al*., 2009). However, the hypoglycemic effect of this extract has not been reported yet.

Phenol-Sulfuric acid method

The phenol-sulfuric acid reaction is a colorimetric method that is widely used to quantify the quantity of sugar in a definite medium. Provided the sugar is in the attendance of various medium such as salts, protein residues, or connected to a polymer, this method can be performed. In hot acidic condition sulfuric acid have to break disaccharide and oligosaccharide to monosaccharide, the sulfuric acid causes all non-reducing sugars converted to reducing sugars then glucose is dehydrated to hydroxymethyl furfural. This form coloured product with phenol and solution turns a yellow-orange color as a result of the interaction between the carbohydrates and phenol. Determination of sugars using this method is based on the absorbance at 490 nm of a colour aromatic complex among of carbohydrate and phenol. The quantity of sugar presented is determined by comparing to a standard curve of glucose using a spectrophotometer. Under the standard conditions, this method is accurate to ± 2% (Fournier, 2001).

In this study we aim to develop Thai silkworm as a model for screening glucose lowering effect of the substances using hyperglycemic Thai silkworm induced by 10% glucose and 10% sucrose diet. Human insulin and some of the oral hypoglycemic agents used in clinical such as glibenclamide, metformin abd acarbose were used to lower the glucose level in silkworm hemolymph. Then a model was tested for glucose lowering effect with the Standardized extract of *Centella asiatica,* ECa 233. In general, the sugars in silkworm hemolymph concentrations were measured by phenol-sulfuric acid method.

CHAPTER III

MATERIALS AND METHODS

Thai silkworm (Nang-lai) 5th instar larva weighing 0.8-1.2 g were supplied from The Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives. The silkworms were kept under laboratory conditions of temperature (27 ± 2 ˚C) for at least 24 - 48 hrs prior to start of the experiments, and Silkmate 2S, Artificial diet, Nihon. Nosan, Japan, was used for feeding and mixing with either glucose or sucrose for the experiment.

Chemicals

1. Standardized Extract of *Centella asiatica*, ECa 233 obtained from Assist. Prof. Chamnan Patarapanich, Ph.D. Department of Pharmaceutical Chemistry, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.
2. D-Glucose MW 180.16 (Univar, Australia)
3. 70% Perchloric acid MW 100.46 (Univar, Australia)
4. Sulfuric acid MW 98.08 (J.T. Baker, Thailand)
5. 98.5% Phenol (Lipton, England)
6. 0.9% Normal saline
7. Human insulin (Wako, USA)
8. Distilled water
9. Glibenclamide (Government Pharmaceutical Organization, Thailand)
10. Metformin (Government Pharmaceutical Organization, Thailand)
11. Acarbose (Sigma,WGK, Germany)
12. Sucrose (Sigma, WGK, Germany)
13. 99.5% DMSO (Sigma, St. Louis, MO, USA)

Instruments

1. Micropipet size 20, 200 and 1,000 microliter (Gilson, France)
2. Microcentrifuge tubes size 1.5 microliter (Biologix, US)
3. Pipette tip size 0.1-10 microliter and 100-1,000 microliter (Corning incorporated, Mexico)
4. 96 Well microtiter plate (Corning incorporated, US)
5. Disposable syringe size 1 microliter (Nipro, Thailand)
6. Hypodermic needle 27G x 1" (Nipro, Thailand)
7. Centrifuge model EBA 20 (Hettich, Germany)
8. Microplate reader model Perkin Elmer (MTX Lab system, Inc., USA)

Methods

Time profiles study

Thai silkworms were induced to become hyperglycemia with a high glucose and sucrose in various concentrations. In addition, time course of total sugar in Thai silkworm hemolymph and the optimum concentrations of both glucose and sucrose that can increase sugar levels in Thai silkworm hemolymph also were evaluated. Process of time profile study was shown briefly in Figure 9.

Determinination of time to peak of the sugar levels in Thai silkworm hemolymph after feeding with a normal diet for 1 hr

To determine the time that sugar level increase to the peak in Thai silkworm hemolymph. The first day 5^{th} instar larva Thai silkworms weighing between 0.8-1.2 grams were divided into 7 groups of 10 larvae/group. Normal diet (Silkmate 2S) 5 g/group was given for 1 hr, the diet then was taken out. Each group of silkworms was kept at 27 ± 2 ^{o}C for the different times (0, 1, 2, 3, 4, 5 and 6 hr) and silkworm hemolymph was collected. Samples were prepared and analyzed using phenol-sulfuric acid method and sugar levels were calculated from standard curve of the serially diluted glucose solution.

Determination of time to peak of the sugar levels in Thai silkworm hemolymph after feeding with 10% glucose diet for 1 hr

To determine the time that sugar level increase to the peak in Thai silkworm hemolymph. The first day 5^{th} instar larva Thai silkworms weighing between 0.8-1.2 grams were divided into 7 groups of 10 larvae/group. 10% glucose diet 5 g/group was given for 1 hr, the diet then was taken out. Each group of silkworms was kept at 27 ± 2 ^{o} C for the different times (0, 1, 2, 3, 4, 5 and 6 hr) and silkworm hemolymph was collected. Samples were prepared and analyzed using phenol-sulfuric acid method and sugar levels were calculated from standard curve of the serially diluted glucose solution.

Thai silkworm glucose concentration profile

To determine the optimum concentration of glucose diet that could to prominent the sugar levels in Thai silkworm hemolymph. The first day 5^{th} instar larva Thai silkworms 0.8-1.2 grams were divided into 4 groups (10 larvae/group) and several of glucose diet concentrations as following: normal diet, 5%, 10% and 20% glucose diet were given 5 g/group for 1 hr, the diet then was taken out. Each group of silkworms was kept at 27 ± 2 ^{o} C for 5 hrs and silkworm hemolymph was collected. Samples were prepared and analyzed using phenol-sulfuric acid method and sugar levels were calculated from standard curve of the serially diluted glucose solution.

Determination of time to peak of the sugar levels in Thai silkworm hemolymph after feeding with 10% sucrose diet for 1 hr

To determine the time that sugar level increase to the peak in Thai silkworm hemolymph. The first day 5^{th} instar larva Thai silkworms weighing between 0.8-1.2 grams were divided into 7 groups (10 larvae/group). 10% sucrose diet 5 g/group was given for 1 hr, the diet then was taken out. Each group of silkworms was kept at 27 ± 2 ^{o} C for the different times (0, 1, 2, 3, 4, 5 and 6 hr) and silkworm hemolymph was collected. Samples were prepared and analyzed using phenol-sulfuric acid method and sugar levels were calculated from standard curve of the serially diluted glucose solution.

Thai silkworm sucrose concentration profile

To determine the optimum concentration of sucrose diet that could to prominent the sugar levels in Thai silkworm hemolymph. The first day 5th instar larva Thai silkworms 0.8-1.2 grams were divided into 4 groups (10 larvae/group) and several of sucrose diet concentrations as following: normal diet, 5%, 10% and 20% sucrose diet were given 5 g/group for 1 hr, the diet then was taken out. Each group of silkworms was kept at 27 ± 2 ^{o} C for 4 hrs and silkworm hemolymph was collected. Samples were prepared and analyzed using phenol-sulfuric acid method and sugar levels were calculated from standard curve of the serially diluted glucose solution.

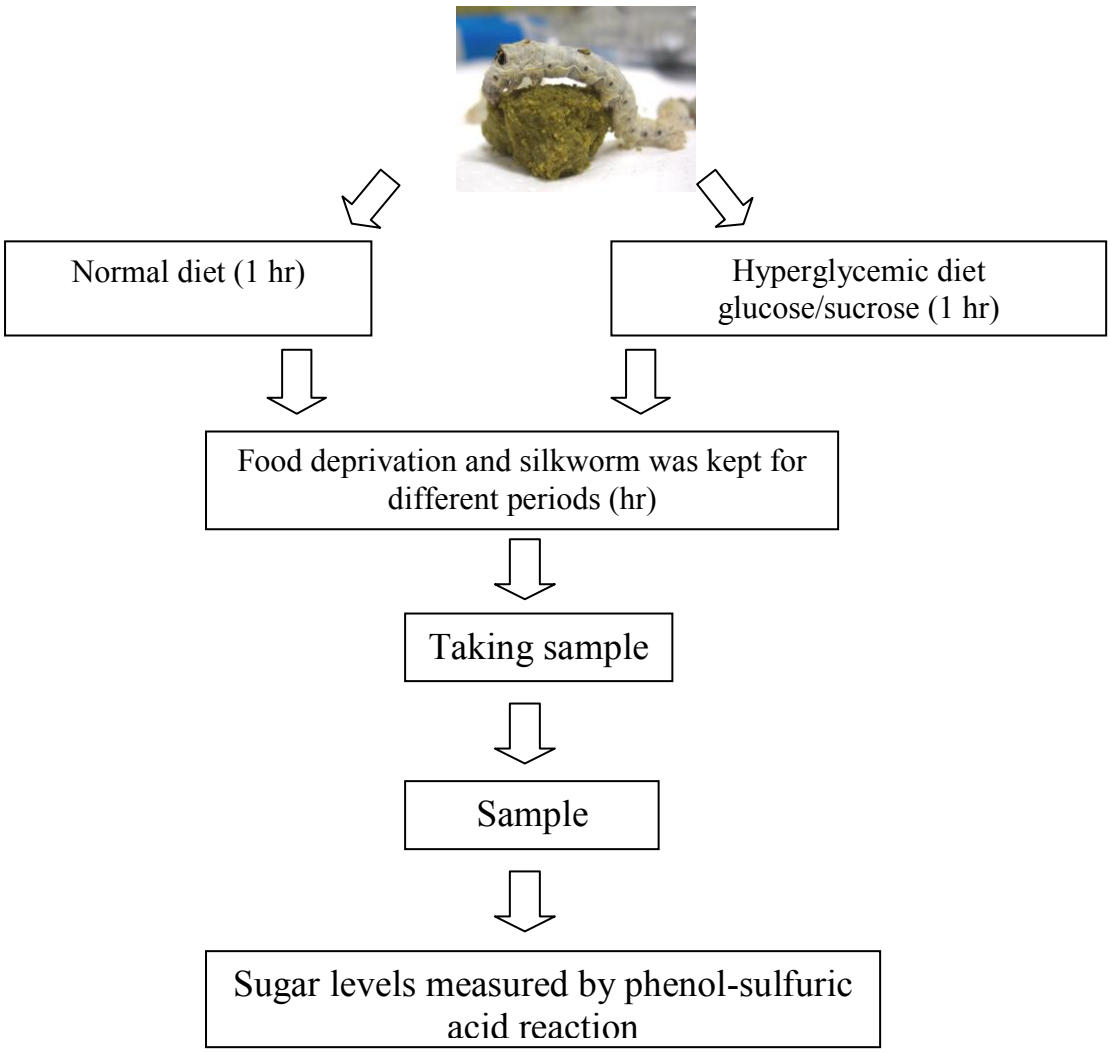


Figure 9 Process of time profile study

Toxicity profile of glibenclamide in Thai silkworm

Toxicity of glibenclamide in Thai silkworm was studied using 0.8-1.2 g/larva of the 5th instar larva Thai silkworms and divided into 7 groups (10 larvae/group). The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 10 % v/v DMSO in 0.9% NaCl and the treatments group were also injected via intra-hemolymph of silkworm with 50 µl of glibenclamide solutions containing glibenclamide of various concentrations of 0.125, 0.25, 0.5, 1, 2 and 3 mg/ml. After the treatments, all of silkworms were kept at 27 ± 2 ^{o}C the mortality of Thai silkworm were observed at 24 and 48 hrs after the treatments. Median lethal dose (LD_{50}) of glibenclamide in Thai silkworm was determined by the regression Probit analysis (SPSS version 16) between Probit unit of lethality of Thai silkworm versus log concentrations of glibenclamide (mg/ml). Process of LD_{50} determination was shown in Figure 10.

Toxicity profile of metformin in Thai silkworm

Toxicity of metformin in Thai silkworm was studied using 0.8-1.2 g/larva of the 5th instar larva Thai silkworms and divided into 7 groups (10 larvae/group). The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments groups were also injected via intra-hemolymph of silkworm with 50 µl of metformin solutions containing metformin of various concentrations of 0.0625, 0.125, 0.25, 0.5, 1 and 1.5 mg/ml. After the treatments, all of silkworms were kept at 27 ± 2 ^{o}C the mortality of Thai silkworm were observed at 24 and 48 hrs after the treatments. Median lethal dose (LD_{50}) of metformin in Thai silkworm was determined by the regression Probit analysis (SPSS version 16) between Probit unit of lethality of Thai silkworm versus log concentrations of metformin (mg/ml). Process of LD_{50} determination was shown in Figure 10.

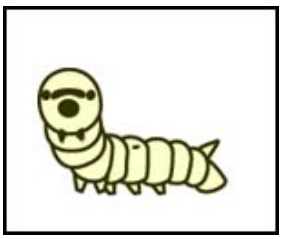
Toxicity profile of acarbose in Thai silkworm

Toxicity of acarbose in Thai silkworm was studied using 0.8-1.2 g/larva of the 5th instar larva Thai silkworms and divided into 8 groups (10 larvae/group). The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments groups were also injected via intra-hemolymph of silkworm with 50 µl

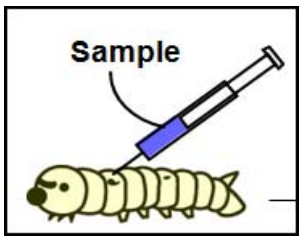
of acarbose solutions containing acarbose of various concentrations of 6.25, 12.5, 25, 50, 100, 200 and 500 mg/ml. After the treatments, all of silkworms were kept at 27 ± 2 ^{o}C the mortality of Thai silkworm were observed at 24 and 48 hrs after the treatments. Median lethal dose (LD_{50}) of acarbose in Thai silkworm was determined by the regression Probit analysis (SPSS version 16) between Probit unit of lethality of Thai silkworm versus log concentrations of acarbose (mg/ml). Process of LD_{50} determination was shown in Figure 10.

Toxicity profile of ECa 233 in Thai silkworm

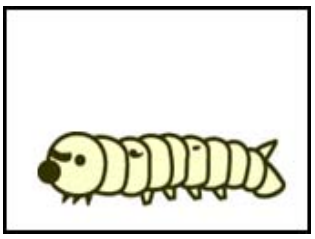
Toxicity of ECa 233 in Thai silkworm was studied using 0.8-1.2 g/larva of the 5th instar larva Thai silkworms and divided into 10 groups (10 larvae/group). The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments groups were also injected via intra-hemolymph of silkworm with 50 µl of ECa 233 solutions containing ECa 233 of various concentrations of 2.5, 5, 10, 20, 40, 60, 80, 100 and 200 mg/ml. After the treatments, all of silkworms were kept at 27 ± 2 ^{o}C the mortality of Thai silkworm were observed at 24 and 48 hrs after the treatments. Median lethal dose (LD_{50}) of ECa 233 in Thai silkworm was determined by the regression Probit analysis (SPSS version 16) between Probit unit of lethality of Thai silkworm versus log concentrations of ECa 233 (mg/ml). Process of LD_{50} determination was shown in Figure 10.



The 5th instar larva Thai silkworm



And amount of 50 µl of sample solution was injected into silkworm hemolymph



% Mortality of Thai silkworm was observed after 24 and 48 hr after the treatment

Probit analysis was performed to obtain LD_{50}

Figure 10 Process of LD_{50} measurement of the test samples in Thai silkworm

Hypoglycemic effect of glibenclamide in Thai silkworm hemolymph

The optimum concentration of glibenclamide that could lower the sugar levels was determined in Thai silkworm hemolymph. The first day 5th instar larva silkworms weighing between 0.8-1.2 grams were divided into 5 groups of 10 larvae/group. The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 10% v/v DMSO in 0.9% NaCl and the treatments group were also injected via intra-hemolymph of silkworm with 50 µl of glibenclamide solutions containing glibenclamide of various concentrations of 0.03, 0.06, 0.125 and 0.25 mg/ml 30 min before 10% glucose diet 5 g/group was given. Silkworms were giving with 10% glucose diet for 1 hr, the diet then was deprive, silkworm were kept at 27 ± 2 ^{o}C for 5 hrs and silkworm hemolymph was collected. Sugar levels were analyzed using phenol-sulfuric acid method and the sugar levels were calculated from the standard curve of the serially diluted glucose solution. Process of determination of sugar levels in Thai silkworm hemolymph was shown in Figure 11.

Hypoglycemic effect of metformin in Thai silkworm

The optimum concentration of metformin that could lower the sugar levels was determined in Thai silkworm hemolymph. The first day 5th instar larva silkworms weighing between 0.8-1.2 grams were divided into 5 groups of 10 larvae/group and 10% glucose diet was given 5 g/group for 1 hr, then the diet was taken out. The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments group were also injected via intra-hemolymph of silkworm with 50 µl of metformin solutions containing metformin of various concentrations of 0.03, 0.06, 0.125 and 0.25 mg/ml. Silkworms were kept at 27 ± 2 ^{o}C for 5 hrs and silkworm hemolymph was collected. Sugar levels were analyzed using phenol-sulfuric acid method and the sugar levels were calculated from the standard curve of the serially diluted glucose solution. Process of determination of sugar levels in Thai silkworm hemolymph was shown in Figure 11.

Hypoglycemic effect of acarbose in Thai silkworm

The optimum concentration of acarbose that could lower the sugar levels was determined in Thai silkworm hemolymph. The first day 5^{th} instar larva silkworms weighing between 0.8-1.2 grams were divided into 5 groups of 10 larvae/group. The negative control group was injected via intra-midgut of silkworm with 50 µl of 0.9% NaCl and the treatments groups were also injected via intra-midgut of silkworm with 50 µl of acarbose solutions containing acarbose of various concentrations of 2.5, 5, 10 and 20 mg/ml 10 min before 10% sucrose diet 5 g/group was given. Silkworms were giving with 10% sucrose diet for 1 hr, the diet then was deprive, silkworms were kept at 27 ± 2 ^{o}C for 4 hrs and silkworm hemolymph was collected. Sugar levels were analyzed using phenol-sulfuric acid method and the sugar levels were calculated from the standard curve of the serially diluted glucose solution. Process of determination of sugar levels in Thai silkworm hemolymph was shown in Figure 11.

Hypoglycemic effect of human insulin in Thai silkworm hemolymph

The optimum concentration of human insulin that could lower the sugar levels was determined in Thai silkworm hemolymph. The first day 5^{th} instar larva silkworms weighing between 0.8-1.2 grams were divided into 4 groups of 10 larvae/group and 10% glucose diet was given 5 g/group for 1 hr, then the diet was taken out. The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments groups were also injected via intra-hemolymph of silkworm with 50 µl of human insulin solutions containing human insulin of various concentrations of 1.75, 3.5 and 7 mg/ml. Silkworms were kept at 27 ± 2 ^{o}C for 5 hrs and silkworm hemolymph was collected. Sugar levels were analyzed using phenol-sulfuric acid method and the sugar levels were calculated from the standard curve of the serially diluted glucose solution. Process of determination of sugar levels in Thai silkworm hemolymph was shown in Figure 11.

Hypoglycemic effect of ECa 233 in Thai silkworm model

The optimum concentration of ECa 233 that could lower the sugar levels was determined in Thai silkworm hemolymph. The first day 5th instar larva silkworms weighing between 0.8-1.2 grams were divided into 5 groups of 10 larvae/group and 10% glucose diet was given 5 g/group for 1 hr, then the diet was taken out. The negative control group was injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments group were also injected via intra-hemolymph of silkworm with 50 µl of ECa 233 solutions containing ECa 233 of various concentrations of 0.005, 0.05, 0.5 and 5 mg/ml. Silkworms were kept at 27 ± 2 ^{o}C for 5 hrs and silkworm hemolymph was collected. Sugar levels were analyzed using phenol-sulfuric acid method and the sugar levels were calculated from the standard curve of the serially diluted glucose solution. Process of determination of sugar levels in Thai silkworm hemolymph was shown in Figure 11.

Hypoglycemic effect of ECa 233 in Thai silkworm model fed with diet for 12 hr before the study started

To determine the hypoglycemic effect of the Standardized Extract of *Centella asiatica*, ECa 233 in Thai silkworm model. The first day 5th instar larva silkworms weighing between 0.8-1.2 grams were divided into 7 groups of 10 larvae/group. All silkworms were fed with normal diet 12 hrs before the study started. Then normal diet 5 grams was continuously giving 1 hr in the first group and 10% glucose diet 5 g/group was given to the 2nd - 7th group for 1 hr, then the diet was taken out. The negative control group (1st and 2nd group) were injected via intra-hemolymph of silkworm with 50 µl of 0.9% NaCl and the treatments group were also injected via intra-hemolymph of silkworm with 50 µl of human insulin solution 3.5 mg/ml and ECa 233 solutions containing ECa 233 of various concentrations of 0.005, 0.05, 0.5 and 5 mg/ml. Silkworms were kept at 27 ± 2 ^{o}C for 5 hrs and silkworm hemolymph was collected. Sugar levels were analyzed using phenol-sulfuric acid method and the sugar levels were calculated from the standard curve of the serially diluted glucose solution. Process of determination of sugar levels in Thai silkworm hemolymph was shown in Figure 11.

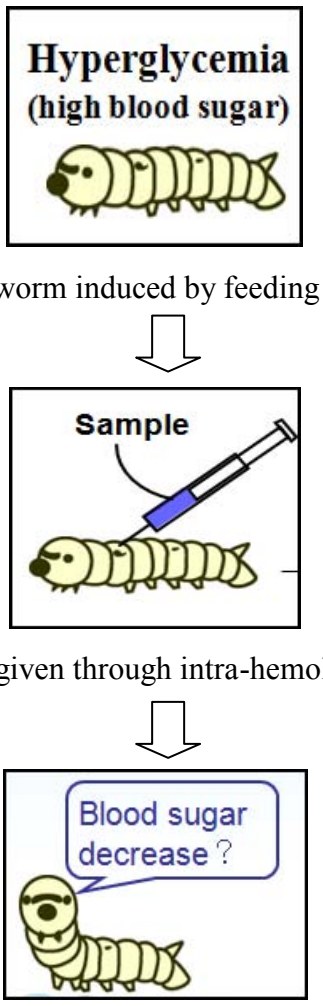


Figure 11 Process of glucose lowering effect screening using Thai silkworm model

Analytical Method

Phenol-Sulfuric Acid Method

This study use phenol-sulfuric acid to determine the amount of sugar levels in Thai silkworm hemolymph**.** This reaction has been used to quantify the amount of sugar in a definite medium such as salts, protein residues, or connected to a polymer. Determination of sugars using this method is based on the absorbance at 490 nm of a color aromatic complex among of carbohydrate and phenol. The quantity of sugar present is determined by compared to a standard curve of the serially diluted glucose solution by used a spectrophotometer.

Standard curve of glucose

Glucose stock solution of 100 mg/100 ml was prepared and the solution was aliquoted to make various concentrations of glucose of 1.25, 2.5, 5, 10 and 20 µg/100 µl. For sugar quantification phenol-sulfuric acid method was performed by mixing 100 µl of each concentrations with 100 µl of 5% phenol followed by 500 µl of concentrated sulfuric acid, samples were mixed by vortexing for 5 sec and incubated at room temperature for 20 min. 200 µl of samples transferred into 96 well microtiter plate and sugar levels were measured by used microplate reader model Perkin Elmer photometry at the wavelength 490 nm. Glucose concentrations were calculated from standard curve of the serially diluted glucose solution.

STATISTICAL ANALYSIS

All results were presented as mean ± S.E.M. Each experiment were performed in duplicate. Statistical analysis was performed by SPSS version 16. Differences among means were analyzed using one way analysis of variance (ANOVA) followed by turkey test for multiple comparisons. The data were considered significant, if *P- value* was less than 0.05

CHAPTER IV
RESULTS

Thai silkworm glucose profiles study

Time profile of sugar levels in Thai silkworm hemolymph after receiving normal diet

After feeding with normal diet 5 g/group for 1 hr, the diet then was taken out. Each group of silkworms was kept for different times. Silkworm hemolymph was collected and sugar levels in silkworm hemolymph were analyzed. The result showed that sugar levels in hemolymph of Thai silkworm reached the peak at 5 hr with the concentration of 2.59 ± 0.08 mg/ml (concentrations of sugar of 0, 1, 2, 3, 4, 5 and 6 hr were 1.13 ± 0.17, 1.48 ± 0.09, 1.66 ± 0.15, 2.19 ± 0.16, 2.27 ± 0.22, 2.59 ± 0.08 and 2.33 ± 0.31 mg/ml, respectively) (Figure 12).

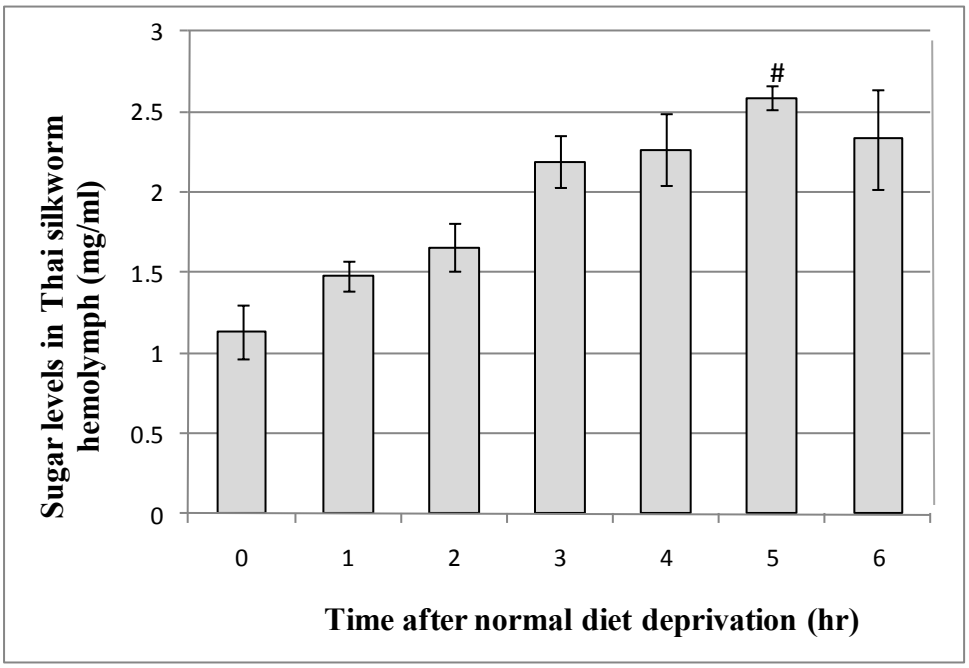


Figure 12 Time profile of sugar levels in Thai silkworm hemolymph after receiving normal diet. Each bar represents the mean ± S.E.M. (N=10), # $P < 0.01$; 5 hr after food deprive versus the other periods

Time profile of sugar levels in Thai silkworm hemolymph after receiving 10% glucose diet

After feeding with 10% glucose diet 5 g/group for 1 hr, the diet then was taken out. Each group of silkworms was kept for different times. Silkworm hemolymph was collected and sugar levels in silkworm hemolymph were analyzed. The result showed that sugar levels in hemolymph of Thai silkworm reached the peak at 5 hr with the concentration of 7.37 ± 0.07 mg/ml (concentrations of sugar of 0, 1, 2, 3, 4, 5 and 6 hr were 2.68 ± 0.12, 3.56 ± 0.14, 4.07 ± 0.04, 4.82 ± 0.34, 6.85 ± 0.31, 7.37 ± 0.07 and 6.86 ± 0.08 mg/ml, respectively) (Figure 13).

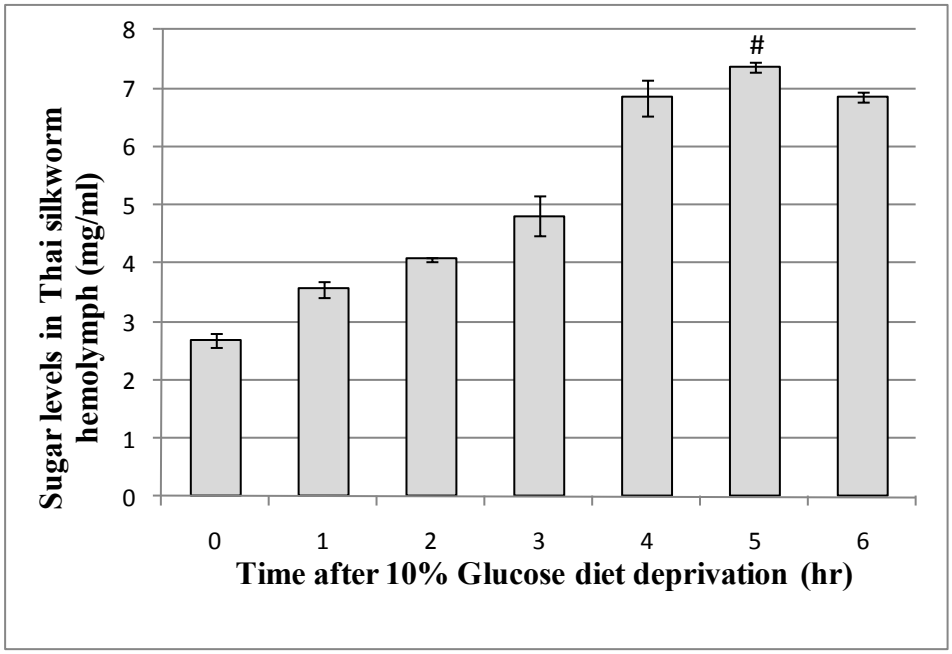


Figure 13 Time profile of sugar levels in Thai silkworm hemolymph after receiving 10% glucose diet. Each bar represents the mean ± S.E.M. (N=10),
# $P < 0.01$; 5 hr after food deprive versus the other periods

Glucose concentration profile

To determine the optimum concentration of glucose diet that could to induce the hyperglycemia, 5%, 10% and 20% glucose diet 5 g/group was fed to silkworm for 1 hr and then diet was taken out, each group of silkworm was kept for 5 hr. Silkworm hemolymph was collected and sugar levels in silkworm hemolymph were analyzed. The result showed that sugar levels in Thai silkworm hemolymph reached the peak with concentration 2.51 ± 0.08, 4.70 ± 0.16, 7.22 ± 0.05 and 12.20 ± 0.22 mg/ml in normal diet, 5% glucose diet group, 10% glucose diet group and 20% glucose diet group, respectively (Figure 14).

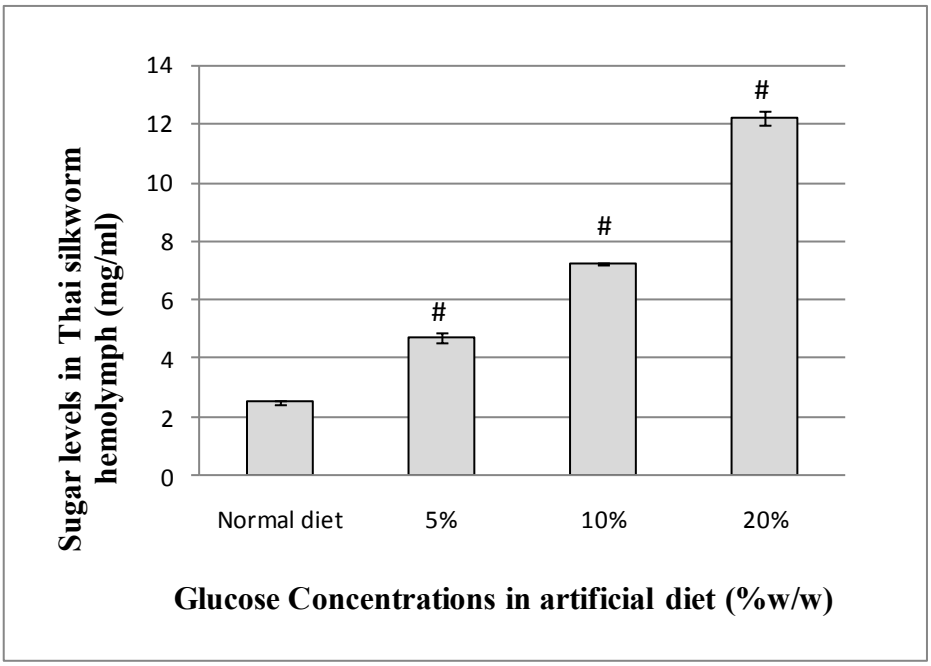


Figure 14 Glucose concentration profile in Thai silkworm hemolymph after receiving artificial diet at different glucose concentrations. Each bar represents the mean ± S.E.M. (N=10), # $P < 0.01$ compared to normal diet group

Time profile of sugar levels in Thai silkworm hemolymph after receiving 10% sucrose diet

After feeding with 10% sucrose diet 5 g/group for 1 hr, the diet then was taken out. Each group of silkworms was kept for different times. Silkworm hemolymph was collected and sugar levels in silkworm hemolymph were analyzed. The result showed that sugar levels in hemolymph of Thai silkworm reached the peak at 4 hr with the concentration of 7.70 ± 0.04 mg/ml (concentrations of sugar of 0, 1, 2, 3, 4, 5 and 6 hr were 5.67 ± 0.15, 6.63 ± 0.10, 7.03 ± 0.12, 7.34 ± 0.10, 7.70 ± 0.08, 7.01 ± 0.08 and 6.72 ± 0.09 mg/ml, respectively) (Figure 15).

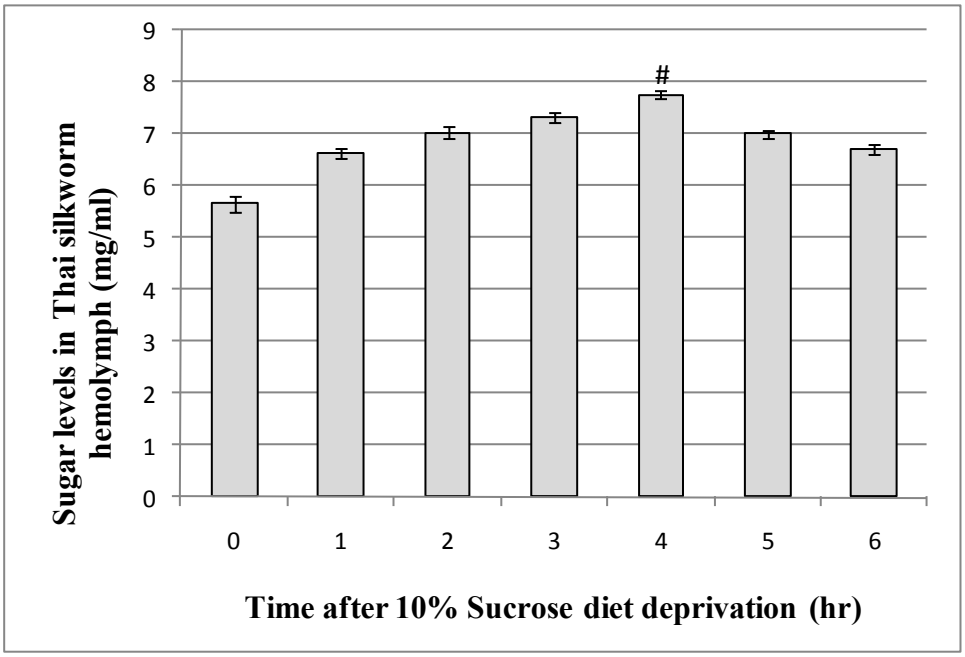


Figure 15 Time profile of sugar levels in Thai silkworm hemolymph after receiving 10% sucrose diet. Each bar represents the mean ± S.E.M. (N=10),
# $P < 0.01$; 4 hr after food deprive versus the other periods

Sucrose concentration profile

To determined the optimum concentration of sucrose diet that could to induce the hyperglycemia, 5%, 10% and 20% glucose diet 5 g/group was fed to silkworm for 1 hr and then diet was taken out, each group of silkworm was kept for 4 hr. Silkworm hemolymph was collected and sugar levels in silkworm hemolymph were analyzed. The result showed that sugar levels in Thai silkworm hemolymph reached the peak with concentration 2.41 ± 0.04, 4.97 ± 0.15, 7.76 ± 0.07 and 9.73 ± 0.25 in the normal diet group, 5% sucrose diet group, 10% sucrose diet group and 20% sucrose diet group, respectively (Figure 15).

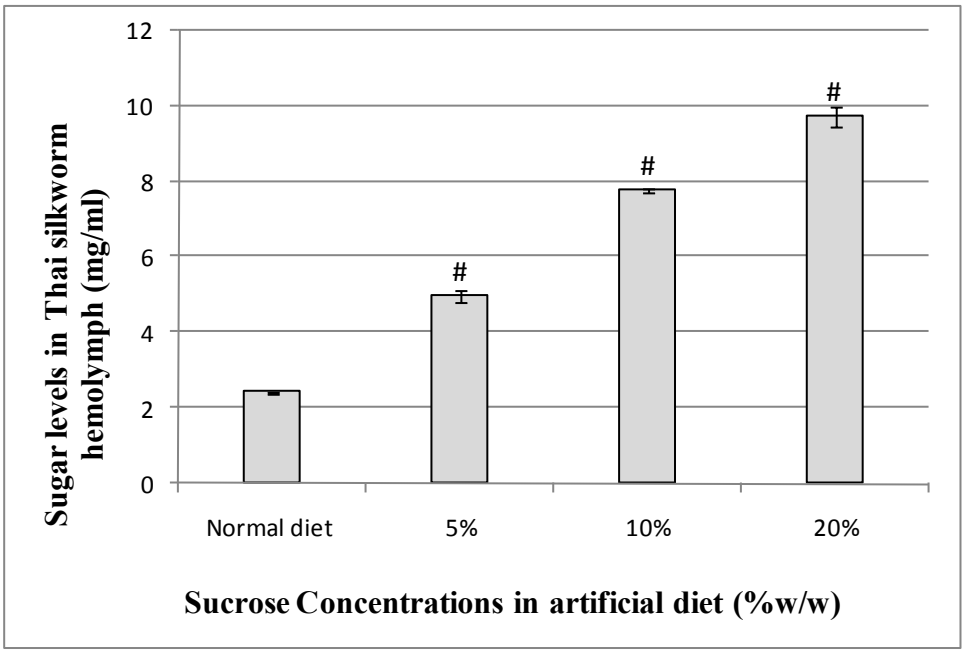


Figure 16 Sucrose concentration profile in Thai silkworm hemolymph after receiving artificial diet at different sucrose concentrations. Each bar represents the mean ± S.E.M. (N=10), # $P < 0.01$ compared to normal diet given group

Toxicity profile of glibenclamide in Thai silkworm

After the 5th instar larva Thai silkworm were injected with 50 µl of glibenclamide solution via intra-hemolymph (0.125, 0.25, 0.5, 1, 2, 3 mg/ml and 0.9% NaCl as a negative control), then the mortality and survival of Thai silkworm were observed at 27 ± 2 ^{o}C after 24 and 48 hrs. The results was shown that the lethal (LD_{50}) of glibenclamide in Thai silkworm was 0.95 mg/ml or 0.047 mg/g (Figure 17).

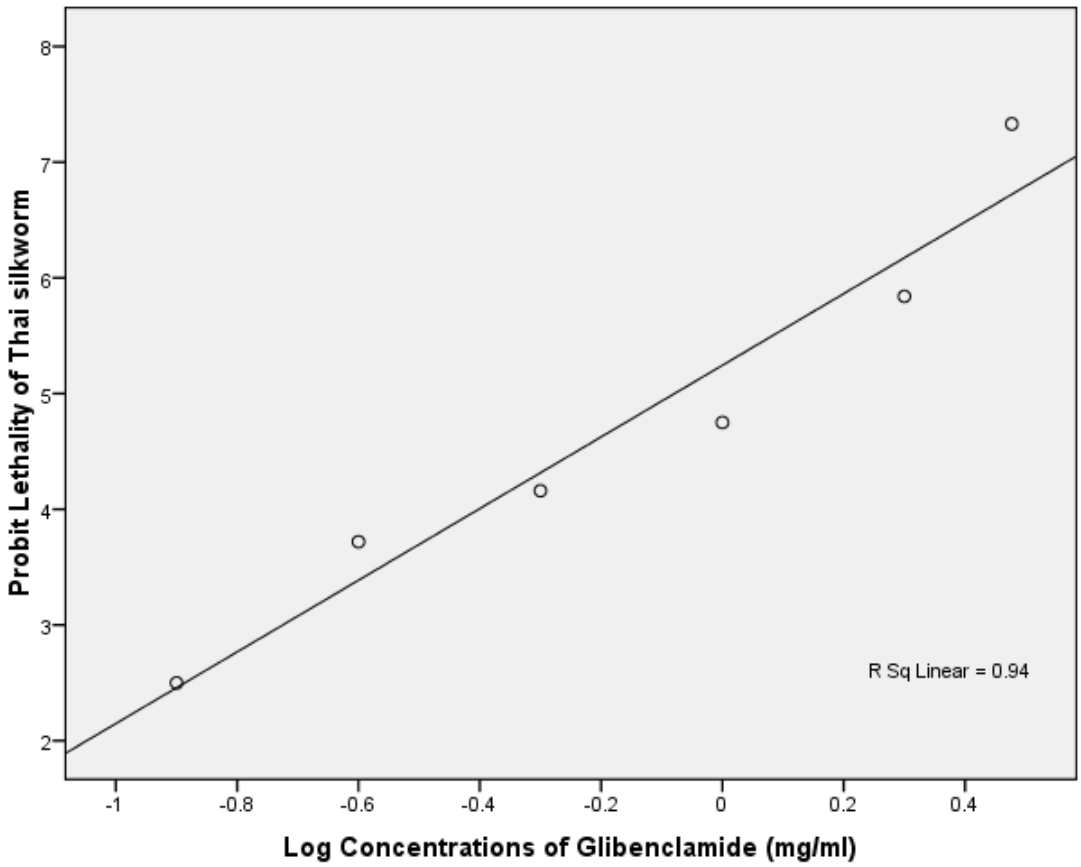


Figure 17 Median lethal dose (LD_{50}) of glibenclamide in Thai silkworm

Toxicity profiles of metformin in Thai silkworm

After the 5th instar larva Thai silkworm were injected with 50 µl of metformin solution via intra-hemolymph (0.0625, 0.125, 0.25, 0.5, 1, 1.5 mg/ml and 0.9% NaCl as a negative control), then the mortality and survival of Thai silkworm were observed at 27 ± 2 ^{o}C after 24 and 48 hrs. The results was shown that the lethal (LD_{50}) of metformin in Thai silkworm was 0.82 mg/ml or 0.041 mg/g (Figure 18).

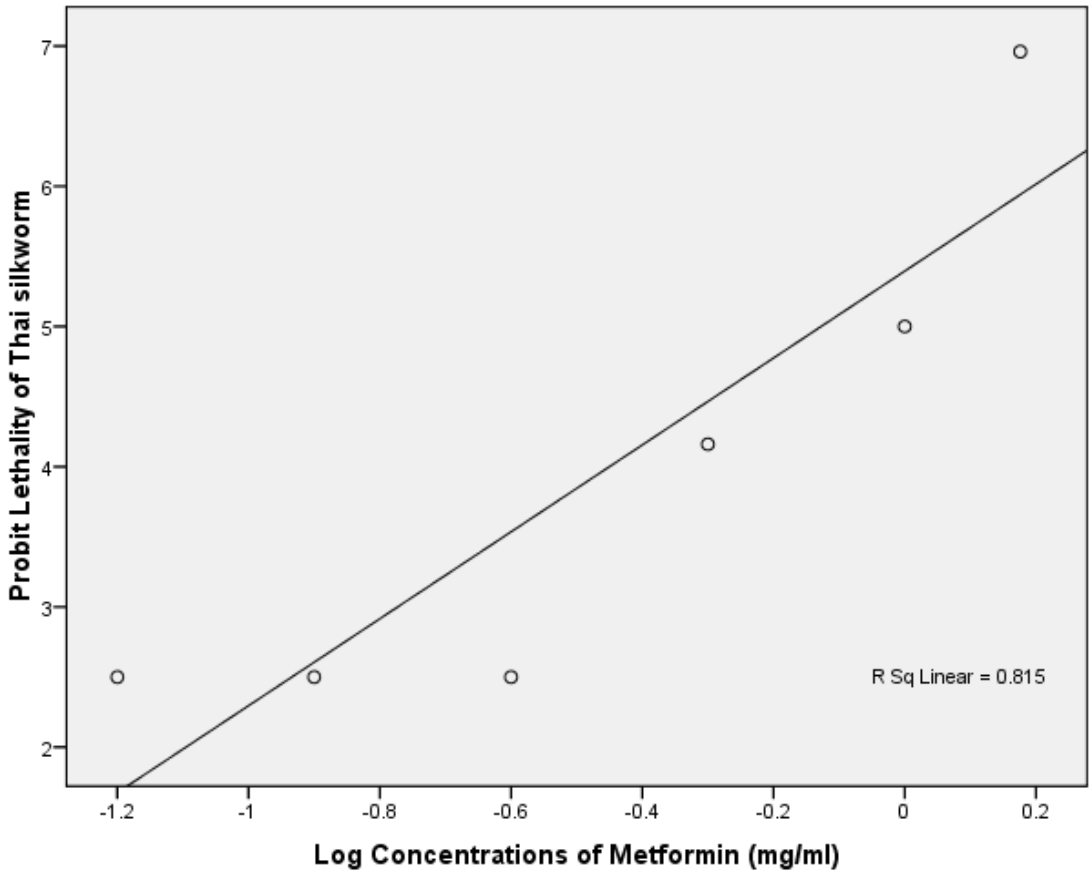


Figure 18 Median lethal dose (LD_{50}) of metformin in Thai silkworm

Toxicity profile of acarbose in Thai silkworm

After the 5th instar larva Thai silkworm were injected with 50 µl of acarbose solution via intra-hemolymph (6.25, 12.5, 25, 50, 100, 200, 500 mg/ml and 0.9% NaCl as a negative control), then the mortality and survival of Thai silkworm were observed at 27 ± 2 °C after 24 and 48 hrs. The result was shown that acarbose up to 500 mg/ml or 25 mg/g did not have the toxic effect to the Thai silkworm (Figure19).

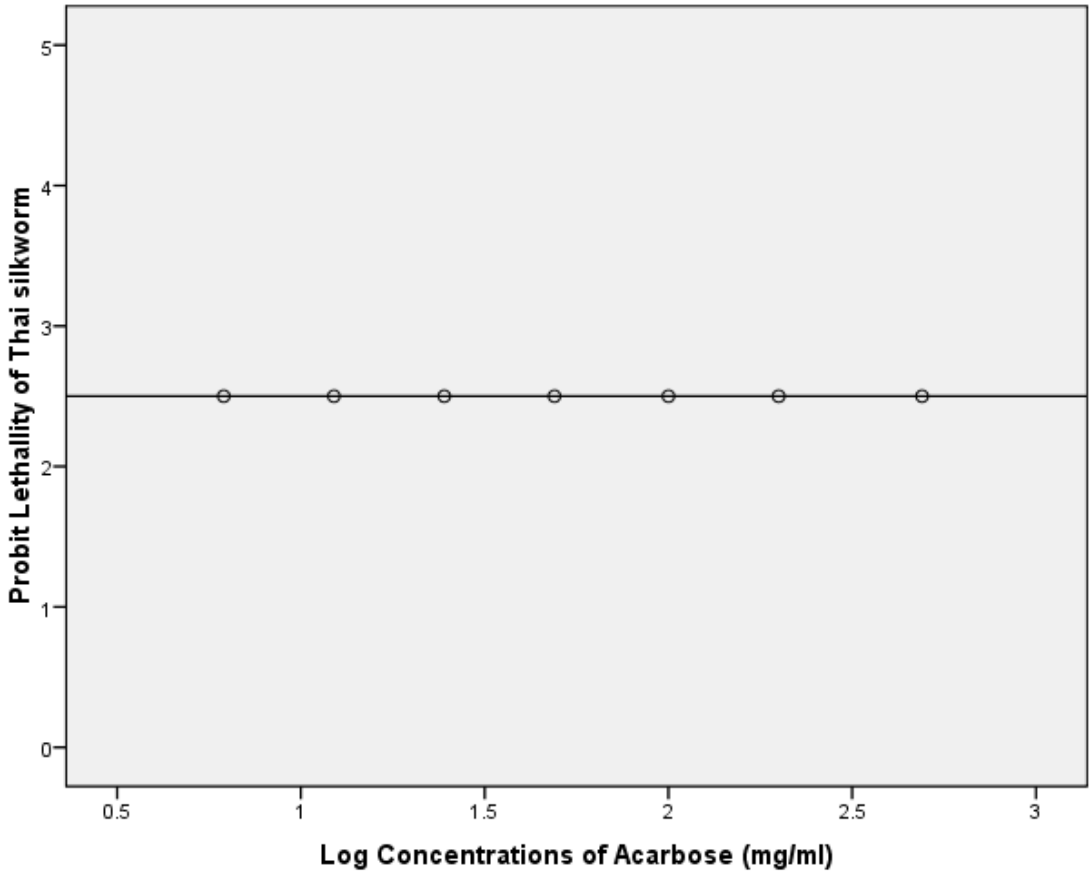


Figure 19 Median lethal dose (LD_{50}) of acarbose in Thai silkworm

Toxicity profile of ECa 233 in Thai silkworm model

After the 5th instar larva Thai silkworm were injected with 50 µl of Standardized extract of *Centella asiatica*, ECa 233 via intra-hemolymph (2.5, 5, 10, 20, 40, 60, 80, 100, 200 mg/ml and 0.9% NaCl as a negative control), then the mortality and survival of Thai silkworm were observed at 27 ± 2 °C after 24 and 48 hrs. The result was shown that the lethal dose (LD50) of ECa 233 in Thai silkworm was 29.29 mg/ml or 1.46 mg/g (Figure 20).

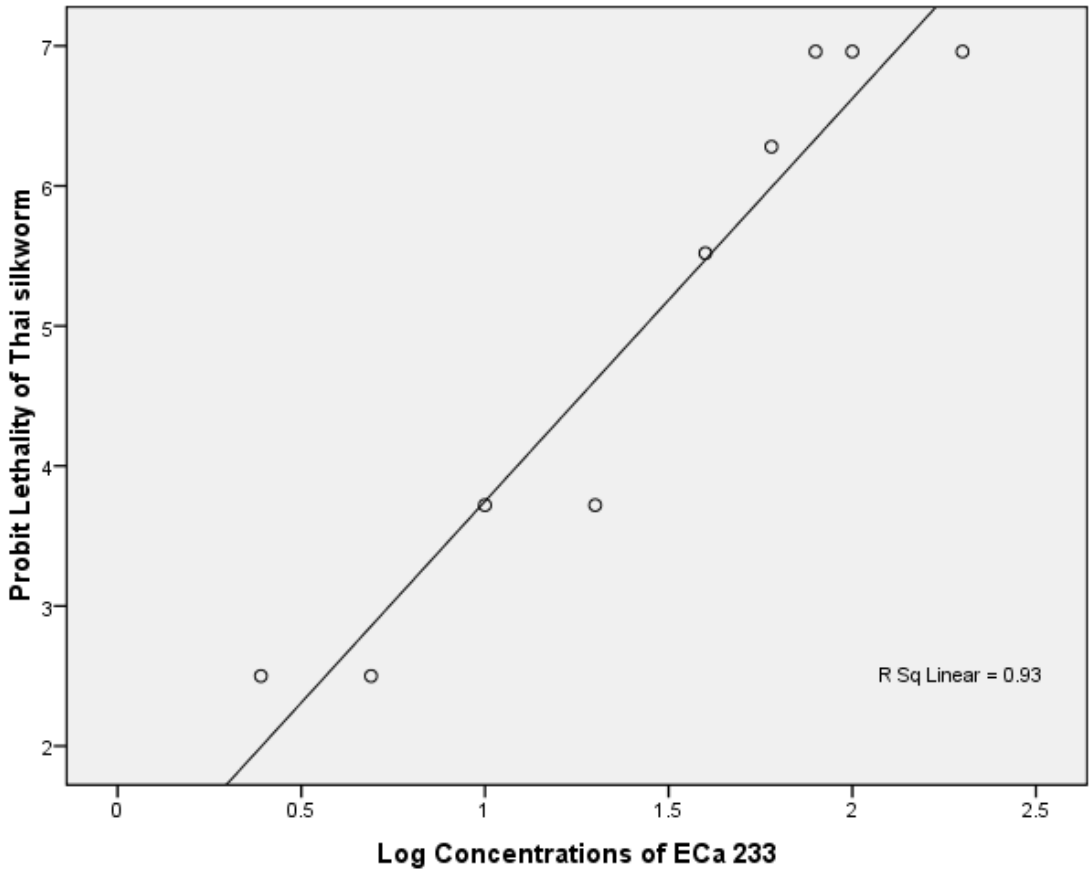


Figure 20 Median lethal dose (LD_{50}) of Standardized Extract of *Centella asiatica*, ECa 233 in Thai silkworm

Hypoglycemic effect of glibenclamide in a Thai silkworm model

After glibenclamide solution in several concentrations (0.03, 0.06, 0.125, 0.25 mg/ml and 10% v/v DMSO in 0.9% NaCl as a negative control) volume 50 µl were injected via intra-hemolymph 30 min before feeding the 5th instar larva silkworm with 10% glucose diet 5 g/group for 1 hr, then the diet was taken out. Silkworm were kept at 27 ± 2 ^{o}C for 5 hrs, then hemolymph was collected and sugar levels were analyzed. The result showed that glibenclamide has hypoglycemic effect in Thai silkworm hemolymph with statistical significance (P< 0.01) in almost all of doses except in the dose 0.03 mg/ml (concentrations of sugar of control group, glibenclamide 0.03, 0.06, 0.125, and 0.25 mg/ml were 7.40 ± 0.14, 7.38 ± 0.10, 6.97 ± 0.16, 6.72 ± 0.14 and 6.38 ± 0.13 mg/ml, respectively) (Figure 21).

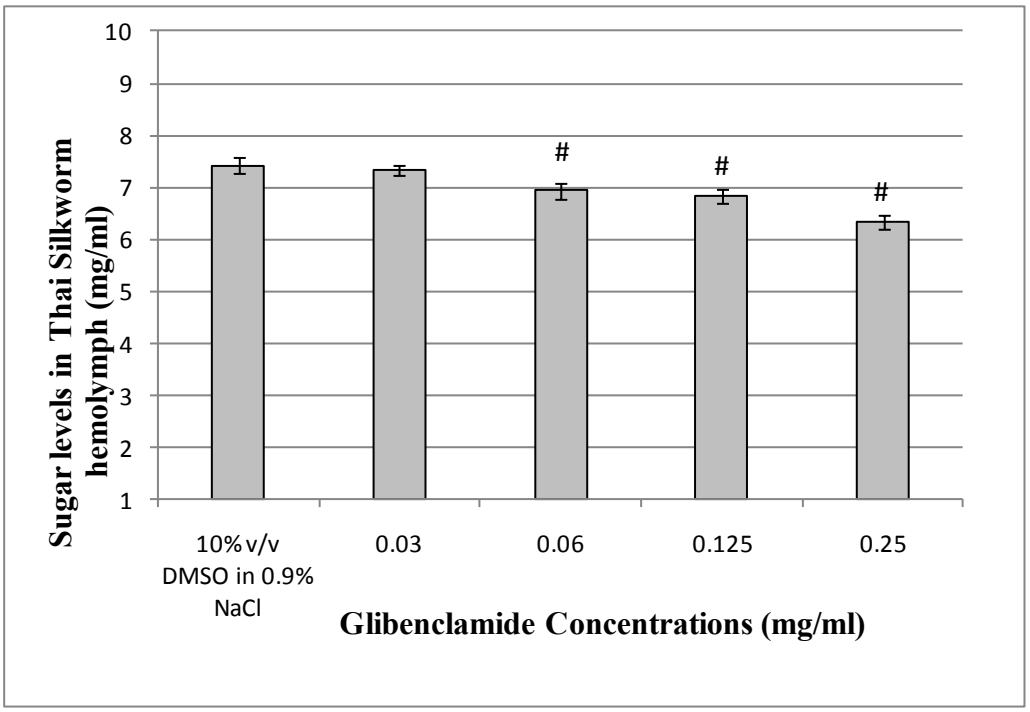


Figure 21 Hypoglycemic effect of glibenclamide in Thai silkworm hemolymph. Each bar represents the mean ± S.E.M. (N=10), # P< 0.01 compared to control group

Hypoglycemic effect of metformin in a Thai silkworm model

After feeding the 5th instar larva silkworm with 10% glucose diet 5 g/group for 1 hr, then the diet was taken out and metformin solution in several concentrations (0.03, 0.06, 0.125, 0.25 mg/ml and 0.9% NaCl as a negative control) volume 50 µl were injected via intra-hemolymph. Silkworm were kept at 27 ± 2 °C for 5 hrs, then hemolymph was collected and sugar levels were analyzed. The result showed that metformin could not lower the sugar levels in Thai silkworm hemolymph in all of doses (concentrations of sugar of control group, metformin 0.03, 0.06, 0.125 and 0.25 mg/ml were 7.27 ± 0.13, 7.30 ± 0.22, 7.24 ± 0.21, 7.19 ± 0.30 and 7.26 ± 0.19 mg/ml, respectively) (Figure 22).

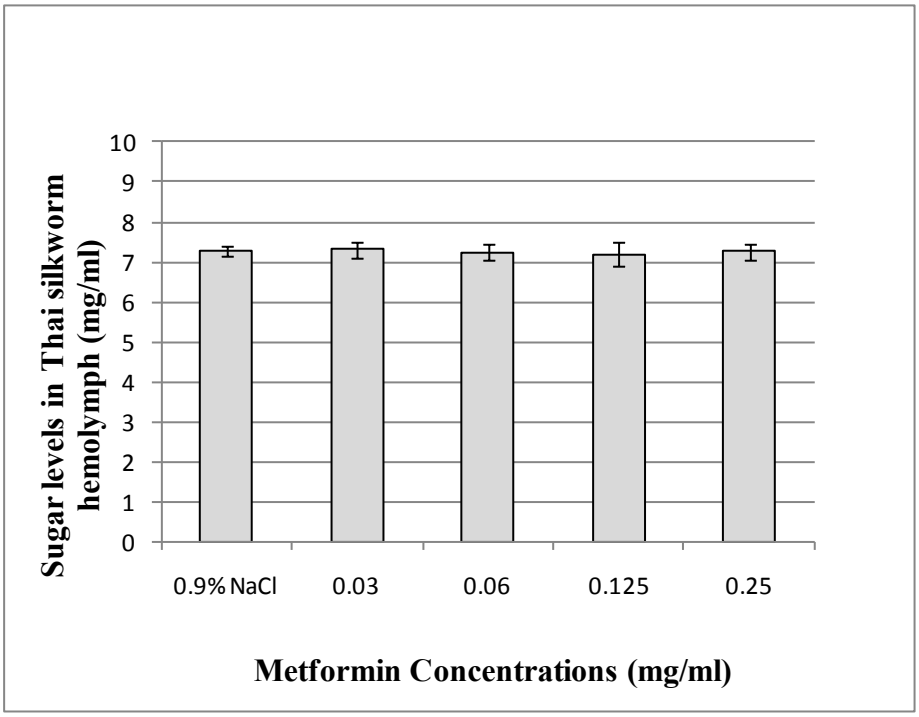


Figure 22 Hypoglycemic effect of metformin in Thai silkworm hemolymph

Hypoglycemic effect of acarbose in a Thai silkworm model

After acarbose solution in several concentrations (2.5, 5, 10, 20 mg/ml and 0.9% NaCl as a negative control) volume 50 µl were injected via intra-midgut 10 min before feeding the 5th instar larva silkworm with 10% sucrose diet 5 g/group for 1 hr, then the diet was taken out. Silkworm were kept at 27 ± 2 ^{o}C for 4 hrs, then hemolymph was collected and sugar levels were analyzed. The result showed that acarbose has hypoglycemic effect in Thai silkworm hemolymph with statistical significance (P< 0.01) in all of doses (concentrations of sugar of control group, acarbose 2.5, 5, 10 and 20 mg/ml were 8.03 ± 0.13, 7.58 ± 0.10, 6.88 ± 0.13, 6.15 ± 0.15 and 4.74 ± 0.10 mg/ml, respectively) (Figure 23).

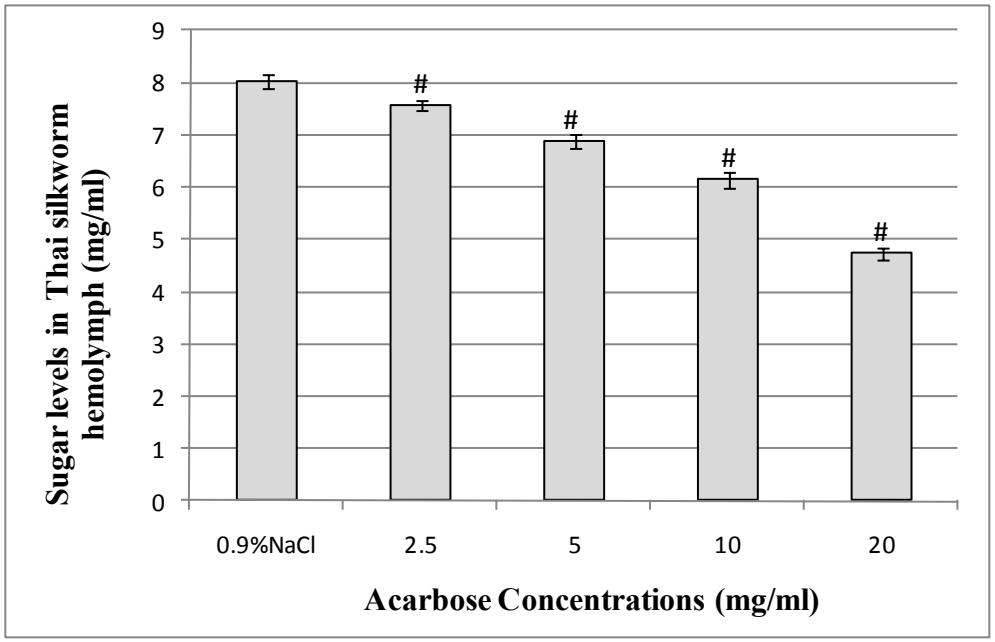


Figure 23 Hypoglycemic effect of acarbose in Thai silkworm hemolymph. Each bar represents the mean ± S.E.M. (N=10), # P< 0.01 compared to control group

Hypoglycemic effect of human insulin in a Thai silkworm model

After feeding the 5th instar larva silkworm with 10% glucose diet 5 g/group for 1 hr, then diet was taken out and human insulin solution in several concentrations (1.75, 3.5, 7 mg/ml and 0.9% NaCl as a negative control) volume 50 µl were injected via intra-hemolymph. Silkworm were kept at 27 ± 2 °C for 5 hrs, then hemolymph was collected and sugar levels were analyzed. The result showed that human insulin has hypoglycemic effect in Thai silkworm hemolymph with statistical significance ($P< 0.01$) in all of doses (concentrations of sugar of control group, human insulin 1.75, 3.5 and 7 mg/ml were 7.37 ± 0.07, 6.84 ± 0.10, 4.10 ± 0.13 and 3.54 ± 0.10 mg/ml, respectively) (Figure 24).

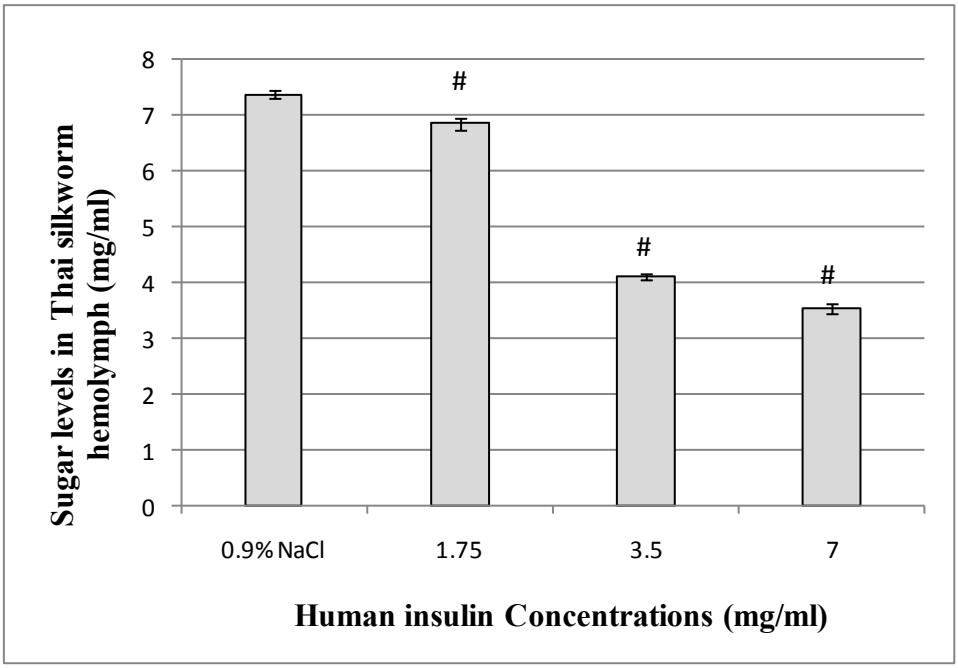


Figure 24 Hypoglycemic effect of human insulin in Thai silkworm hemolymph. Each bar represents the mean ± S.E.M. (N=10), # $P< 0.01$ compared to control group

Hypoglycemic effect of Standardized Extract of *Centella asiatica*, ECa 233 in a Thai silkworm model

After feeding the 5th instar larva silkworm with 10% glucose diet 5 g/group for 1 hr, then diet was taken out and the Standardized Extract of *Canella Asiatic*, ECa 233 solution in several concentrations (0.005, 0.05, 0.5, 5 mg/ml and 0.9% NaCl as a negative control) volume 50 µl were injected via intra-hemolymph. Silkworm were kept at 27 ± 2 ^{o}C for 5 hrs, then hemolymph was collected and sugar levels were analyzed. The result showed that ECa 233 has hypoglycemic effect in Thai silkworm hemolymph with statistical significance (P< 0.01) in almost of doses except in the dose 0.005 mg/ml (concentrations of sugar of 0.9%NaCl, ECa 233 0.005, 0.05, 0.5, 5 mg/ml were 7.34 ± 0.04, 7.14 ± 0.12, 6.52 ± 0.12, 6.27 ± 0.20 and 5.28 ± 0.07 mg/ml, respectively) (Figure 25).

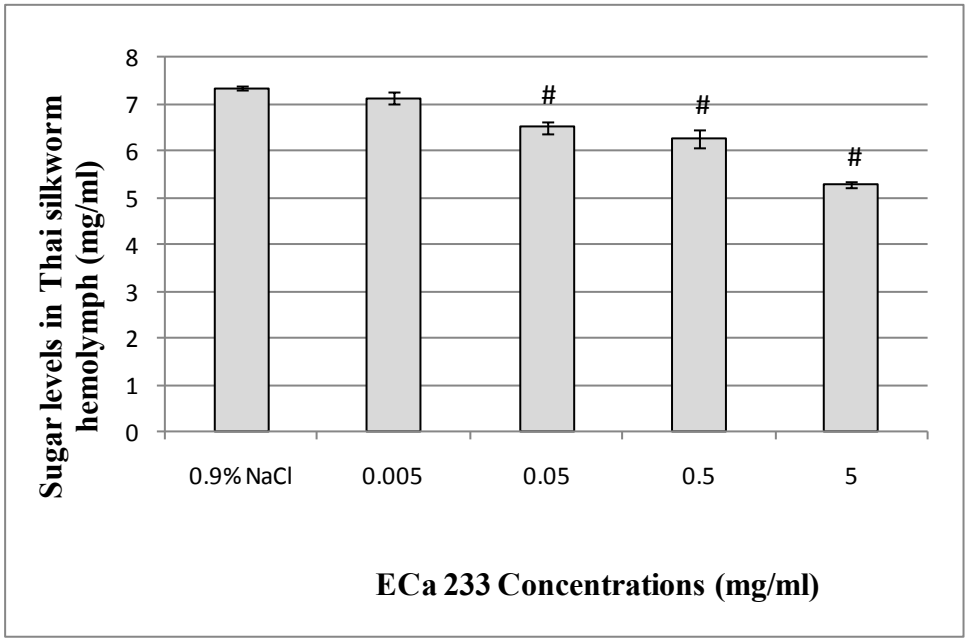


Figure 25 Hypoglycemic effect of Standardized Extract of *Centella asiatica*, ECa 233 in Thai silkworm hemolymph. Each bar represents the mean ± S.E.M. (N=10), # P< 0.01 compared to control group

Hypoglycemic effect of ECa 233 in Thai silkworm model fed with diet 12 hr before the study started

After the 5th instar larva silkworms were fed with normal diet 12 hr before the study started. Then normal diet 5 grams was continuously giving to the first group and 10% glucose diet 5 g/group was given to the 2nd - 7th group for 1 hr, then diet was taken out. The negative control group (1st and 2nd group) were injected via intra-hemolymph with 50 µl of 0.9% NaCl and the treatments group were also injected via intra-hemolymph with 50 µl of human insulin solution 3.5 mg/ml and ECa 233 solutions containing ECa 233 of various concentrations of 0.005, 0.05, 0.5 and 5 mg/ml. Silkworm were kept at 27 ± 2 ^{o}C for 5 hrs, then hemolymph was collected and sugar levels were analyzed. The result showed that human insulin and ECa 233 has hypoglycemic effect in Thai silkworm hemolymph with statistical significance (P< 0.01) in almost of doses (concentrations of sugar of normal diet, 10% glucose diet, human insulin 3.5 mg/ml, ECa 233 0.005, 0.05, 0.5 and 5 mg/ml were 11.46 ± 0.24, 20.06 ± 0.27, 3.69 ± 0.19, 10.20 ± 0.29, 10.09 ± 0.32, 7.57 ± 0.23 and 4.80 ± 0.18 mg/ml, respectively) (Figure 26).

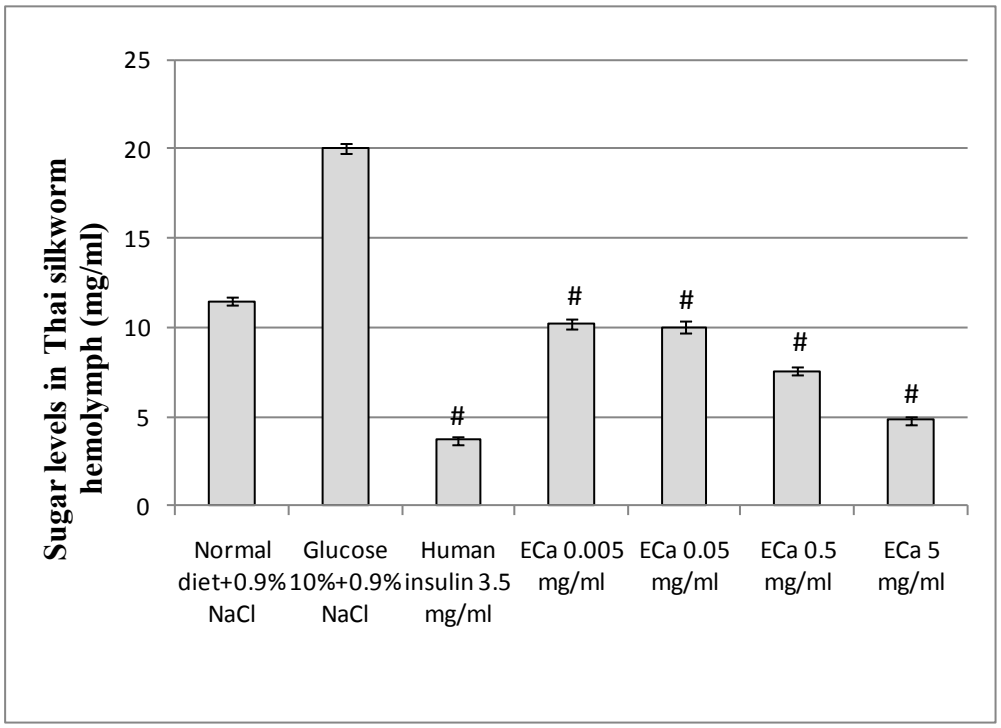


Figure 26 Hypoglycemic effect of ECa 233 in Thai silkworm model diet were taking 12 hr before the studied started. Each bar represents the mean ± S.E.M. (N=10), # $P < 0.01$ compared to control group

CHAPTER V
DISCUSSION AND CONCLUSIONS

Diabetes mellitus is increasing worldwide. In order to obviate this problem, several research topics are focusing on the development of new effective hypoglycemic agent. Laboratory animals are necessary for the effective evaluation of new hypoglycemic agent. However, the use of the laboratory animals has shown some of limitations concerning cost of the sufficient animal cultivation, the availability of qualified staff such as the cost of cage, feed, transportation fee and bedding. This is also including group sizes of animal since the smallest group selected often without a powerful to analyze and the consequential respect is the ethical issues. Furthermore, the use of laboratory animal models especially mammalian or vertebrate model is of the great expenses. In the meantime invertebrate animal model, therefore, has been reported to be utilized as a laboratory animal model including silkworm (*Bombyx mori*). The silkworm model has been developed by the researchers of The University of Tokyo, Japan. Use of silkworm model is of many benefits as followings: size of the silkworm body is large enough to be able to inject sample solutions into hemolymph. The mid-gut and other organs of silkworm can be isolated. Silkworm is less costly to cultivate and can be maintained easily in a small space. Moreover, it is easy to handling and less ethical issues. Silkworm has been reported as an infection model. The model can be utilized to evaluate antibacterial and antiviral agents (Hamamoto *et al*., 2004, 2005; Kaito *et al*., 2002; Orihara *et al*., 2008). Pharmacokinetics and pharmacodynamics of antibiotics in silkworm have shown to be consistent with mammals (Hamamoto *et al*., 2009). Recently silkworm has also been used as a model for screening of anti-diabetic drug (Matsumoto *et al*., 2011).

Silkworm has bombyxin, an insulin-related peptide synthesized in the brain of the silkworm. Bomxyxin has been reported as the hetero-dimer of insulin-like A and B chains whose amino acid sequences show about 50% and 40% identity to the A and B chains, respectively of human insulin (Figure 3 and Figure 4). The effect of bombyxin is to lower the concentration of sugar levels in silkworm hemolymph. When the diet is taken, this will stimulate the insulin signaling via bombyxin Akt phosphorylation resulting in the reduction of sugar level in silkworm hemolymph (Figure 5). Thus, in this

study we aimed to demonstrate the use of the Thai silkworm as a model for screening the anti-diabetic effects of substances.

The results have shown that Thai silkworm can be induced to become hyperglycemia by using silkmate 2S (artificial diet) mixing with 10% glucose diet or 10% sucrose diet and sugar levels in the hemolymph can be determined by phenol-sulfuric acid method. Sugar level in hemolymph reached the maximum concentrations at 5 hr after food deprivation whereas taking 10% sucrose diet sugar level in hemolymph reached the maximum concentrations at 4 hr after food deprivation. It also was found that the optimum concentration of glucose and sucrose that mixed in the artificial diet and could be clearly detected was 10% w/w. In general, trehalose is a great majority sugar in silkworm hemolymph whereas glucose usually is not detected. (less than 4% of the total sugar in silkworm hemolymph). Therefore the increase of sugar level determined in Thai silkworm hemolymph should come from a high glucose diet given (Matsumoto *et al*., 2011).

When Thai silkworm was tested as a model for the anti-diabetic screening of the natural products in this study, 4 anti-diabetic drugs used in human were applied as standard treatment namely human insulin, some oral hypoglycemic drugs with different mechanism of action such as glibenclamide, metformin, acarbose. ECa 233 is the natural product used to test for this model. First, the evaluation of the toxicity profiles of all standards including ECa 233 in Thai silkworm, the results have shown that these substances in the concentrations used were safe to silkworm model. The LD_{50} of glibenclamide, metformin and ECa 233 were 0.95 mg/ml or 0.047 mg/g (Oral rat >20,000 mg/kg, Intraperitoneal rat 3750 mg/kg, Oral mouse > 3250 mg/kg, Intraperitoneal mouse 5900 mg/kg; Glyburide (Glibenclamide), 2011) 0.82 mg/ml or 0.041 mg/g (Acute toxicity rabbit 350 mg/kg, metformin hydrochloride rat oral 1000 mg/kg, mouse oral 1450 mg/kg, rat subcutaneous 300 mg/kg; Material safety data sheet, 2009) and 29.29 mg/ml or 1.46 mg/g (acute toxicity study oral administration in the dose up to 10 g/kg did not cause any lethality in mice Tantisira.M.H.,2009) respectively whereas the median lethal dose of acarbose in Thai silkworm model could not be determined up to 500 mg/ml or 25 mg/g (mouse and rat oral 24,000 mg/kg; Material safety data sheet, 2007). For insulin, in this work insulin toxicity study has been omitted since insulin concentration up to 10 mg/ml has been utilized in silkworm model for

glucose lowering effect by Matsumoto et al., 2011 already. As well, our model use insulin concentration only up to 7 mg/ml. In addition, the toxicity profiles of all standards and ECa 233 were used to decide the doses for the hypoglycemic effect study.

From the hypoglycemic effect study, the results have shown that human insulin has hypoglycemic effect in Thai silkworm hemolymph in all doses with statistical significance compared to negative control group (0.9% NaCl) and this effect was shown in a concentration dependent manner. Similar to insulin, glibenclamide and acarbose also have hypoglycemic effect in Thai silkworm hemolymph in all doses with statistical significance compared to negative control group (0.9% NaCl) and this effect was shown in a concentration dependent manner as well. Human insulin 3.5 mg/ml, glibenclamide 0.25 mg/ml and acarbose 20 mg/ml have shown to decrease sugar levels in Thai silkworm hemolymph 44.29%, 13.31% and 35.73% respectively compared to negative control group while metformin did not have this effect. When hypoglycemic effect of ECa 233 was screened using the Thai silkworm hyperglycemic model, the results have shown that ECa 233 in various concentrations 0.005, 0.05, 0.5 and 5 mg/ml has hypoglycemic effect in Thai silkworm hemolymph in almost of doses except in the dose 0.005 mg/ml and this effect also was shown in a concentration dependent manner. The maximum dose of ECa 233 in this study (5 mg/ml) could decrease the sugar levels in Thai silkworm hemolymph 28.26% compared to negative control group (Figure 25).

Thai silkworm model was tested for hypoglycemic effect compared between the optimum dose of standards (human insulin 3.5 mg/ml, glibenclamide 0.25 mg/ml and metformin 0.25 mg/ml), ECa 233 (5 mg/ml) and negative control. The results have shown that human insulin, glibenclamide and ECa 233 could decrease sugar levels in Thai silkworm hemolymph with statistical significance compared to negative control. Glibenclamide 0.25 mg/ml and ECa 233 5 mg/ml could decrease sugar levels 13.31% and 28.26% respectively compared to negative control whereas metformin 0.25 mg/ml did not have this effect in Thai silkworm model.

Upon the results from this study, it has shown that Thai silkworm could be use to determine the sugar levels in hemolymph via the use of phenol-sulfuric acid method. The stage of silkworm suitable to use as a laboratory animal is the first day of the 5^{th} instar larva because this instar larva is not only the mature stage but also has a proper size that easily to handle, to inject the test substances and to collect the blood sample including an

organs involved in drug metabolism and can be isolated. In addition, the first day of the 5th instar larva silkworm was passed from the molted and fasted all the day. Therefore, sugar levels that can be determined in Thai silkworm hemolymph are from glucose or sucrose that mixed in their diet. The suitable standard to use as a positive control for the development of Thai silkworm as a model for screening hypoglycemic effect of the substances namely human insulin 3.5 mg/ml, glibenclamide 0.25 mg/ml (the maximum dose in this study). From this study, the result was shown that glibenclamide could decrease sugar level in Thai silkworm hemolymph with statistical significance and this effect was shown in a concentration dependent manner. However, increase the dose of glibenclamide has the limitation from the toxicity profile (LD_{50} 0.95 mg/ml or 0.047 mg/g.larva). This limitation also applied for acarbose (20 mg/ml, maximum dose in this study). The results from this study also showed the hypoglycemic effect in Thai silkworm model with statistical significance and in a concentration dependent manner. Acarbose is a safe compound in Thai silkworm model (LD_{50} is more than 500 mg/ml or 25 mg/g.larva) so it did not show any limitation for adjusting or increasing the dose of acarbose for the hypoglycemic effect study. Unlike the other drugs, metformin is not suitable to use as a positive control. In mammals insulin decreases blood sugar levels via the activation of insulin signaling pathway and then increases Akt phosphorylation through the activation of PI3K resulted in uptake of sugar into tissues. Silkworm have bombyxin, this peptide not only demonstrate the similar structure to human insulin but also shown to increase phosphorylated Akt in silkworm (Nagata *et al*., 2008). The study done by Matsumoto et al., 2011 suggested that human insulin enhances the uptake of sugar into silkworm fat body by the phosphorylated of Akt via the activation of PI3K as in insulin-stimulated mammalian adipose tissue and the activation of AMPK signaling pathway by given AICAR has shown to decrease sugar level in silkworm hemolymph similar to mammals. Thus, the candidate substances that have been used to screen for the hypoglycemic effect that activate the insulin signaling pathway and/or the AMPK signaling pathway can be evaluated using silkworm hyperglycemic model. This study on the hypoglycemic effect of human insulin in the hyperglycemic Thai silkworm induced by a high glucose or sucrose diet has shown the consistent results compared to the previous study done by Matsumoto et al., 2011. Moreover, we have discovered that glibenclamide and acarbose also exhibited the same effect in hyperglycemic Thai

silkworm too. We suggested that since the hypoglycemic effect of glibenclamide in mammals via the inhibiting ATP-sensitive potassium channels in pancreatic beta cell resulted in depolarization of the cell membrane, which causes to open voltage-dependent calcium channels then to increase intracellular calcium in the beta cell, which stimulate the secretes of insulin from β-cell of pancreas and then also insulin bind to the insulin receptor and activation via increased Akt phosphorylation through the activation of PI3K and finally to decrease the blood sugar levels, glibenclamide, therefore, may stimulate the secretion of bombyxin and leading to activation through insulin signaling pathway resulted in the hypoglycemic effect in Thai silkworm hemolymph. An alpha-glucosidase enzyme in silkworm mid-gut have been isolated and purified (Toshihiro *et al*., 2002). This enzyme actions by breaking disaccharide or polysaccharide to monosaccharide before absorbing to the bloodstream. The reduction of blood sugar level by acarbose is via inhibition the activities of this enzyme. The results from this study have shown that acarbose can decrease blood sugar level in Thai silkworm hemolymph. This outcome is directly to the mechanism of action of this agent. When ECa 233 was used for testing the hypoglycemic effect in hyperglycemic Thai silkworm model, the result showed that ECa 233 also can decrease blood sugar levels with statistical significance and in a concentration dependent manner. However, mechanism of action of ECa 233 for hypoglycemic effect is unknown. In order to explain this mechanism of action, more research topics are required to elucidate it clearly.

As known for many years, an invertebrate model especially silkworm model has been established as an animal models for the life sciences research in several topics such as infection model for screening of the antibiotic candidate from the natural substances, for evaluating the resistance and the efficacy of the recent antibiotics used in clinical, for evaluating the drug toxicity and metabolism, including the hyperglycaemic model for anti-diabetic screening from the natural product. The study done by Matsumoto *et al*., 2011 suggested that this hyperglycemic silkworm model can be used for the screening of candidate substances that exert their hypoglycemic effect via the activation of insulin signaling pathway and/or the AMPK signaling pathway. Furthermore, results from this study have shown that both glibenclamide and acarbose also decreased the sugar levels in Thai silkworm hemolymph. Thus, it is possible to put more suggestion that in the use of hyperglycemic silkworm as a model for the anti-diabetic screening, it is possible that the

candidate substances will exert their activites via the stimulation of the insulin secretion from β-cell of pancreas in mammals as well as it can stimulate the release of bombyxin in silkworm and then also insulin/bombyxin activation via increased Akt phosphorylation through the activation of PI3K resulted in the decrease of blood sugar level. In addition, it is also possible that the candidate substances will exert their activities via the inhibition of the activity of alpha-glucosidase enzyme causing disaccharide or polysaccharide not breaks down to monosaccharide and delay the absorption of glucose to the bloodstream.

The hypoglymemic Thai silkworm model in this study can facilitate to screen the potential candidates with hypoglycemic effect before continuing the study in vertebrate model or the clinical study. Thai silkworm model is of many advantages to use as a laboratory animal model because silkworm is less of ethical issues, less of a cost than vertebrate or mammals, uncomplicated to cultivation, ease to maintained a large number of larvae including can be able to use a large of sample size to make a trustworthy of the statistical significant in data analysis. In addition, Thailand has plenty of the herbals which have been used in the traditional medicine for anti-diabetic. Thus, this model not only can save the time but also shorten the research process to clarify and screen several herbals to give the end point and develop the valuable of this herbal in order to make the profit for the future.

However, Thai silkworm model for the hypoglycemic effect screening have shown some limitations such as the physical appearance of Thai silkworm. A small size and restricted of body volume result in the limitation of the volume of the test solutions not more than 100 μl, the solubility of the test substances, if the compound is less soluble, some solubilising agents are necessary. Sometimes these agents can be toxic to silkworm, the suitable age of Thai silkworm to use for the laboratory animal is limit to the first day of the 5^{th} instar larva because this stage are a proper size and ease to handle, to inject the test solutions and to collect the sample and in this stage silkworm have a first day past from the starvation. Thus, the sugar levels detected are from the high glucose diet alone. Moreover, the anti-diabetics that use for the positive control or standard agents have to exert their activities within the life cycle of Thai silkworm. The favorable time is 5-7 days of the 5^{th} instar larva stage which is the short time. The biological effect with the delayed onset of action such as Thiazolidinedione group (glitazone), this agent appear to activate peroxisome proliferators-activated receptor (PPARγ), which is

involved of lipids metabolism and the differentiation of adipocytes. This agents have a very slow onset of action the effects start within 2 weeks and the apparently benefit of treatment have show more about 3 months (Maclsaac *et al*., 2004). Metformin is one of hypoglycemic agent of the biguanide group, this agent action by reduce gluconeogenesis from the liver of mammals leading to the lower glucose levels in bloodstream. Concerning this mechanism, mammals and silkworm have differences of anatomical and physiological, thus, hypoglycemic effect of this agent could not be observed in this model. Therefore, this study suggested that the suitable positive control for anti-diabetic drugs screening using Thai silkworm model are insulin, AICAR (Matsumoto *et al*.,2011), glibenclamide and acarbose whereas glitazone (Thiazolidinedione group) or metformin (Biguanide group) have some limitations.

Finally, Thai silkworm model can be proposed as an alternative for screening of anti-diabetic drugs in some extents. However, the importance of further study in the conventional model such as mouse model cannot be excluded.

REFFERENCES

Thai

เอกรินทร์ สายฟ้า, สุวรรณา เหลืองชลธาร, ชำนาญ ภัตรพานิช และ รุทธ สุทธิศรี. การวิจัยและพัฒนาผลิตภัณฑ์ยาและเครื่องสำอางที่มีมาตรฐานจากสมุนไพรบัวบกสู่การผลิตระดับอุตสาหกรรม. รายงานโครงการเรื่องการวิจัยและพัฒนาผลิตภัณฑ์ยาและเครื่องสำอางที่มีมาตรฐานจากสมุนไพรบัวบกสู่การผลิตระดับอุตสาหกรรม. คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย ; 2549.

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APPENDICES

APPENDIX A

This thesis work was presented as a poster presentation, entitled HYPOGLYCEMIC EFFECT OF ECa 233 IN A SILKWORM MODEL, NRCT-JSPS Core University Program on Natural Medicine in Pharmaceutical Sciences The 9th Joint Seminar Natural Medicine Research for the Next Decade: New Challenges and Future Collaboration, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand on 8-9 December 2010. The poster number of participant was 93.


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Jointly organized by
Faculty of Pharmaceutical Sciences, Chulalongkorn University
Chulabhorn Research Institute
National Research Council of Thailand (NRCT)
Institute of Natural Medicine, University of Toyama
Japan Society for the Promotion of Science (JSPS)
PROCEEDINGS

HYPOGLYCEMIC EFFECT OF ECa 233 IN A SILKWORM MODEL

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Introduction

Diabetes mellitus is a group of chronic metabolic diseases in which a person has high blood sugar level, either because the conditions in which the pancrease does not produce enough insulin, or because tissues do not respond and/or fail to use insulin that is produced properly(1). Chronic hyperglycemia that may causes to damage microvascular and produces many complications to major organs(2). To maintain blood sugar level several of anti-diabetic drugs have been used, however it was shown some adverse effects(3). *Centella asiatica* has been studied for many diseases in animal model including hypoglycemic effect(4-9). Its LD_{50} as the extract was > 8 g/kg in rat model(4). ECa 233 is a standardized extract of *Centella asiatica* characterized with well-defined ratio of the active ingradient(4). In this study the effects of ECa 233 on glucose levels in silkworm haemolymph were evaluated in hyperglycemic silkworm larvae comparing with human insulin.

Objectives

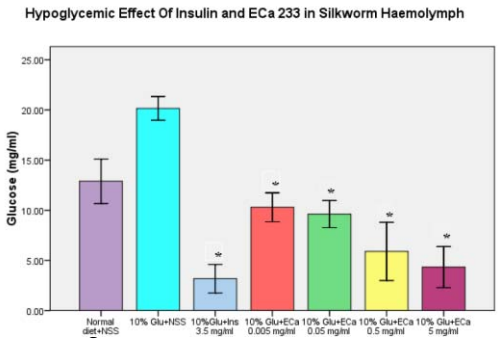
To evaluated the hypoglycemic effect of ECa 233 in silkworms. Furthermore, to evaluated toxicity profiles of ECa 233 in silkworms

Materials and Methods

Silkworm larvae were supplied from The Queen Sirikit Department of Sericulture, Ministry of Agriculture and Cooperatives. ECa 233 were obtained from Assist. Prof. Dr. Chamnan Patarapanich. For acute toxicity study, silkworm larvae were injected with ECa 233 in various concentrations. The mortality rate of silkworm larvae was observed for 48 hours and the LD_{50} was then calculated. The blood glucose-lowering effect of ECa 233 were studied in hyperglycemic silkworm larvae induced by feeding 10% glucose diet 1 hour before injection. Then each group of silkworm larvae was injected with ECa 233 in various concentrations (0.005-5 mg/ml), insulin 3.5 mg/ml and 0.9% NaCl as placebo respectively and kept for another 5 hours. Silkworm haemolymph was taken. The phenol-sulfuric acid reaction was performed and the color intensity was measured by microplate reader model Perkin Elmer at wavelength 490 nm.

Results

From this study, LD_{50} of Eca 233 in silkworm was 29.29 mg/ml or 1.46 mg/g. larva and the hypoglycemic effects of Eca 233 have shown in the graph



* P-*value* <0.05

Discussion

In toxicity study, the LD_{50} was 29.92 mg/ml or 1.46 mg/g. larva. It can be concluded that ECa 233 is rather safe for silkworm larva as it is quite safe in rat model. Various concentrations of ECa 233 were chosen for hypoglycemic effect study regarding the LD_{50} value (0.005, 0.05, 0.5 and 5 mg/ml). The results have shown that ECa 233 in all concentrations rendered glucose-lowering effects in a dose-dependent manner and it was statistically significant comparing with 0.9% NaCl group (p-value < 0.05). Furthermore, insulin, a positive control, also showed the hypoglycemic effect as expected in this model.

Conclusions

From the study, ECa 233 has proven safe and efficacious in lowering the glucose level in silkworm model. However, the importance of further study in the conventional model such as mouse model can not be excluded. In the meantime silkworm model can be proposed as an alternative for screening of antidiabetic drugs in some extents.

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APPENDIX B

1. The preparation of 10% glucose diet and 10% sucrose diet

10% glucose or 10% sucrose diet 5 grams was prepared by mixing of 4.5 grams Silk Mate with 0.5 gram of glucose or sucrose.

2. The preparation of 0.6 M perchloric acid ($HClO_4$) stock solution

0.6 M Perchloric acid ($HClO_4$) stock solution was prepared by weighing 70% perchloric acid solution 8.6 grams and diluting the solution to 100 ml with purified water.

3. The preparation of 5% phenol stock solution

5% Phenol stock solution was prepared by mixing 98.5% phenol solution 5.07 ml and diluting the solution to 100 ml with purified water.

4. The preparation of 10 mg/ml human insulin solution

10 mg/ml Human insulin solution was prepared by dissolving 10 mg human insulin with 0.9 % NaCl and diluting the solution to 1 ml. Human insulin stock solution was then diluted to 1.75, 3.5 and 7 mg/ml respectively for insulin profile and hypoglycemic effect study in Thai silkworm model.

5. The preparation of 5 mg/ml glibenclamide stock solution

5 mg/ml Glibenclamide stock solution was prepared by dissolving10 mg of glibenclamide with 10% v/v DMSO and diluting the solution to 2 ml with 0.9% NaCl. Glibenclamide stock solution was then diluted to several concentrations such as 0.03, 0.06, 0.125, 0.25, 0.5, 1, 2 and 3 mg/ml respectively for toxicity profile and hypoglycemic effect study in Thai silkworm model.

6. The preparation of 5 mg/ml metformin stock solution

5 mg/ml Metformin stock solution was prepared by dissolving 10 mg of metformin with 0.9% NaCl and diluting the solution to 2 ml. Metformin stock solution was then diluted to several concentrations such as 0.03, 0.06, 0.125, 0.25, 0.5, 1 and 1.5

mg/ml respectively for toxicity profile and hypoglycemic effect study in Thai silkworm model.

7. The preparation of 500 mg/ml acarbose stock solution

500 mg/ml Acarbose stock solution was prepared by dissolving 1000 mg of acarbose with 0.9% NaCl and diluting the solution to 2 ml. Acarbose stock solution was then diluted to several concentrations such as 200, 100, 50, 25, 12.5 and 6.25 mg/ml respectively for toxicity profile study and 20, 10, 5 and 2.5 mg/ml for hypoglycemic effect study in Thai silkworm model.

8. The preparation of 500 mg/ml ECa 233 stock solution

500 mg/ml ECa 233 stock solution was prepared by dissolving of 500 mg ECa 233 with 0.9% NaCl and diluting the solution to 1 ml. ECa 233 stock solution was then diluted to several concentrations such as 0.005, 0.05, 0.5 and 5 mg/ml respectively for hypoglycemic effect study in a silkworm model and 2.5, 5, 10, 20, 40, 60, 80, 100 and 200 mg/ml respectively for toxicity study in Thai silkworm.

9. Samples preparation

Thai silkworm hemolymph were collected as a sample by taken 5 µl through a cut on the first pro-leg of Thai silkworm, transferred samples to the microcentrifuge tube and mixed with 45 µl of 0.6 M $HClO_4$ then vortex by vortex mixtures for 5 second. Precipitated protein were removed by centrifugation at 3000 rpm for 10 min. 10 µl of supernatant were diluted with the appropriate volume of distilled water 90 µl. For sugar quantification phenol-sulfuric acid method was performed by mixing 100 µl of hemolymph extract with 100 µl of 5% phenol followed by 500 µl of sulfuric acid, samples were mixed by vortexing for 5 sec. and incubated at 25±2 °C for 20 min. 200 µl of samples transferred into 96 well microtiter plate and sugar levels were measured by used microplate reader model Perkin Elmer photometry at the wavelength 490 nm. Glucose concentrations were calculated from standard curve of the serially diluted glucose solution (1.25, 2.5, 5, 10 and 20 mg/ml respectively).

Glucose concentration (µg/100 µl)	OD_{490}
1.25	0.095
2.5	0.182
5	0.227
10	0.413
20	0.844

Table 2 Glucose serial dilution for standard curve and absorbance

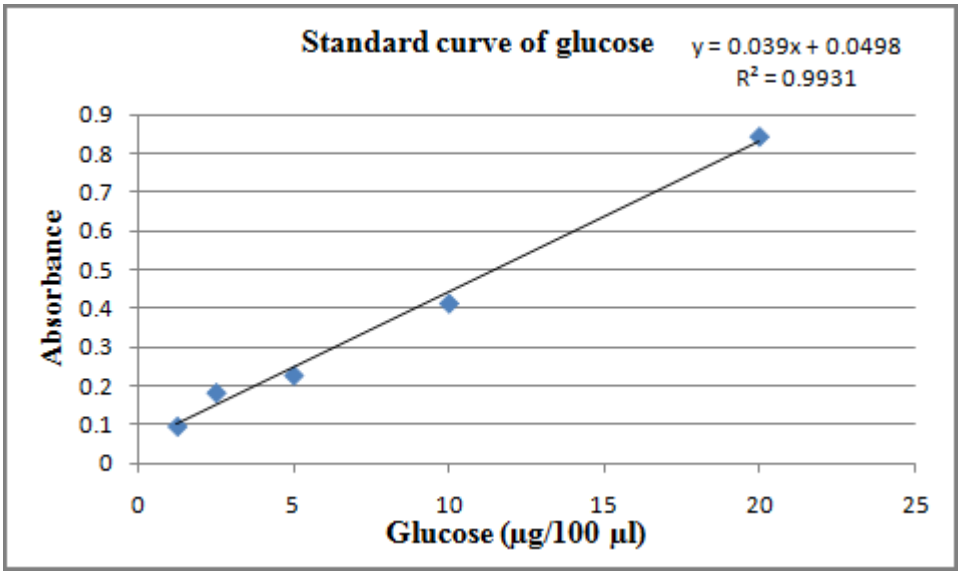

Figure 27 Standard curve of glucose

VITAE

Mr. Abhiruj Chiangsom was born on March 27, 1979 in Mahasarakham, Thailand. He graduated with Bachelor Degree of Pharmacy from Faculty of Pharmaceutical Sciences, Ubon Ratchathani University, Ubon Ratchathani, Thailand in 2005. He had started his work at Ladprao General Hospital, Bangkok from 2005 to 2009. In 2009, he enrolled to Pharmacology Master Degree Program in Chulalongkorn University.