การคัดกรองสารออกฤทธิ์ชีวภาพที่ผลิตจาก *Streptomyces* spp. ที่แยกได้จากผึ้งโพรง (*Apis cerana*) และชันโรง (*Tetragonula laeviceps*)

นางสาวอรพรรณ ตั้งวิชัย

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SCREENING OF BIOACTIVE COMPOUNDS PRODUCED BY *Streptomyces* spp. ISOLATED

FROM EASTERN HONEY BEE (*Apis cerana*) AND STINGLESS BEE (*Tetragonula laeviceps*)

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Thesis Title	SCREENING OF BIOACTIVE COMPOUNDS PRODUCED BY *Streptomyces* spp. ISOLATED FROM EASTERN HONEY BEE (*Apis cerana*) AND STINGLESS BEE (*Tetragonula laeviceps*)
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อรพรรณ ตั้งวิชัย : การคัดกรองสารออกฤทธิ์ชีวภาพที่ผลิตจาก *Streptomyces* spp. ที่แยกได้จากผึ้ง โพรง (*Apis cerana*) และชันโรง (*Tetragonula laeviceps*) (SCREENING OF BIOACTIVE COMPOUNDS PRODUCED BY *Streptomyces* spp. ISOLATED FROM EASTERN HONEY BEE (*Apis cerana*) AND STINGLESS BEE (*Tetragonula laeviceps*)) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. ธนาภัทร ปาลกะ, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: ดร. ชมพูนิกข์ กาญจนพังคะ, 85 หน้า.

ในปัจจุบันการเพิ่มขึ้นของเชื้อก่อโรคสายพันธุ์ต้านยาปฏิชีวนะนั้น เกิดขึ้นอย่างรวดเร็วกว่าการที่มียาชนิด ใหม่ที่มีการนำมาใช้ในการรักษา ดังนั้น จึงจำเป็นต้องมีการค้นหาสารปฏิชีวนะชนิดใหม่ ซึ่งนอกเหนือจากโรคติดเชื้อ แล้ว โรคอ้วนและภาวะน้ำหนักเกินยังเป็นอีกสาเหตุหนึ่งของการเสียชีวิตทั่วโลก การหาสารที่มีฤทธิ์ในการลดการ สะสมไขมันจึงมีความสำคัญทางสาธารณสุข โดยแหล่งของสารปฏิชีวนะที่เป็นที่นิยม ได้แก่ แบคทีเรียในกลุ่ม Actinomycetes โดยเฉพาะจีนัส *Streptomyces* ถึงแม้จะมีการแยกเชื้อ *Streptomyces* จากดินตามธรรมชาติ กันอย่างแพร่หลาย แต่การศึกษาที่ผ่านมากลับเป็นการพบสารชนิดเดิมที่มีการรายงานไว้แล้ว ดังนั้น การแยก *Streptomyces* จากแหล่งที่ยังไม่มีการค้นหา เช่น แมลง ซึ่งมีการอาศัยอยู่ร่วมกับจุลินทรีย์ในลักษณะพึ่งพาอาศัย กัน (symbionts) อาจนำไปสู่การค้นพบสารออกฤทธิ์ชีวภาพชนิดใหม่ได้ ในงานวิจัยนี้ได้ทำการแยก *Streptomyces* จากผึ้งโพรง (Eastern honey bee: *Apis cerana*) และชันโรง (stingless bee: *Tetragonula laeviceps*) โดย สามารถแยกเชื้อจากภายในและภายนอกลำตัวผึ้งได้จำนวนทั้งสิ้น 125 ไอโซเลต โดยพบว่ามี 5 ไอโซเลตที่มี ประสิทธิภาพในการผลิตสารที่ออกฤทธิ์ต้านจุลินทรีย์ทดสอบได้กว้าง ต่อแบคทีเรียแกรมบวก แบคทีเรียแกรมลบ และยีสต์ที่ใช้ทดสอบ จากแผนภูมิต้นไม้ (phylogenetic tree) ที่ได้จากการวิเคราะห์ลำดับ 16S rRNA ของเชื้อทั้ง 5 ไอโซเลตนี้ พบว่ามีความใกล้เคียงกับ *Streptomyces* spp. ในฐานข้อมูลที่มีการรายงานก่อนนี้ และ ยังพบว่า *Streptomyces* ที่แยกได้จากแมลงชนิดเดียวกัน ไม่ได้มีสายวิวัฒนาการที่ใกล้เคียงกันทั้งหมด เมื่อนำสารสกัดที่ได้ จากเชื้อเหล่านี้ไปทดสอบฤทธิ์ในการยับยั้งกระบวนการเปลี่ยนสภาพของเซลล์ไขมัน (adipocyte differentiation) พบว่า สารสกัดจากไอโซเลต I-EHB-18 ซึ่งมีความใกล้เคียงกับ *Streptomyces andamanensis* sp. Nov มีฤทธิ์ใน การยับยั้งระยะเริ่มต้นของกระบวนการเปลี่ยนสภาพของเซลล์ไขมันในเซลล์ 3T3-L1 โดยที่ไม่มีพิษอย่างเฉียบพลัน ต่อเซลล์ และจากการศึกษาอย่างละเอียดพบว่าสารสกัดอย่างหยาบนี้สามารถยับยั้งการแสดงออกของ Peroxisome proliferator-activated receptor gamma (PPARγ) ซึ่งเป็นโปรตีนควบคุมสำคัญของกระบวนการเปลี่ยนสภาพ ของเซลล์ไขมัน ดังนั้น งานวิจัยครั้งนี้จึงเป็นการรายงานสารสกัดอย่างหยาบที่มีฤทธิ์ในการยับยั้งกระบวนการเปลี่ยน สภาพของเซลล์ไขมัน ที่ผลิตโดย *Streptomyces* ที่แยกได้จากผึ้งโพรงเป็นครั้งแรก ซึ่งจากผลการวิจัยครั้งนี้อาจ นำไปสู่การพัฒนายาชนิดใหม่เพื่อใช้ในไปใช้ในอนาคตต่อไปได้

ภาควิชา	จุลชีววิทยา	ลายมือชื่อนิสิต ....
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ORAPUN TANGWICHAI: SCREENING OF BIOACTIVE COMPOUNDS PRODUCED BY *Streptomyces* spp. ISOLATED FROM EASTERN HONEY BEE (*Apis cerana*) AND STINGLESS BEE (*Tetragonula laeviceps*). ADVISOR: ASSOC. PROF. TANAPAT PALAGA, Ph.D., CO-ADVISOR: CHOMPOONIK KANCHANABANCA, Ph.D., 85 pp.

In recent years, antibiotic-resistant pathogens have been increasing faster than the numbers of new therapeutic compounds entering clinical use, thus the search for new antibiotics is in need. Apart from diseases caused by pathogenic microorganisms, obesity and overweight are linked to deaths worldwide. The search for drugs that can reduce the number of fat cell is also necessary in public health. One of the widespread sources of antibiotics is Actinomycetes, especially *Streptomyces* genus. *Streptomyces* spp. have been extensively isolated from soil but the discovery of novel compound is rare. Therefore, *Streptomyces* isolation from rare niche, i.e. symbionts of insects, could potentially lead to the discovery of novel bioactive compounds. In this study, Eastern honey bees (*Apis cerana*) and stingless bees (*Tetragonula laeviceps*) were used as sources to isolate *Streptomyces* and total of 125 isolates were obtained from internal and external. We found that five isolates produced the metabolites with broad antimicrobial activity against tested species of Gram positive bacteria, Gram negative bacteria and yeast. The phylogenetic tree based on 16s rRNA gene sequences showed high similarity with previously discovered microorganisms. Moreover, the results implied that the evolution which *Streptomyces* spp. isolated from the same insect were not root together. We have investigated the effects of the crude metabolites on preadipocytes cell line 3T3-L1 differentiation and found that crude extracted from I-EHB-18, closely related to *Streptomyces andamanensis* sp. Nov, inhibited adipocyte differentiation of 3T3-L1 cells at the early stage of differentiation with no acute cytotoxicity. Detailed study revealed that this crude extract suppressed expression of Peroxisome proliferator-activated receptor gamma (PPARγ), a master regulator of adipocyte differentiation. This is the first report of bioactive compound possessing anti-adipocyte differentiation activity produced by *Streptomyces* isolated from Eastern honey bees. Hence, the result may lead to a new compound for developing therapeutic drugs in the future.

Department:	Microbiology	Student's Signature	
Field of Study:	Microbiology and Microbial Technology	Advisor's Signature	
		Co-Advisor's Signature	
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LIST OF ABBREVIATIONS

A	Ampere
A_{540}	Absorbance at 540 nm
Ab	Antibody
ANOVA	Analysis of variance
ATCC	American Type Culture Collection
BCA	Bicinchoninic Acid
BLAST	Basic Local Alignment Search Tool
CA	Crude aqueous extract
C/EBP	CCAAT- enhancer-binding proteins
CREB	AMP response element-binding protein
DEX	Dexamethasone
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
EHB	Eastern honey bee
EMBL-EBI	The European Bioinformatics Institute
FABP4	Fatty acid binding protein 4
FBS	Fetal bovine serum
Fwd	Forward
GATA	GATA binding protein
GLUT4	Glucose transporter 4
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSD11β1	11β-hydroxysteroid dehydrogenase type 1
HRP	Horse radish peroxidase
IBMX	3-isobutyl-1-methylxanthine
IC_{50}	The 50% inhibition concentration
KLF	Krüppel-like factor

LPL	Lipoprotein lipase
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NA	Nutrient broth
NB	Nutrient agar
NCBI	National Center for Biotechnology Information
ORO	Oil red O
PBS	Phosphate buffered saline
PBS-T	Phosphate buffered saline-Tween
PCR	Polymerase chain reaction
PDA	Potato dextrose agar
PPAR	Peroxisome proliferator-activated receptor
PVDF	Polyvinylidine fluoride
Rev	Reverse
RNA	Ribonucleic acid
rpm	Round per minute
SDS	Sodium dodecyl sulfate
rRNA	Ribosomal ribonucleic acid
SY	Starch-Yeast
SB	Stingless bee
SREBP1c	Sterol regulatory element binding transcription factor 1

CHAPTER I

INTRODUCTION

1.1 Background and rationale

Actinomycetes, especially genus *Streptomyces*, are among of the major bioactive compound producers responsible for producing more than 45% of known microbial metabolites. To date, 80% of the antibiotics such as streptomycin, lincomycin, tetracycline, kanamycin and chloramphenicol are produced from *Streptomyces*. Moreover, *Streptomyces* spp. have been the sources of medically important drugs such as antitumor, anti-hypertensive drugs, immunosuppressants and other therapeutic agents. Thus, *Streptomyces* isolated from unexplored environment could potentially leads to the discovery of new bioactive compounds.

Due to the continuing misuses of antibiotics, the number of antibiotic-resistant pathogenic bacteria have been increasing faster than the numbers of new therapeutic compounds entering clinical use in recent years. As the attempt to discover new drug candidates, *Streptomyces* have been intensively isolated and screened from soil as a source of bioactive compounds. However, it was often discovered the same species and the rate of discovering novel lead compounds is low. Thus, the search for new antibiotics is still needed. *Streptomyces* isolation from rare niche, i.e. symbionts of insects, could potentially lead to the discovery of novel bioactive compounds.

Apart from diseases caused by pathogenic microorganisms, obesity and overweight are linked to deaths worldwide. The development of mature fat cells from fibroblastic preadipocytes are usually involved in chronic disease such as obesity, type II diabetes, hypertension and cardiovascular diseases. Therefore, the bioactive compounds with the ability to inhibit adipocyte differentiation are in urgent need.

There are reports show symbiotic relationships between insects and the diverse group of microorganisms including *Streptomyces* species. The microorganisms are beneficial to their hosts by directly or indirectly providing nutrients, and protecting them from antagonists or natural enemies. Among symbiotic associations insect-*Streptomyces* symbioses that is interesting source for bioactive compounds discovery. For example, the

study of actinomycetes associated with two species of solitary mud dauber wasps, *Sceliphron caementarium* and *Chalybion californicum,* showed at least 15 *Streptomyces* spp. were isolated from the insects. Moreover, the chemical analysis indicates a novel antimicrobial compound, sceliphrolactam, from these *Streptomyces* spp. In fungus-growing pine beetle (*Dendroctonus frontalis*), the novel antifungal mycangimycin was isolated from *Streptomyces* sp. SPB74 associated with the insect. Moreover, the endophenazine compounds A–D produced by *Streptomyces* endosymbiont species taken from four different arthropods were discovered.

There are reports suggested that *Streptomyces* spp. were frequently found in pollen, provisions and alimentary canals of the bees and these bacteria are also considered as part of resident microflora of alfalfa leafcutter bees (*Megachile rotundata*). Bees are highly diverse social insects and considered to play a vital role in pollination of economic crops as well as indigenous plants in Thailand. There are reports show that most of the actinomycetes isolated from bee hives in Thailand belongs the genus *Streptomyces*. There also a report about novel *Streptomyces* species from the South-East Asian stingless bee (*Tetragonilla collina*), *Streptomyces chiangmaiensis* sp. nov. and *Streptomyces lannensis* sp. nov. Furthermore, microorganisms associated with stingless bee and honey bee have been isolated from other regions outside the northern Thailand. Hence, the bioactive compounds produced by these bee symbiont *Streptomyces* spp. remain largely unexplored.

In this study, Eastern honey bee (*Apis cerana*) and stingless bee (*Tetragonula laeviceps*) were used as sources for *Streptomyces* isolation. The antimicrobial cross-streak assay was used for primary screening and the phylogenetic tree of the selected *Streptomyces* spp. was generated based on 16S rRNA gene sequences. The crude compounds extracted from *Streptomyces* spp. were screened for cytotoxicity in preadipocytes cell lines, 3T3-L1, and anti-adipocyte differentiation assay was used for secondary screening.

1.2 Research objectives

1) To isolate and identify *Streptomyces* spp. with the ability to produce bioactive compounds from eastern honey bee (*Apis cerana*) and stingless bee (*Tetragonula laeviceps*)

2) To screen for metabolites with antimicrobial and anti-adipocyte differentiation activity produced from the isolated *Streptomyces* spp.

1.3 Hypothesis

Streptomyces spp. isolated from bees produce bioactive compounds with anti-microbial and/or anti-adipocyte differentiation activity.

1.4 Benefit

Metabolites produced by *Streptomyces* spp. from Eastern honey bee and stingless bee with antimicrobial and/or anti-adipocyte differentiation activity from the novel sources may lead to new antibiotics or lead compound for drugs to treat obesity in the near future.

CHAPTER II

LITERATURE REVIEWS

2.1 Bioactive from microbial metabolites

The bioactive compounds isolated from microorganisms can be derived from primary metabolism or secondary metabolism. The primary metabolites are common in all biological systems, directly involved in the normal growth, reproduction and development such as proteins, polysaccharides, nucleic acids and fatty acids. The secondary metabolites are often low molecular weight compounds with biological activities including antimicrobial, antitumor and antiviral activities (Berdy, 2005). Secondary metabolites differ from primary metabolites in four manners; (1) they are not essential for growth; (2) their production is dependent on growth conditions such as type of culture media; (3) they are often produced as groups of closely related molecules; and (4) it is often possible to overproduce these components (Drew, 1977). There are more than 20,000 bioactive compounds discovered from microorganisms. Actinomycetes, especially genus *Streptomyces*, are one of the major producers responsible for more than 45% of known bioactive microbial metabolites produced today (Berdy, 2005).

2.2 *Streptomyces*

2.2.1 Characteristics of *Streptomyces*

Streptomyces, the filamentous gram-positive bacteria is the largest genus of the Actinobacteria with over 500 known species (Ikeda *et al.*, 2003). Their genome contain high G-C content around 69-78% (Dehnad, 2010). *Streptomyces* species are nonmotile and catalase positive; besides they are able to reduce nitrates to nitrites, and degrade adenine, casein, gelatin, hypoxanthine, starch, and L-tyrosine. The species can produce an extensive branching substrate and aerial mycelium. The substrate hyphae are branched and very small approximately 0.5-1.0 μm in diameter. The colonies are slow-growing and relatively smooth surface but later they develop a weft of aerial mycelium that further differentiate to spores. This give a morphological distinct of *Streptomyces* as powdery and velvety colonies;

moreover, they produce a wide variety of pigments responsible for the color of the colonies (Flardh and Buttner, 2009).

Most *Streptomyces* spp. live as saprophytes, lives on decaying organic matter, in the soil, although *Streptomyces* spp. successfully inhabit in a wide range niches, both terrestrial and aquatic. *Streptomyces* produce secondary metabolite called geosmin (known as 'earth smell') which gives the soil its characteristic smell and provides the indication of how widespread these bacteria are in the soil (Jüttner and Watson, 2007).

2.2.2 Life cycle of *Streptomyces*

The *Streptomyces* life cycle (Figure 1) begins with spore germination under favorable conditions (Seipke *et al.*, 2012). Germination of a spore involves swelling, polarization of growth and emergence of a germ tube that further develops into a hypha. Hyphae grow by tip extension and branch into a vegetative mycelium that grows across and deep down into the solid medium. In response to nutritional and other stress signals, *Streptomyces* produce reproductive aerial hyphae. The secondary metabolites production is usually linked to this stage of differentiation. The aerial hyphae differentiate into a long chain of pre-spore compartments, which then develop thick spore walls, synthesize a grey polyketide spore pigment and acquire other characteristics of mature spores (Flardh and Buttner, 2009).
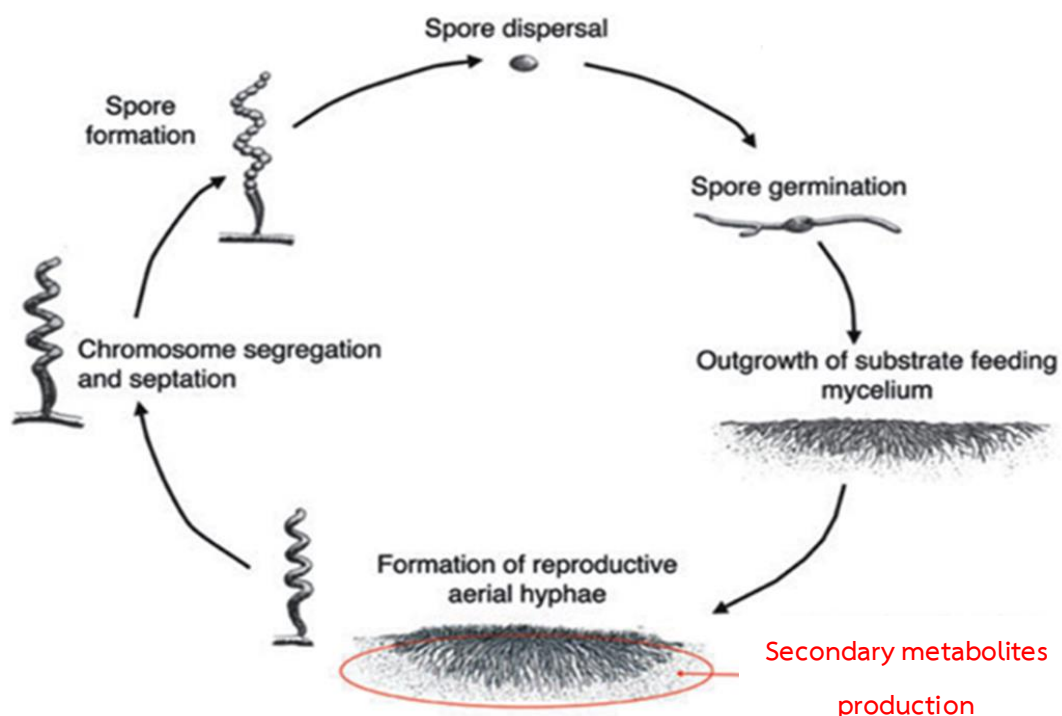
Figure 1 Life cycle of *Streptomyces*

(Modified from Seipke *et al.*, 2012)

2.2.3 *Streptomyces* metabolites

The most important characteristic of *Streptomyces* is the ability to produce secondary metabolites with antibacterial, antifungal, antiviral and other therapeutic properties. Since the discovery of streptomycin in 1943, intensive drug discovery, has been mainly on soil-derived Actinomycete. The discovery of chloramphenicol, tetracyclines and macrolides demonstrated that *Streptomyces* are a rich source of structurally unique bioactive substances. *Streptomyces griseus* is the first *Streptomyces* to be used for industrial production of streptomycin. To date, 80% of the antibiotics used in clinical such as streptomycin, chloramphenicol, kanamycin, lincomycin and tetracycline are produced these genus (Table 1) *(de Lima Procópio et al.*, 2012).

Table 1 Representative antibiotics produced from *Streptomyces* species (Adapted from de Lima Procópio *et al*., 2012)

Antibiotic	Strain	Years of discovery
Platensimycin	*Streptomyces platensis*	2006
Daptomycin	*Streptomyces roseosporus*	2003
Ribostamycin	*Streptomyces ribosidificus*	1970
Fosfomycin	*Streptomyces fradiae*	1969
Kanamycin	*Streptomyces kanamyceticus*	1957
Noviobiocin	*Streptomyces niveus*	1956
Vancomycin	*Streptomyces orientalis*	1956
Cycloserine	*Streptomyces garyphalus*	1955
Lincomycin	*Streptomyces lincolnensis*	1952
Nystatin	*Streptomyces noursei*	1950
Viomycin	*Streptomyces vinaceuse*	1951
	Streptomyces capreolus	
Tetracycline	*Streptomyces aureofaciens*	1950
Neomycin	*Streptomyces fradiae*	1949
Chloramphenicol	*Streptomyces venezuelae*	1949
Cephalosporins	*Streptomyces clavuligerus*	1945
Streptomycin	*Streptomyces griseus*	1944

2.3 Insect–*Streptomyces* mutualistic symbioses

Insects are the most abundant and diverse class of animals on earth and they are also associated with a variety of symbiotic microorganisms including *Streptomyces* species (Malke, 1967; May, 1988). Several studies indicate that *Streptomyces* mostly play a protective role of producing antibiotics used to defend the larvae of insect or food source against pathogens infections (Figure 2) (Kaltenpoth, 2009). In addition, mutualistic microorganisms

are probably considered as factor for the enormous success of insects in the adaptation to new environments and food sources (Cavalier-Smith, 1992).
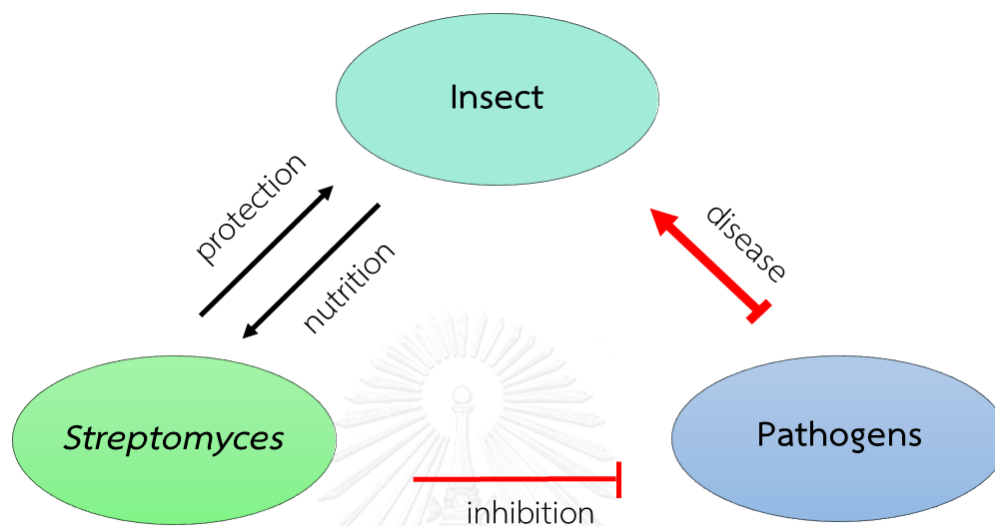
Figure 2 The symbiotic relationships between insects and *Streptomyces* species

Kaltenpoth *et al*. (2005) reported that female beewolves digger wasp (*Philanthus coronatus*) have evolved a unique mutualistic symbiosis with *Streptomyces* species, *Candidatus* Streptomyces philanthi grows inside the specialized antennal glands of the female wasps and apply them to the brood cell prior to oviposition (Kaltenpoth *et al*., 2005). The symbiotic *Streptomyces* bacteria are taken up by the larva and then locate to the walls of cocoon. Bioassays indicate that these *Streptomyces* protect the cocoon from fungal infestation and significantly enhance the survival probability of the larva by producing antibiotics. Subsequently, they also reported that these *Streptomyces* produce a cocktail of nine antibiotic substances. The complementary action of all symbiont-produced antibiotics confers a potent antimicrobial defense for the wasp larvae (Kroiss *et al*., 2010).

Poulsen *et al*. (2011) suggested that at least fifteen *Streptomyces* spp. associated with Philanthus digger wasps, the solitary mud dauber wasps *Sceliphron caementarium* (black and yellow mud daubers) and *Chalybion californicum* (blue mud daubers). The chemical analysis revealed a novel antifungal and antibacterial compound, sceliphrolactam, from these *Streptomyces* spp. (Figure 3A)(Poulsen *et al*., 2011).

Schoenian *et al.*, and Seipke *et al.* (2011) revealed that *Streptomyces* symbionts isolated from leaf-cutter ant (*Acromyrmex octospinosus* and *Acromyrmex volcanus*) produced multiple antifungal compounds, including candicidin and antimycins (Figure 3C and D). Moreover, the same authors also reported antibacterial valinomycin isolated from *Streptomyces* living on the surface of attine worker ants (Figure 3E)(Schoenian *et al.*, 2011; Seipke *et al.,* 2011).

Oh *et al.* (2009) reported the novel antifungal, mycangimycin isolated from *Streptomyces* sp. SPB74 associated within fungus-growing pine beetle (*Dendroctonus frontalis*) (Figure 3B) (Oh *et al.*, 2009).
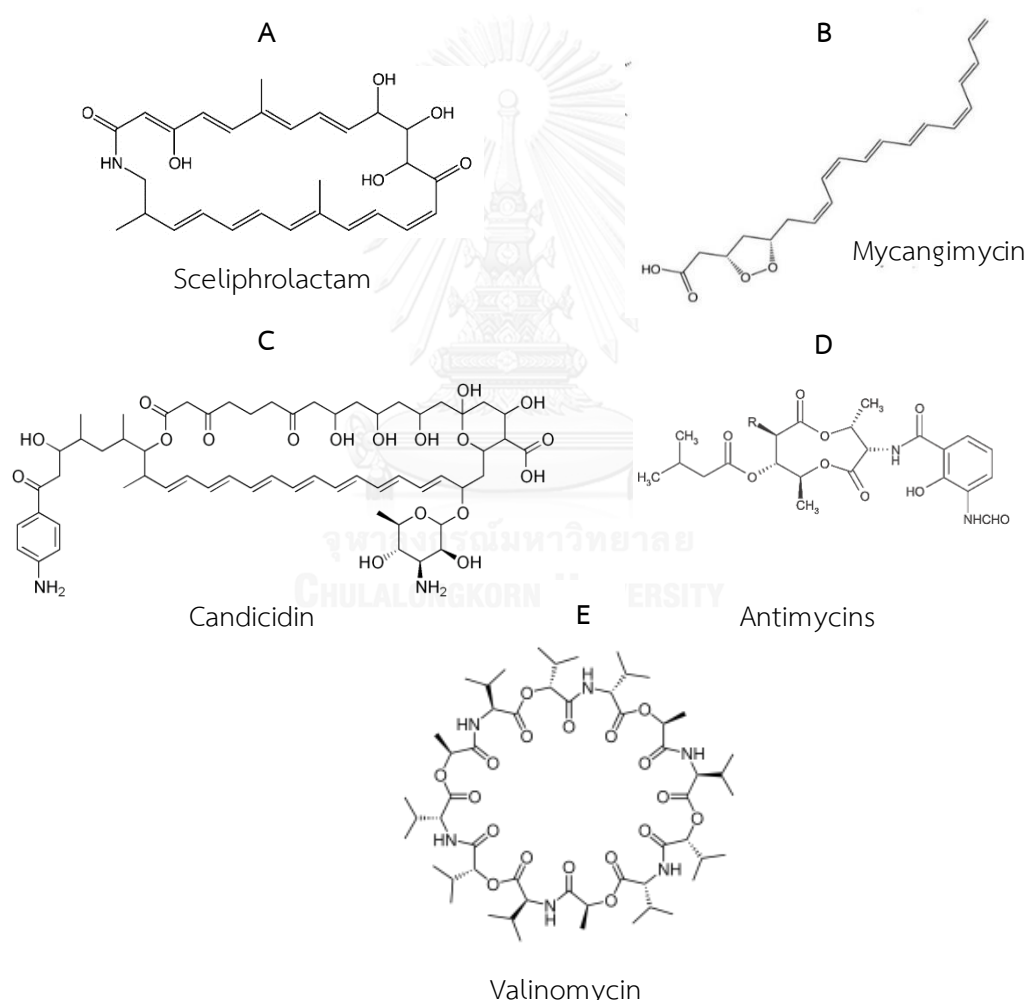
Figure 3 Structures of novel antibiotics isolated from *Streptomyces* spp. living in symbiosis with insects. A: from solitary mud dauber wasps (Poulsen *et al.*, 2011), B: from fungus-growing pine beetle (Oh *et al.*, 2009), C-D: from leaf-cutter ant and E: from attine worker ants (Schoenian *et al.*, 2011; Seipke *et al.,* 2011)

2.4 Bee-associated *Streptomyces*

Bees are highly diverse social insects and considered to play a vital role in pollination of economic crops as well as indigenous plants in Thailand. The microflora associated with bees are numerous, and vary from molds, yeasts, to bacteria. There are reports suggested that *Streptomyces* spp. were frequently found in pollen, provisions and alimentary canals of the bees. These bacteria are also considered as part of resident microflora of alfalfa leafcutter bees (*Megachile rotundata*) (Inglis *et al.*, 1993).

Promnuan *et al.* (2009) suggested that most of actinomycetes isolates from bee hives in Thailand belonged to the genus *Streptomyces*. There is also a report about a novel *Streptomyces* species from South-East Asian stingless bee (*Tetragonilla collina*), *Streptomyces chiangmaiensis* sp. nov. and *Streptomyces lannensis* sp. nov. (Promnuan *et al.*, 2009; Promnuan *et al.*, 2013).

Furthermore, microorganisms associated with stingless bee and honey bee have been isolated from other regions outside the northern Thailand. However, the bioactive compounds produced by these bee symbiont *Streptomyces* spp. remain largely unexplored.

2.5 Obesity and adipocyte differentiation

The majority health problems, such as diabetes type 2, cardiovascular diseases, obstructive sleep apnea, certain types of cancer and osteoarthritis are related to obesity which is caused by an overabundance of fat cells accumulating in the body. Moreover, obesity is a causal factor of various diseases (Haslam and James, 2005). Expanded adipose tissue shows an increased number and size of adipocytes due to the enhancement of the adipocytes differentiation (Spalding *et al.*, 2008). However, *Streptomyces toxytricini* produce lipstatins, compounds with a potent lipase inhibitory activity and has been proven useful for used in diabetes and obesity treatment and are available on the market as a component of a drug termed orlistat (Solecka *et al.*, 2012).

Adipocytes, also known as fat cells, are the main cellular components of adipose tissue. Their primary function is to control energy balance by storing triacylglycerol in periods of energy excess and mobilizing it during energy deprivation (Ali *et al.*, 2013). Therefore, they also secrete numerous protein and lipid factor that control the metabolism of other tissue such as glucose metabolism, immunological responses, inflammatory responses and blood pressure regulation (Lefterova and Lazar, 2009). Adipocyte tissue expands when the number

and size of adipocyte increases. The mature adipocyte, is characterized by a large internal fat droplet, which distends the cell so that the cytoplasm is compacted to a thin layer surrounding the lipid droplet while the nucleus is displaced to the outer edge of the cell.

3T3-L1 cells, derived from disaggregated 17-19-day-old Swiss 3T3 mouse embryos, are commonly used as preadipocytes in the study of adipocyte differentiation (Green and Kehinde, 1976). The distinct stages of differentiation consist of early, intermediate and late stages. Confluent 3T3-L1 preadipocytes can differentiate to adipocyte upon the addition of early inducing cocktail for 48 hours with a combination of insulin, dexamethasone (DEX), and 3-isobutyl-1-methylxanthine (IBMX), in the presence of fetal bovine serum (Figure 4).
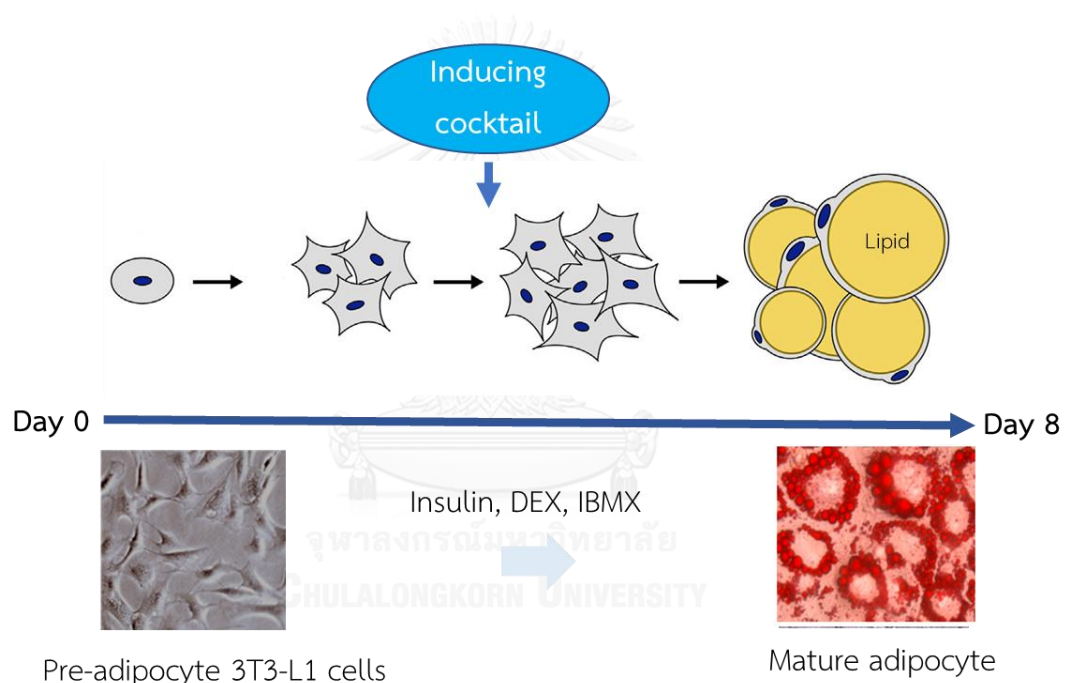
Figure 4 Procedures of adipocyte differentiation in *vitro*

Insulin acts through the insulin-like growth factor 1 (IGF-1) receptor. DEX, a synthetic glucocorticoid agonist, is traditionally used to stimulate the glucocorticoid receptor pathway. IBMX, a cAMP-phosphodiesterase inhibitor, is traditionally used to stimulate the cAMP-dependent protein kinase pathway. After 48 hours of induction, insulin alone is required to continue the differentiation process. Although, DXM is a powerful inductor at early stages of differentiation, but displays anti-adipocyte differentiation effects when added at later stages

of adipose maturation, indicating that the effects of hormones are strictly time dependent (Figure 5) (Caprio *et al.*, 2007).
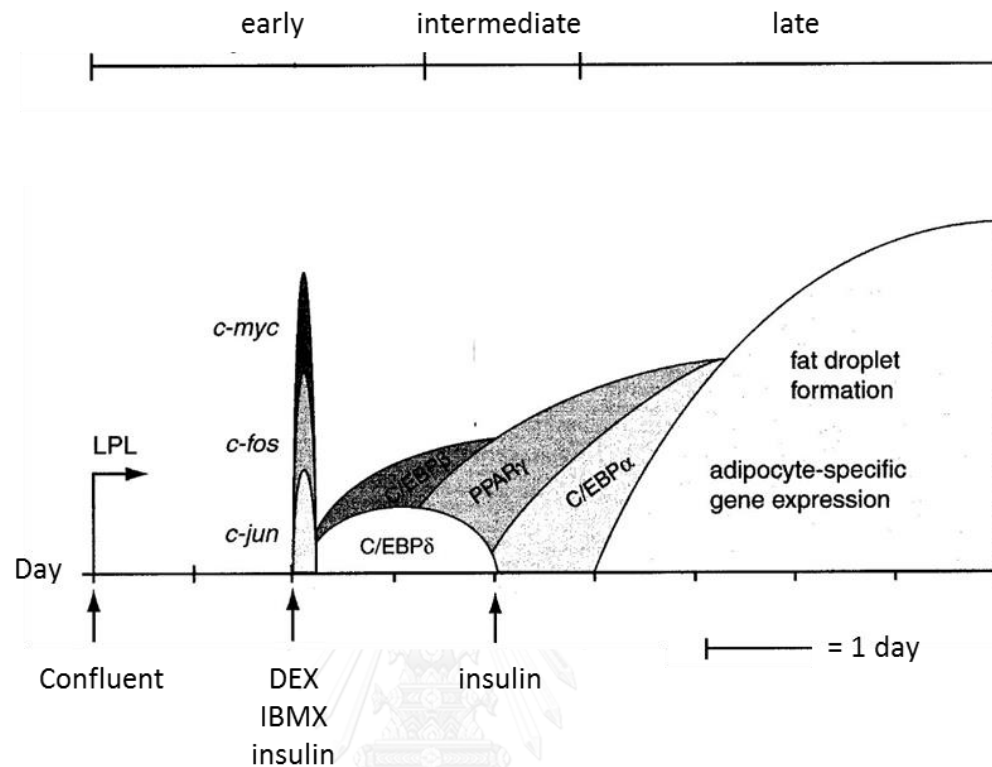
Figure 5 Stages and regulators of 3T3-L1 preadipocyte differentiation

(Modified from Ntambi and Young-Cheul, 2000)

2.6 Transcriptional regulation of adipocyte differentiation

The current model for adipocyte differentiation is shown in Figure 6. Based on this model, it is speculated that during the entire differentiation process there are several essential molecular interactions that occur among members of the CCAAT- enhancer-binding proteins (C/EBPs) and the peroxisome proliferator-activated receptor (PPAR) families. In the early stage, C/EBPβ and δ are the first transcription factors induced within 24 hours after induction. C/EBPβ is responsive primarily to IBMX, whereas C/EBPδ is responsive primarily to DEX. After removal of IBMX and DEX from the culture medium, the expression of C/EBPβ and δ declines within 48 hours. The upstream of C/EBPβ and C/EBPδ directly induce expression of PPARγ and C/EBPα, which is maximum on day 3-4 (Ntambi and Young-Cheul, 2000). PPARγ and C/EBPα are necessary for adipocyte differentiation and positively cross-regulate each other to maintain their gene expression despite a reduction in the expression

of C/EBPβ and δ. PPARγ and C/EBPα alone or in cooperation with each other induce the transcription of many adipocyte specific genes, such as lipoprotein lipase (LPL), fatty acid binding protein 4 (FABP4), glucose transporter 4 (GLUT4), and 11β-hydroxysteroid dehydro-genase type 1 (HSD11β1), and enzymes involved in creating and maintaining the adipocyte phenotype. LPL hydrolyzes triglyceride to free fatty acids and monoacylglycerol, and is present mostly in adipocytes. FABP4, as adipocyte protein 2, is expressed in differentiated adipocytes, and regulates insulin sensitivity, fat metabolism, and sugar metabolism. GLUT4, which is stimulated by insulin, translocate to the plasma membrane, and facilitates uptake of glucose into cells. HSD11β1 is highly expressed in adipocytes (Seckl and Walker, 2001).

Furthermore, all the studies suggested that PPARγ is both necessary and sufficient for adipocyte differentiation (Farmer, 2006). The PPARγ expression can induce differentiation in C/EBPα deficient mouse embryonic fibroblasts, but C/EBPα cannot rescue when PPARγ is not expressed, showing that PPARγ is a key regulator of adipocyte differentiation (Rosen *et al.*, 2002).
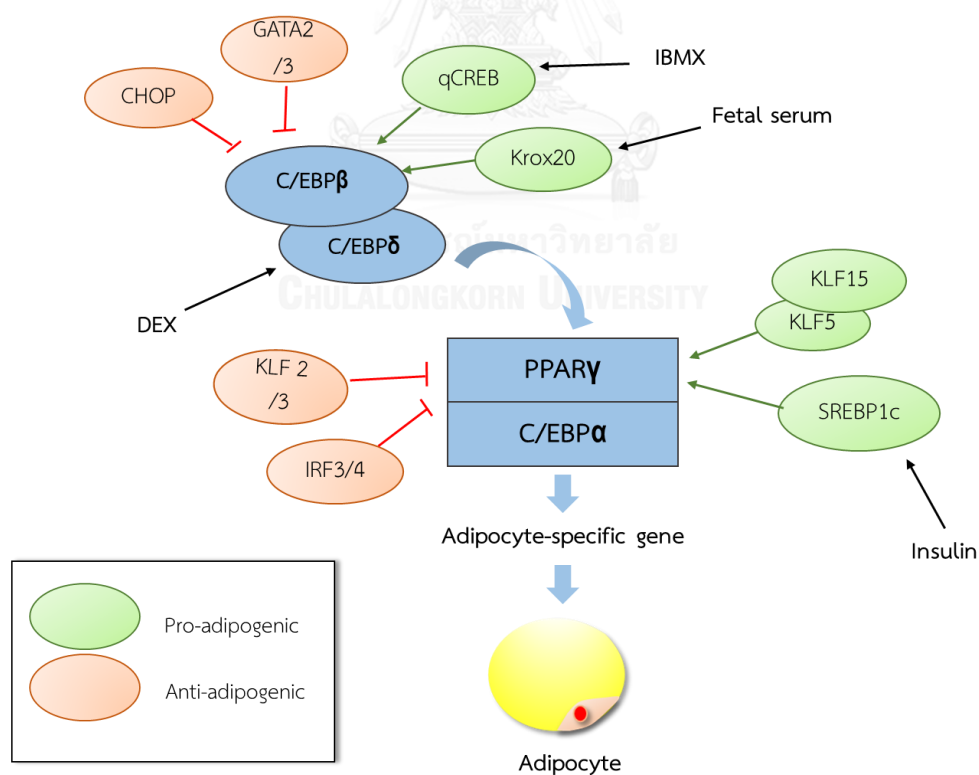
Figure 6 Adipocyte differentiation transcriptional cascade

(Modified from Ali *et al.*, 2013)

Some regulators associated with the early stages of adipocyte differentiation have been identified, including members of Krüppel-like factor (KLF) family, Sterol regulatory element binding transcription factor 1 (SREBP1c), Cyclic-AMP response element-binding protein (CREB), GATA binding protein (GATA) and forkhead families.

Members of the KLFs are characteristic zinc-finger proteins that regulate apoptosis, proliferation and differentiation (Brey *et al.*, 2009). Among these family members, KLF2, 3, 4, 5, 6, 7, and 15 have been reported to play roles in adipocyte differentiation. KLF2, 3 and 7 act as anti-adipogenic regulatory factors of differentiation because over-expression of these regulators inhibit differentiation in 3T3-L1 cells. In contrast, KLF5, 6, and 15 promotes adipocyte differentiation, and induced early during adipocyte differentiation, these regulators act as pro-adipogenic factors of differentiation (Matsuo *et al.*, 2015a).

2.7 Metabolites from *Streptomyces* with anti-adipocyte differentiation activity

Matsuo *et al.* (2015) reported that berrelidin isolated from *Streptomyces* sp. TK08330 inhibit adipocyte differentiation in 3T3-L1 cells by upregulation of GATA 2 and GATA-3 and cineromycin B isolated from *Streptomyces cinerochromogenes* is reported to inhibit differentiation in 3T3-L1 cells mainly via upregulation of KLF 2 and KLF3 (Matsuo *et al.*, 2015a; Matsuo *et al.*, 2015b).

However, the bioactive compounds produced by bee symbiont *Streptomyces* spp. remain largely unexplored. Hence, in this study, we aimed to screen bioactive compounds with anti-microbial and anti-adipocyte differentiation activity produced by *Streptomyces* spp. isolated from eastern honey bees and stingless bees.

2.8 Research Overview
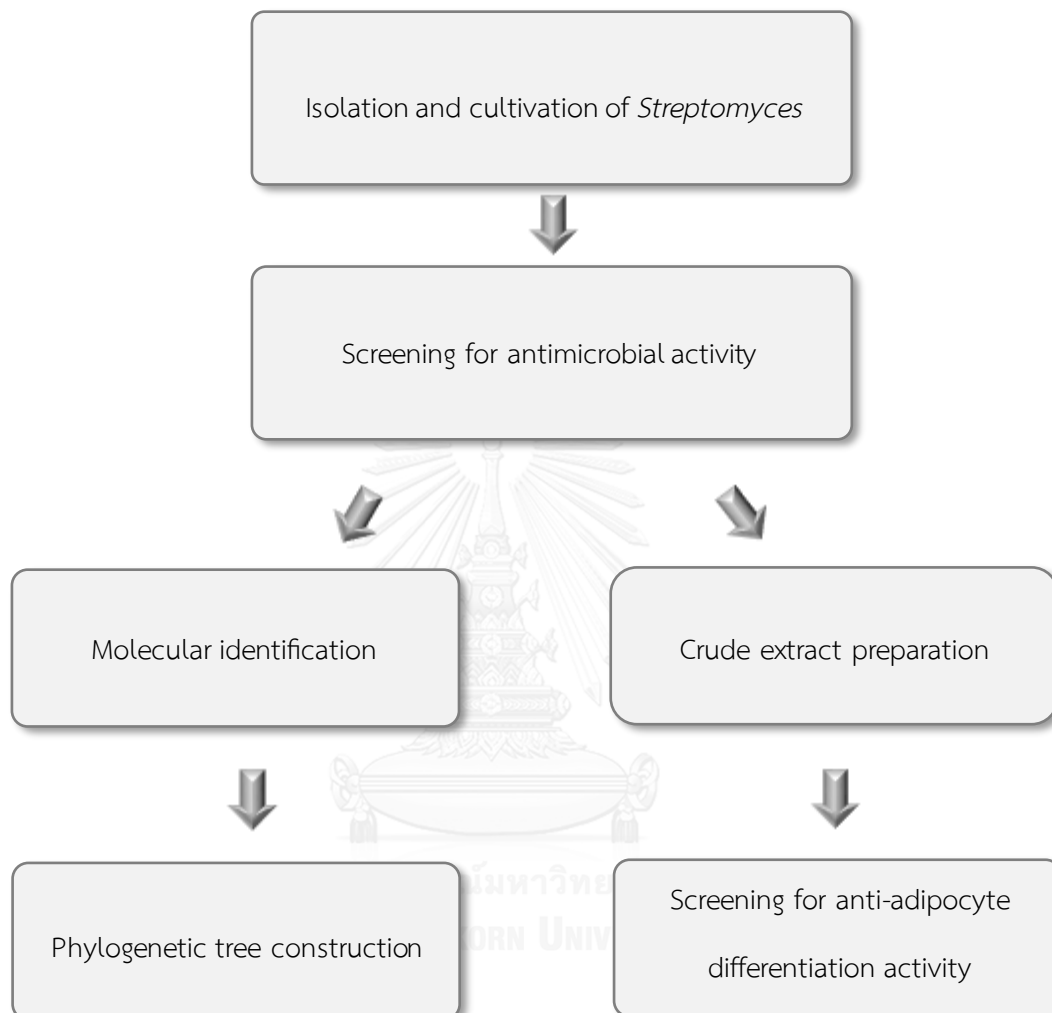
CHEPTER III

MATERIALS AND METHODS

3.1 Reagents and bacteria

3.1.1 Chemical reagents

All chemical was purchased from, Thermo Scientific, (UK) and Sigma-Aldrich, (USA) unless otherwise stated. The water used in this study was sterilized and deionized.

3.1.2 Biological reagents

The gel extraction kit and The Bicinchoninic Acid (BCA) Protein Assay Kit were purchased from Qiagen (Germany) and Thermo Scientific (USA).

3.1.3 Bacterial strains and growth

All antimicrobial testing bacteria *Staphylococcus aureus* ATCC 6538P, *Bacillus subtilis* ATCC 16633, *Escherichia coli* ATCC 8739 were obtained from Department of Microbiology culture collection, and the yeast, *Candida albicans* was kindly provided by Associate Professor Chulee Yompakdee, Ph.D. All culture medium was prepared using deionized water and sterilized before use by autoclaving at 15 minutes at 15 psi pressure 121ºC. The bacteria were grown in Nutrient broth (NB) or Nutrient agar (NA) at 37ºC. Yeast was grown in Potato dextrose agar (PDA) at 30ºC.

3.2 Sample collection

Eastern honey bee (*Apis cerana*) and stingless bee (*Tetragonula laeviceps*) samples were kindly given from Associate Professor Chanpen Chanchao, Department of Biology, Faculty of Science, Chulalongkorn University. The samples were kept in sterile plastic bags and stored at -20 ºC until use.

3.3 *Streptomyces* isolation and cultivation

3.3.1 Isolation from external part of bees

The samples were rinsed with 200 µl of phosphate buffered saline (PBS) (Appendix C) and vortexed. The liquid fractions that obtained from this step were plated on Humic-Acid Vitamin agar (Appendix A) and incubated at 30 ºC for 4-7 days. After that, the presumptive *Streptomyces* colonies with dry and powdery spore were picked and maintained in Starch-Yeast (SY) medium (Appendix A).

3.3.2 Isolation from internal part of bees

The samples that obtain from 3.3.1 were washed with 70% ethyl alcohol and then with PBS for 3 times. After the sample were grinded, 200 µl of PBS were added. The liquid fractions obtained from this step were plated on Humic-Acid Vitamin agar and incubated at 30 ºC for 4-7 days. After that the presumptive *Streptomyces* colonies were selected and maintained in SY medium.

The name assigned for *Streptomyces* spp. isolated from eastern honey bees and stingless bees were as followed:

X-XXX-00

The first alphabets represent part of bees that the *Streptomyces* spp. were found (E for external part and I for internal part of bees). The next alphabets group represent species of bees (EHB for Eastern honey bee and SB for stingless bee). The numbers represent the order of isolation.

3.4 Antimicrobial activity screening

The antimicrobial activity of *Streptomyces* spp. isolated from samples were examined using modified cross-streak method (Carvajal, 1947; Velho-Pereira and Kamat, 2011) using *S. aureus* ATCC 6538P, *B. subtilis* ATCC 16633, *E. coli* ATCC 8739 and *C. albicans* as susceptibility testing microorganism. *Streptomyces* were cultured in NB at 30 ºC and shaken at 200 rpm overnight. After that, a single streak of *Streptomyces* spp. culture was applied on the center of the NA plate and incubated at 30 ºC for 4-7 days. The tested microorganisms were streaked at a 90° angle to the *Streptomyces* and incubated at 37ºC overnight. The cross-streak method was performed by keeping the distance between *Streptomyces* streak and the margin of agar plate at 1 cm and the length and width of the

Streptomyces streak line was fixed at 7 and 0.5 cm, respectively. The distance between the tested microorganism streak was fixed at 1.5 cm and the length of the tested microorganism streak line at 2.5 cm with streak width at 0.5 cm. The markings were drawn on a ruled paper, which act as a template and was attached under the plates to facilitate streaking (Figure 7). After incubation, the visible inhibition zones indicating antimicrobial activity were observed.
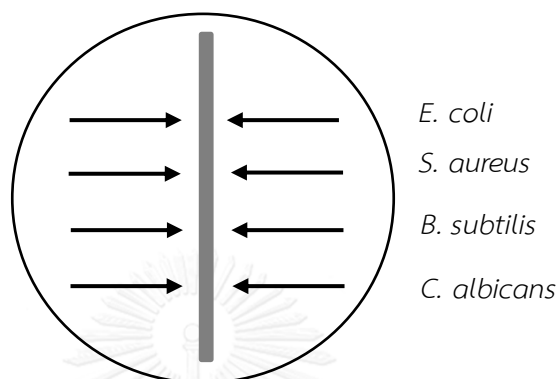
Figure 7 Cross-streak method on NA plate

(Modified from Carvajal, 1947; Velho-Pereira and Kamat, 2011)

3.5 Identification of *Streptomyces* spp.

3.5.1 Colony polymerase chain reaction (colony PCR)

The tested *Streptomyces* spp. that showed antimicrobial against all tested stains were grown on NA and incubated at 30 °C for 2-3 days. The 16S rRNA gene of each isolate was detected by using colony PCR (Ishikawa *et al.*, 2000). Microscopic amounts of *Streptomyces* mycelia were picked using the tip of sterile toothpicks and dipped directly into 20 µl of PCR reaction mixture containing 2x EmeraldAmp® GT PCR Master Mix (TAKARA, Japan), 10µM of Forward (Fwd), 10µM of Reverse (Rev) Primers (Table 2) and sterilized deionized water. The primers used for amplification were 27F and 1492R universal primer (Intergrated DNA Technologies, Singapore). The PCR reaction was performed at 95 °C for 3 minutes, each of 35 cycles thermal consisted of 95 °C for 30 second, 54.4 °C for 30 second and 72 °C for 1 minute. The final extension was at 72 °C for 10 minutes. PCR reactions without DNA were used as negative control. Amplified products were confirmed by electrophoresis with 1.5% agarose gel (Appendix C) at 135 volts for 65 minutes and visualizing with ethidium bromide stain under Gel Documentation System (Biorad, USA).

Table 2 The primer sequences used in this study

Primer	Sequences
27F	Fwd 5' AGAGTTTGATCMTGGCTCAG 3'
518F	Fwd 5' CCAGCAGCCGCGGTAATACG 3'
800R	Rev 5' TACCAGGGTATCTAATCC 3'
1492R	Rev 5' TACGGYTACCTTGTTACGACTT 3'

3.5.2 DNA extraction and purification

The successfully amplified DNA of *Streptomyces* spp. were extracted from the agarose gel by QIAquick® gel extraction kit according to the instruction of manufacturer. Briefly, the DNA fragments (~1,500 bp) were excised from the agarose gel by a clean and sharp scalpel into 1.5 ml centrifuge tube. The gel slices were completely dissolved with Buffer QC at 50 °C for 10 minutes. The samples were added with 100% isopropanol and then transferred to a QIAquick® spin column. This column is membranes that permit DNA binding. Total DNA was washed with Buffer PE. For the elution step, 30 µl of Buffer EB was added to the center of column then incubated for 4 minutes and centrifuged at 13,000 rpm for 1 minute.

3.5.3 DNA Quantification

DNA sample concentration was measured by using Nanodrop 1000 Spectrophotometer (Thermo Scientific, USA). To determine concentration and check purity of DNA sample, 2 µl of DNA samples were subjected to absorbance measurement at 260 nm (A_{260}) and 280 nm (A_{280}) in the spectrophotometer. The concentration of DNA was calculated from A_{260} reading. The purity of DNA was evaluated from a ratio of A_{260}/A_{280}. The DNA sample was sent to 1st base Laboratories Sdn Bhd (Malaysia) for sequenced using the amplification primers (Table 2).

3.5.4 Construction of phylogenetic tree

The 16S rRNA gene sequences of the *Streptomyces* spp. were blasted with available sequences in databases using NCBI BLAST. The reference sequences downloaded from GenBank and MEGA v7.0 (Kumar *et al.,* 2016) was used for the multiple alignment and

the construction of phylogenetic trees using neighbor-joining method (Saitou and Nei, 1987). The bacterial 16S rRNA gene sequence of *Escherichia coli* (J01859.1) was used as an outgroup.

3.6 Preparation of metabolite crude extract

Streptomyces spp. were cultured in 50 ml C4 medium which has been the classical fermentation medium for bioactive compound production, containing e.g. soybean meal as an effective source of nitrogen for antibiotic formation, glucose as carbon source, and sodium chloride which might be involved in release of the antibiotic from the mycelia (Demain and Inamine, 1970) (Appendix A) in 250 ml flask on refrigerated incubator shaker (New Bruswick Scientific, UK) at 30 °C and shaken at 200 rpm for 7 days. After incubation, the culture was centrifuged at 5,000 rpm for 5 minutes, and the supernatant was extracted twice with two volume of ethyl acetate because it has slightly polar its almost immiscible with water, high enough boiling point and less toxicity in centrifuge tube for 20 minutes (Sadigh-Eteghad *et al*., 2011). The solvent phase and aqueous phase were transferred to different round bottom flask to remove ethyl acetate using rotary vacuum evaporator (Eyela, USA). Aqueous phase was transferred to 1.5 ml centrifuge tube to remove water using concentrator (Eppendorf, Germany). After that, the crude solvent and aqueous phase were dissolved with DMSO (Dimethyl sulfoxide) and tested antimicrobial activity using disc diffusion assay to confirm the activity of crude extract. The tested microorganisms; *S. aureus* ATCC 6538P, *B. subtilis* ATCC 16633, *E. coli* ATCC 8739 and *C. albicans* were dispersed on NA plate using sterile cotton swab. Crude extracts (20 mg/ml) were applied onto 6 mm paper discs (GE Healthcare Life Sciences, UK). The discs containing crude produce were placed on tested microorganism agar plate and incubated at 30 °C overnight then the visible inhibition zones indicated antimicrobial activity were observed.

3.7 Culture of 3T3-L1 preadipocytes cell line

For every assay, a mouse embryonic fibroblasts, 3T3-L1 (ATCC CL-173) were cultured in Dulbecco's modified Eagle's medium (DMEM) (Thermo Fisher Scientific, UK), supplemented with 10% Calf bovine serum, 1% sodium pyruvate, 1% HEPES (4-(2-hydroxyethyl)-1-

piperazineethanesulfonic acid) free acid, and 1% penicillin-streptomycin (Thermo Fisher Scientific, UK) at 37°C in 5% CO_2 (Thermo Electron Corporation, USA) (Appendix B).

3.8 Adipocyte differentiation of 3T3-L1 preadipocytes cell line

3T3-L1 cells ($2x10^4$ cells/well) were cultured in 24-well plates with culture medium for two days, and then medium cultured was replaced to differentiation medium, i.e. DMEM with 10% Fetal bovine serum (FBS), 1% HEPES free acid, 1% sodium pyruvate, 1% penicillin-streptomycin, 1.0 μM DEX, 0.5 mM IBMX and 1.0 μg/ml insulin solution from bovine pancreas (Appendix B), to induce adipocyte differentiation for two days. After induction, the differentiation medium was exchanged for maintenance medium containing DMEM, supplemented with 10% FBS, 1% HEPES free acid, 1% sodium pyruvate, 1% penicillin-streptomycin and 1.0 μg/mL insulin (Appendix B). Incubation was continued for two days, followed by two days of incubation with maintenance medium at 37°C in 5% CO_2 incubator until the end of the differentiation process (Figure 8).
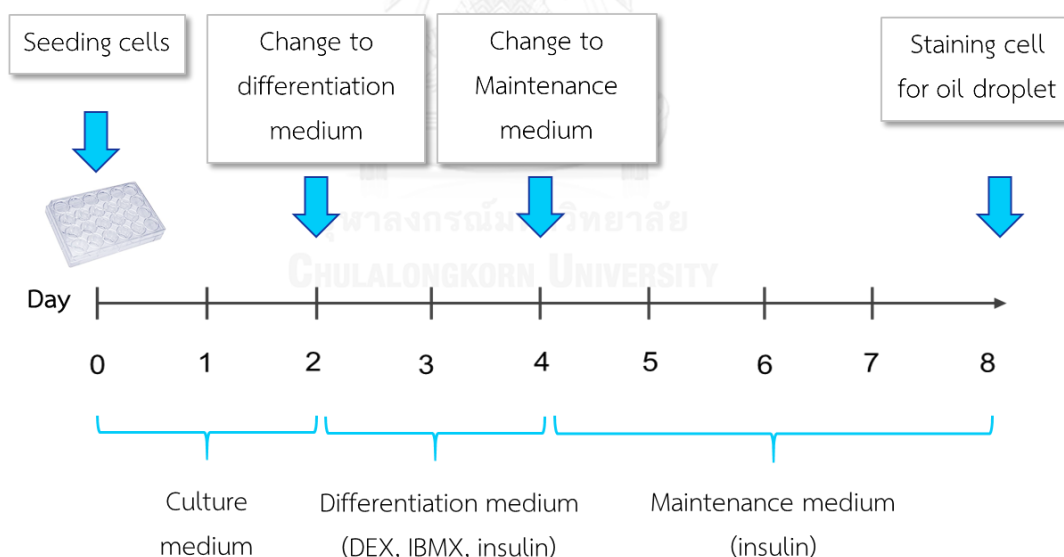
Figure 8 Schematic representation of 3T3-L1 cell line differentiation procedure

3.9 Cytotoxicity test using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide) assay

Crude extracts were dissolved in DMSO and 3T3-L1 cells (4×10^3 cells/well) were cultured in 96-well plates overnight. After that, cells were treated with crude extract at various concentrations, 100, 31.62, 10, 3.162, 1 and 0.316 μg/ml, for 24 hours. MTT solution (Appendix B) (Promega, USA) was used to determine the cell viability for 4 hours at 37°C in 5% CO_2 incubator. The insoluble formazan was dissolved with DMSO. Cell viability was determined from the absorbance at 540 nm using microplate reader (Biochrom Anthos, USA). The relative cell viability (%) was expressed as a percentage relative to the untreated control cells using the following formula. The 50% inhibition concentration (IC_{50}) was calculated using GraphPad Prism 5.03 (GraphPad Software, USA).

$$\%\text{Cell viability} = \left[\frac{\text{Absorbance of treated cells - Absorbance of blank}}{\text{Absorbance of vehicle cells - Absorbance of blank}} \right] \times 100$$

3.10 Oil-Red O (ORO) and Hematoxylin staining

Lipid droplets in cells were stained with ORO at the end of adipocyte differentiation induction process (day 8). The cells were washed with PBS and then fixed with 10% formaldehyde at room temperature for at least 1 hour. In 24-well plates which were wrapped with parafilm and covered with aluminum foil to prevent cells from drying. After fixation, the cells were washed twice with deionized water and dehydrated with 60% isopropanol for 5 minutes. After that, the cells were dried at room temperature and stained with fresh ORO working solution (Appendix C) on Rocker (Labnet International Inc., USA) for 10 minutes. Cells were washed four times with deionized water and stained with Hematoxylin solution for 5 minutes then washed twice with deionized water. The cells were covered with deionized water to avoid drying and visualized under fluorescent microscope. Lipid droplets appear red and nuclei appear blue.

3.11 Screening of anti-adipocyte differentiation activity

3T3-L1 cells (2×10^4 cells/well) were cultured in 24-well plate for two days. The cells were pretreated with crude bioactive compounds at 50 μg/ml for 1 hours and then medium

cultured was immediately replaced to differentiation medium containing 50 µg/ml of crude bioactive compounds to induce preadipocyte differentiated to adipocyte for 48 hours. After induction, the differentiation medium was replaced with maintenance medium and incubated at 37℃ with 5% CO_2 until the end of adipocyte differentiation process (day 8). Cells were stained with ORO and Hematoxylin. Cells that positive for ORO and Hematoxylin staining were counted under microscope.

3.12 Western blot

3.12.1 Protein extraction and quantitation

3T3-L1 cells ($4x10^4$ cells/well) were cultured in 12-well plate. The total cellular proteins of day 4 and day 5 3T3-L1 cells differentiation process with and without crude bioactive compounds (50 µg/ml) were extracted using buffer A and buffer B (Appendix C). 3T3-L1 cells were washed with 1 ml of cold PBS and subsequently with 200 µl of buffer A. After that, 50 µl of the lysis buffer B for extracting protein was added to each sample. Samples were vortexed and centrifuged at 12000 g, 4 ℃ for 10 minutes. Total protein concentration was quantified using a Pierce™ BCA Protein Assay Kit. The concentration of protein in each sample were adjusted with sterile MilliQ water in the ratio at 1:9 in 96-well plate (NuncTM, Thermo Scientific, USA). Bovine albumin serum (BSA) was used a standard protein at 1, 0.5, 0.25, 0.125, 0.063 and 0.031 µg/ml. Each well was added with 200 µl of working reagent (reagent A and reagent B) and incubated at 37℃ for 30 minutes. The absorbance was measured at 540 nm using microplate reader.

3.12.2 SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

The 10% SDS-PAGE gel was made following the formulas shown in Appendix C. Protein samples were mixed with 2 x dye (Appendix C) and sterile deionized water in an equal volume. The samples were heated at 99 ℃ for 5 minutes on Thermomixer (Eppendorf, Germany). The protein samples and prestained protein molecular weight marker (Thermo Scientific, USA) were loaded into the gel that immersed in the running buffer (Appendix C). The electrophoresis was performed at 100 volts constantly for 120 minutes using Protein Ⅲ system instrument (Biorad, USA). After gel electrophoresis was finished, proteins were transferred onto Polyvinylidene fluoride (PVDF) membranes (Merck Millipore, Germany). Firstly, separating gel and two pieces of Whatman filter paper were equilibrated in transfer

buffer (Appendix C) for 5 minutes. PVDF membranes were soaked in absolute methanol for 1 minute and then rinsed with deionized water twice before using. One pieces of equilibrated filter paper were placed in semi-dry Trans-Blot®SD (Biorad, USA). After that, PVDF membrane, separating gel and one piece of filter paper were subsequently placed on filter paper. Air bubbles were eliminated by a gentle rolling glass tube. The semi-dry transfer was carried out under the condition using a constant at 80 mA for 90 minutes.

3.12.3 Antibody probing

After protein transfer was completed, the PVDF membranes were blocked twice with blocking solution (Appendix C) for 5 minutes on rocker. Primary antibodies, rabbit anti PPARγ (1:1000) (Cell signal Technology, USA) and rabbit anti GAPDH (1:5000) (Cell signal Technology, USA) were diluted in blocking solution before using. PVDF membranes were probed with primary antibody on a rocker at least 1 hour before incubating at 4 °C overnight. After incubation, primary antibody was removed then probed membranes were washed with PBS-T (Appendix C) for 5 minutes twice and 15 minutes twice. After washing, the membranes were incubated with secondary antibody, goat anti-rabbit IgG (G α R) conjugated horse radish peroxidase (HRP) (EMD Millipore, USA) for 1 hour on a rocker at 1: 1000 for detecting PPARγ and 1: 4000 for detecting GAPDH. After that, secondary primary was removed and the membranes were washed with PBS-T for 5 minutes twice and 15 minutes three times.

3.12.4 Signal detection

The signal was detected by chemiluminescence and autoradiography. The substrates for detection were composed of solution A and B (Appendix C). Solution A and B were freshly prepared, mixed well and immediately incubated to the membrane for 1 minute at room temperature. The membrane was covered with the plastic wrap and placed in Hypercassette (Amershan Bioscience, UK) to expose Amersham HyperfilmTM ECL (Amershan Bioscience, UK) in the dark at room temperature for 20 seconds to 30 minutes depending on the target protein. After that, the film was developed in X-ray film developer ECL (Carestream, USA) and fixed in the X-ray film fixer (Carestream, USA). The film was dried and then label with the marker.

3.13 Statistical analyses

All experiments were done triplicate with two independent experiments. Results were expressed as the mean ± standard deviation (SD). The statistical significance of difference between an experimental group and its corresponding control will be evaluated by one-way ANOVA (GraphPad Prism 5.03). All comparisons were made as specified, and $p<0.05$ was considered statistical significance.

CHAPTER IV

RESULTS

4.1 Isolation and cultivation of *Streptomyces*

The insect-derived samples were plated on selective medium Humic-Acid Vitamin agar and incubated at 30 °C for 7 days. After incubation, one hundred twenty-five isolates of the putative *Streptomyces* spp. by colony morphology were obtained from Humic-Acid Vitamin agar (Figure 9). The total number of isolates from eastern honey bees and stingless bees were 47 and 78, respectively. In addition, more than 80% of total isolates were from the internal samples. (Table 3).
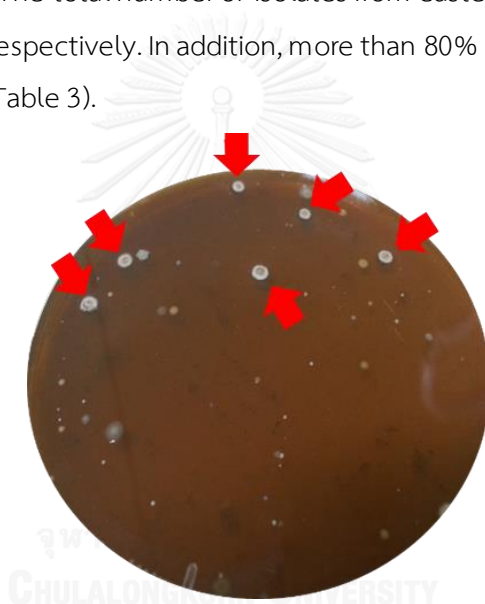
Figure 9 Representative of the putative *Streptomyces* colonies on Humic-Acid Vitamin agar. Red arrows indicate putative *Streptomyces* colonies

Table 3 The total numbers of putative *Streptomyces* isolated from eastern honey bees and stingless bees

Type of bee	Number of isolate	
	External samples	Internal samples
Eastern honey bee	12	35
Stingless bee	5	73
Total	17	108

4.2 Screening for antimicrobial activity

All isolated *Streptomyces* spp. were screened for antimicrobial activity using modified cross-streak method (Velho-Pereira and Kamat, 2011) against human pathogenic representative species of Gram positive bacteria, Gram negative bacteria and yeast including; *S. aureus*, *B. subtilis*, *E. coli* and *C. albicans*. The visible inhibition zones were measured from the margins of inhibition of indicator microorganisms zone to the streak line of *Streptomyces* on the center of agar plate. The antagonistic activities of *Streptomyces* spp. against the tested microorganisms were summarized in Table 4 (Appendix D, Table 1.1).

The results showed that ten isolates produced the metabolites that have broad antimicrobial activity against all tested stain (Appendix D, Figure 1.1). However, some of these isolates have similarity in morphology including color of colony, spore and pigment. Therefore, only five isolates, I-SB-38, I-SB-71, I-EHB-05, I-EHB-06, and I-EHB-18 were selected for further characterization (Figure 10).

Table 4 Antimicrobial activity of *Streptomyces* isolated from bees

Number of suppressed tested microorganism	Tested microorganism				Number of isolates with activity
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*	
0					61
1	/				6
		/			22
				/	8
2	/	/			12
	/			/	1
3	/	/	/		1
	/	/		/	4
4	/	/	/	/	10

The antagonistic activities show as "/"
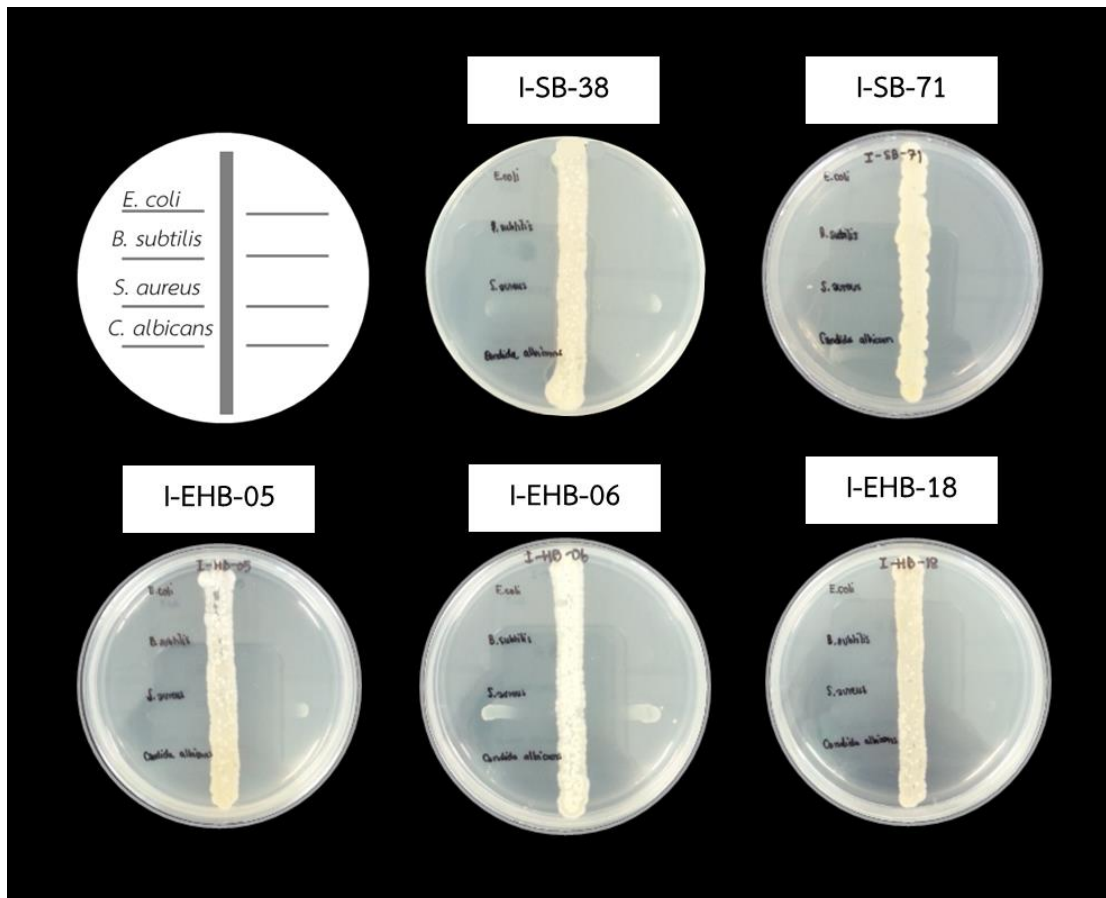
Figure 10 Antimicrobial activity of *Streptomyces* spp. using modified cross-streak method

4.3 Molecular identification of *Streptomyces*

4.3.1 Colony PCR

According to the cross-streak screening assay, five isolates with broad spectrum antimicrobial activity were subjected to strain identification by molecular method. The 16S rRNA gene of these isolates were amplified using colony PCR method (Figure 11). The PCR products with the size around 1500 bp were extracted from the agarose gel using QIAquick® gel extraction kit. The DNA samples (40 ng/µl) were analyzed for sequencing using 27F, 518F, 800R and 1492R as the amplification primers. The 16S rRNA sequences of these isolates were shown in Appendix C.
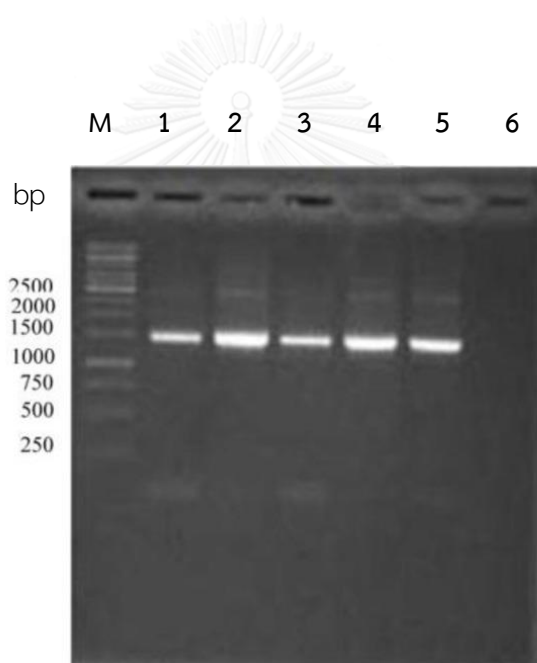
Figure 11 PCR product of 16S rRNA gene (~1500 bp). Lane M: DNA Standard 1 kb ladder markers, lane 1: I-SB-38, lane 2: I-SB-71, lane 3: I-EHB-05, lane 4: I-EHB-06, lane 5: I-EHB-18 and lane 6: negative control

4.3.2 16S rRNA -based phylogenetic tree

The 16S rDNA sequences were compared with the corresponding sequences available in the NCBI database and 16S rRNA sequences of insect associated *Streptomyces* from already published research (Appendix D, Table 2.1). Phylogenetic trees were constructed using the neighbour joining (Saitou and Nei, 1987) and maximum composite likelihood methods (Tamura *et al*., 2004). *E. coli* J01859.1 was used as an out group. Bootstrap values for nodes of the phylogenetic trees were determined based on 1,000 replications. Sequence similarity was calculated manually after pairwise alignment using MEGAv7 (Figure 12).

Based on the phylogenetic tree, all five isolates were identified as *Streptomyces* species. The 16S rRNA sequences of I-SB-38 and I-SB-71 were closely related to *Streptomyces bikiniensis* strain 13661V whereas I-EHB-05, I-EHB-06 and I-EHB-18 were closely related to *Streptomyces andamanensis* sp. nov compared databases using NCBI BLAST (Table 5).

Table 5 *Streptomyces* identification based on 16S rRNA sequences

Isolates	Identification	Similarity
I-SB-38	*S. bikiniensis* strain 13661V	98%
I-SB-71	*S. bikiniensis* strain 13661V	96%
I-EHB-05	*S. andamanensis* sp. nov	97%
I-EHB-06	*S. andamanensis* sp. nov	98%
I-EHB-18	*S. andamanensis* sp. nov	98%
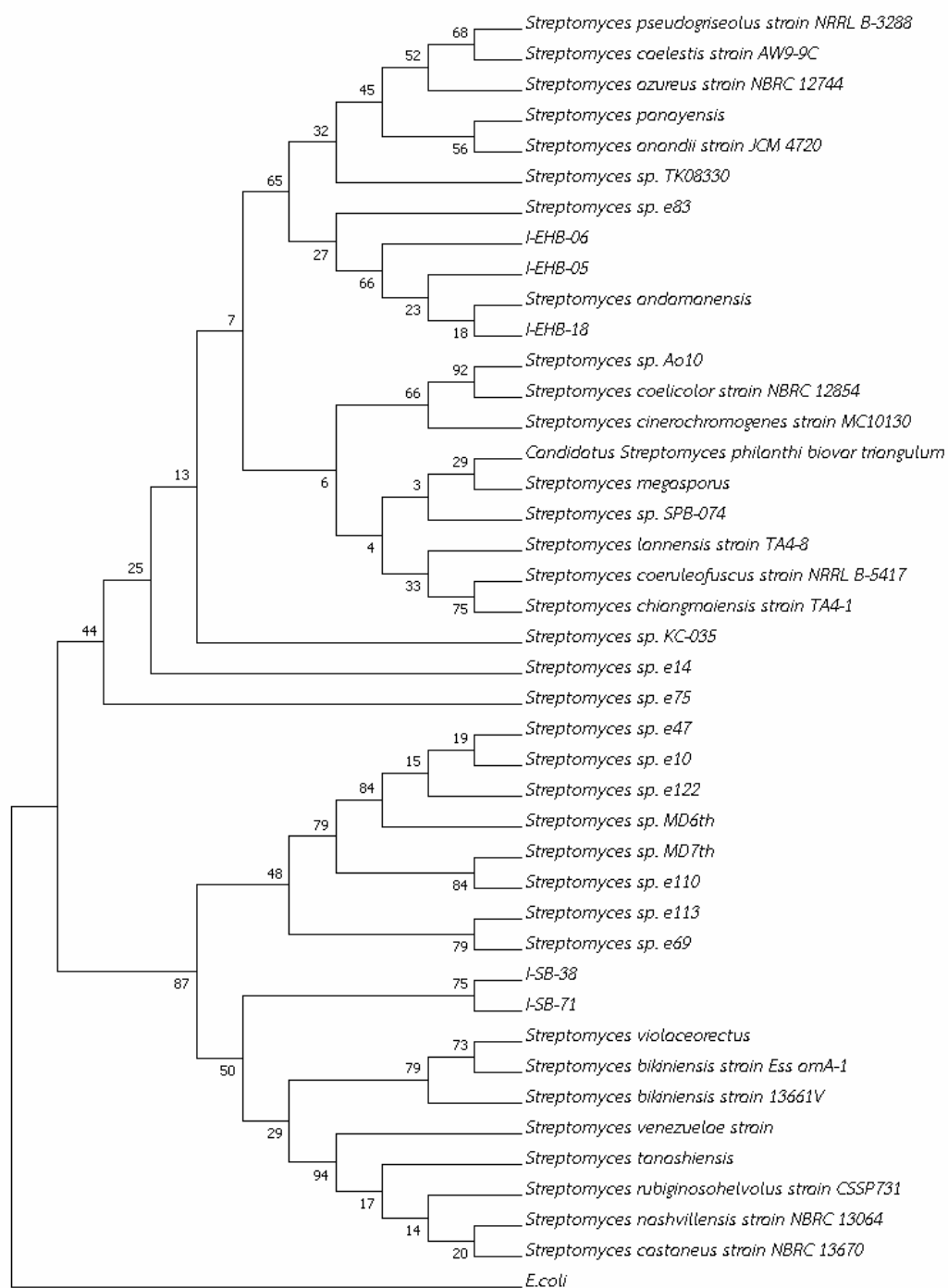
Figure 12 Neighbour-joining tree based on 16S rRNA gene sequences. The phylogenetic tree showing the relationships between isolated strains and type strains of closely related species in the genus *Streptomyces*. Bootstrap values based on 1,000 replications were shown at branch nodes. The evolutionary distances were computed using the maximum composite likelihood method. *E. coli* J01859.1 was used as an out group. Bar, 0.020 substitutions per nucleotide position
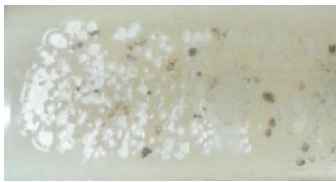
The similarity of 16S rDNA sequence of isolates also were determined by pairwise alignment using the EMBL-EBI web services. The result indicated that both chains isolated of stingless bee, I-SB-38 and I-SB-71 are different. For honey bee isolates, I-EHB-05 is closely related to I-EHB-06 and I-EHB-18 (Table 6) (Appendix D).

For the morphological characteristics, I-SB-38 isolate produced a brownish-gray spore differ from I-SB-71 isolates that produced a white or light gray spore. I-EHB-05 isolate produced a white spore, while I-EHB-06 and I-EHB-18 isolate produced a white or light brown spore (Table 7).

Table 6 Pairwise alignment selected of *Streptomyces* isolates

Aligned sequences		Length	
1 st	2 nd	(bp)	Similarity
I-SB-38	I-SB-71	1514	92.5%
I-EHB-05	I-EHB-06	1457	99.0%
I-EHB-05	I-EHB-18	1468	97.7%
I-EHB-06	I-EHB-18	1469	97.5%

Table 7 Morphology of selected *Streptomyces* isolates

Host	Sampling site	Isolate	Colony and spore on SY medium
Stingless bees	Internal	I-SB-38	
Stingless bees	Internal	I-SB-71	
Eastern honey bees	Internal	I-EHB-05	
Eastern honey bees	Internal	I-EHB-06	
Eastern honey bees	Internal	I-EHB-18	

4.4 Metabolite crude extract preparation

To obtain the crude extract, I-SB-38, I-SB-71, I-EHB-05, I-EHB-06 and I-EHB-18 isolates were cultured in 50 ml of C4 medium at 30 °C, 200 rpm. After seven days, the supernatants were extracted with ethyl acetate to separate non-polar and moderate polar compounds from the highly polar ones. After drying, both ethyl acetate crude and water soluble crude fraction were dissolved in DMSO at the concentration of 20 mg/ml. All crude extracts were subjected to the antimicrobial test using disc diffusion method. Ampicillin (10 µg/ml) and amphotericin B (100 µg/ml) were used as control for bacteria and yeast testing strains. The results showed that only crude extract from aqueous phase of all of isolates contained antimicrobial activity (Figure 13).
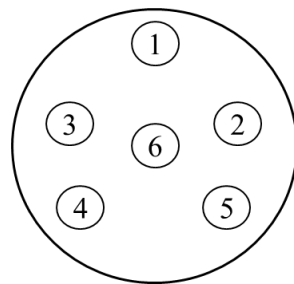
1: C4 medium,

2: DMSO

3: supernatant before extraction

4: aqueous phase (20 mg/ml)

5: ethyl acetate crude (20 mg/ml)

6: ampicillin (10 µg), or

   amphotericin B (100 µg)
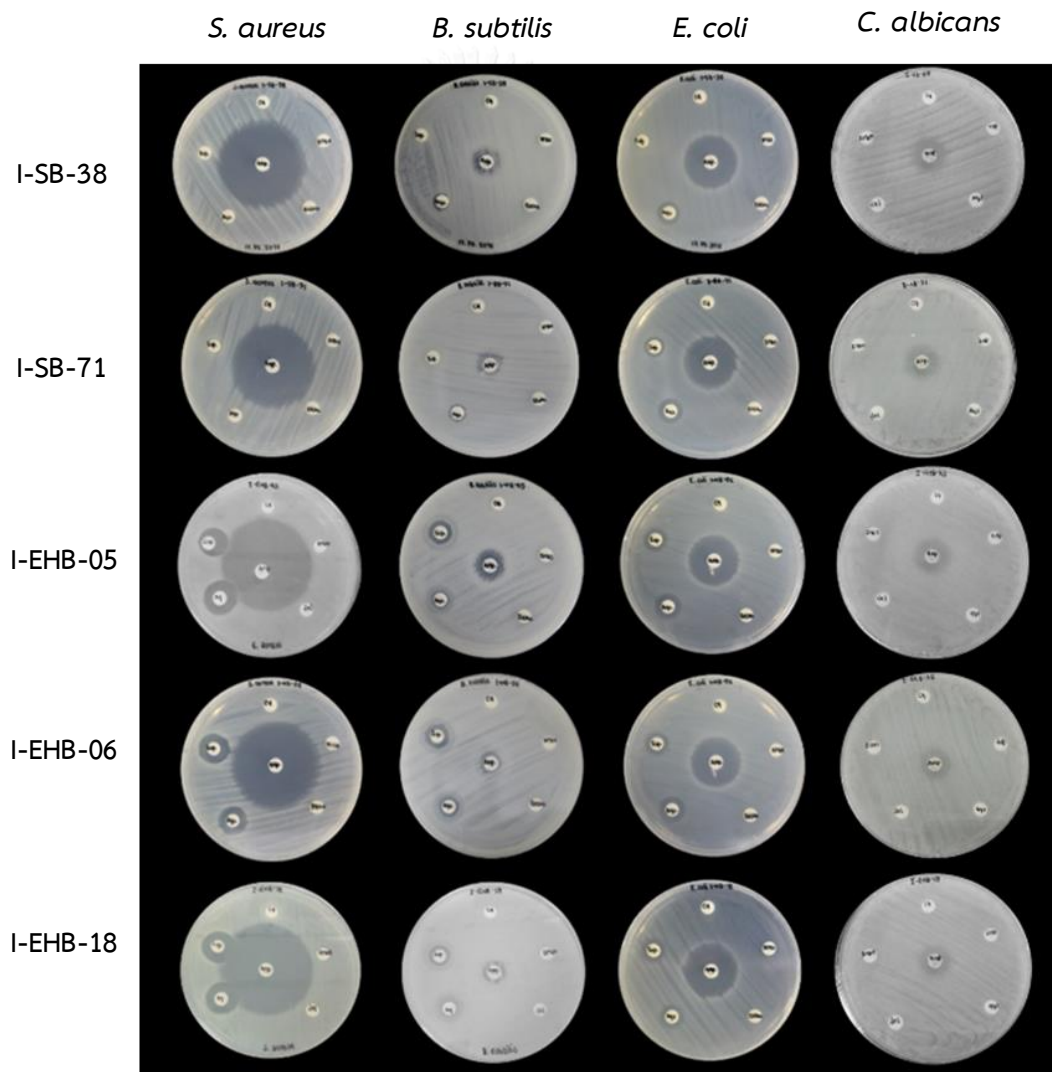
Figure 13 Antimicrobial activity of crude extract by disc diffusion method. Crude extracts from supernatant of I-SB-38, I-SB-71, I-EHB-05, I-EHB-06 and I-EHB-18 isolates, respectively

4.5 Effects of crude extracts on cell viability of pre-adipocyte like cell line 3T3-L1

To check the cytotoxicity of the crude extracts, preadipocyte cell line 3T3-L1 was treated with the crude extracts at various concentrations (0.316, 1, 3.162, 10, 31.62, and 100 µg/ml) for 24 hours and the cell viability were determined by MTT assay. The result indicated that the crude extracts from aqueous and solvent phase of I-SB-38, I-SB-71, I-EHB-05, I-EHB-06 and I-EHB-18 were not toxic to cells at the concentrations up to 100 µg/ml (Table 8) (Appendix D, Figure 3.1 and Figure 3.2).

Table 8 Effect of crude extracts on cell viability of 3T3-L1 cell

Crude extracts	Phase	IC_{50} (µg/ml)
I-SB-38	aqueous	> 100
I-SB-71	aqueous	> 100
I-EHB-05	aqueous	> 100
I-EHB-06	aqueous	> 100
I-EHB-18	aqueous	> 100
I-SB-38	solvent	> 100
I-SB-71	solvent	> 100
I-EHB-05	solvent	> 100
I-EHB-06	solvent	> 100
I-EHB-18	solvent	> 100

4.6 Effects of crude extracts on adipocyte differentiation in 3T3-L1 cell line

To screen for anti-adipocyte differentiation activity, the 3T3-L1 cell line were treated with crude extract at 50 μg/ml and subjected to induce adipocyte differentiation. After seven days, the induced cells were stained for lipid droplet with Oil Red O staining (ORO). The fully differentiated cells exhibited red color droplets in the cell under microscope, indicating lipid accumulation (Figure 14B). The result showed that the crude aqueous extract from I-EHB-18 isolate significantly suppressed lipid accumulation in 3T3-L1 cell line (Figure 14G). Therefore, this crude extract was used for further study. On the other hand, the crude aqueous extracts from I-SB-38, I-SB-71 and I-EHB-06 (Figure 14C, 14D and 14F) and crude solvent extracts from I-SB-38 and I-SB-71 were not suppress adipocyte differentiation as well as lipid accumulation in 3T3-L1 cells (Figure 14H and 14I). Furthermore, crude solvent extract from I-EHB-05 and crude solvent extracts from I-EHB-05, I-EHB-06 and I-EHB-18 showed toxicity on 3T3-L1 cells (Figure 14E, J, K and N).
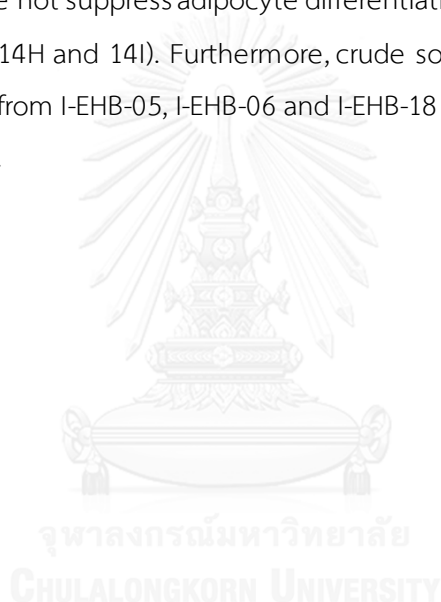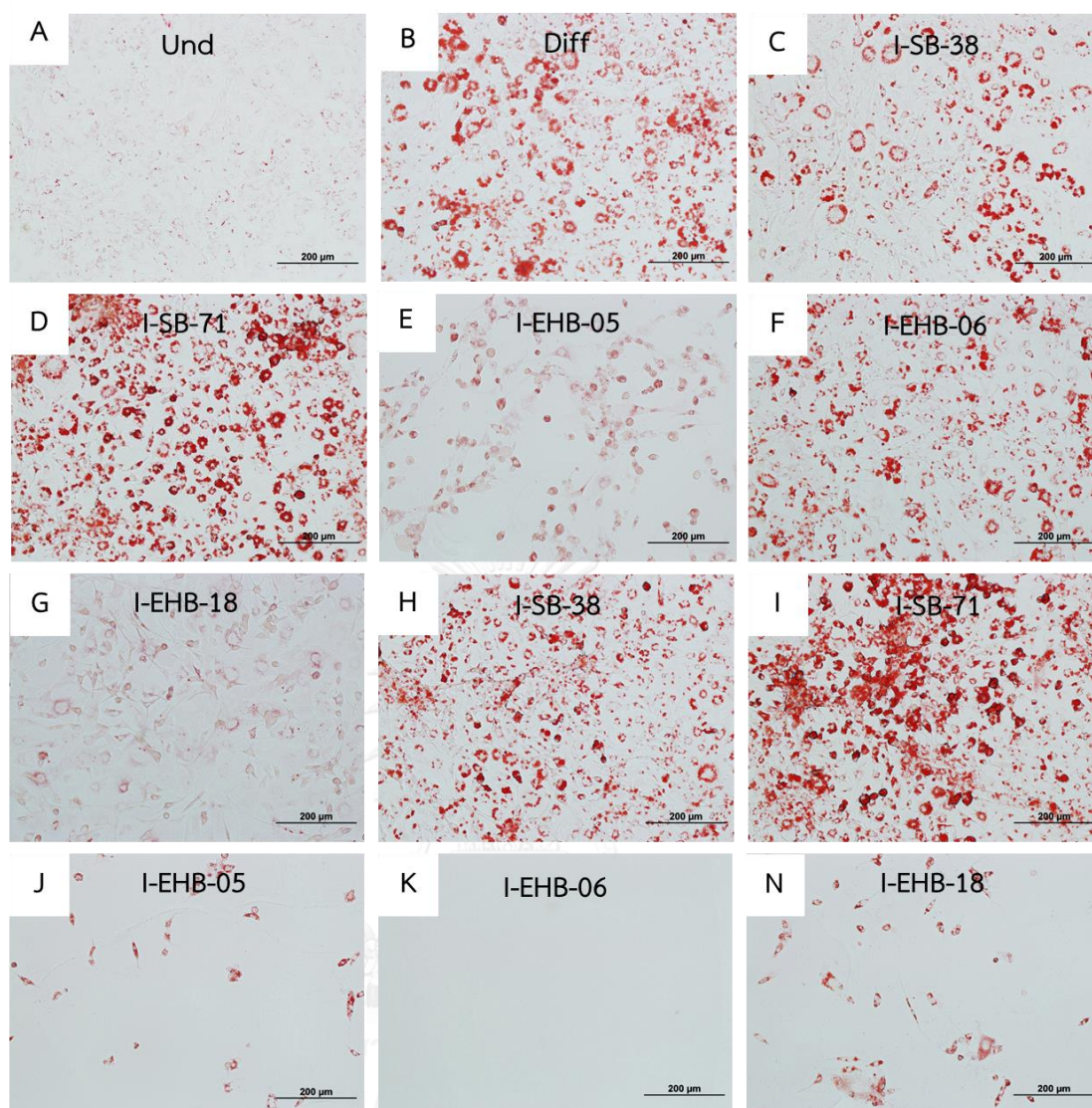
Figure 14 Effects of crude extracts from selected isolates on 3T3-L1 differentiation. A: undifferentiated 3T3-L1 cells (positive control), B: differentiated 3T3-L1 cells (negative control), (C-G): differentiated 3T3-L1 cells treated with crude aqueous extracts, (H-N): differentiated 3T3-L1 cells treated with crude solvent extracts (50 μg/ml)

The initial screening of anti-adipocyte differentiation indicated that crude aqueous extract from I-EHB-18 isolates designated CA18 potentially inhibit adipocyte differentiation and lipid accumulation of 3T3-L1 cell line (Figure 15). To investigate the dose-dependent effect of crude extracts on adipocyte differentiation, 3T3-L1 cells were treated with crude compound at the concentrations of 3.125, 6.25, 12.5, 25, 50 and 100 μg/ml for 7 days and then stained with ORO and hematoxylin stain. Crude extract at 50 and 100 μg/ml markedly suppressed lipid accumulation resulting in much less oil droplet being stained by ORO on microscopic showed significant inhibition of adipocyte differentiation (Figure 16).
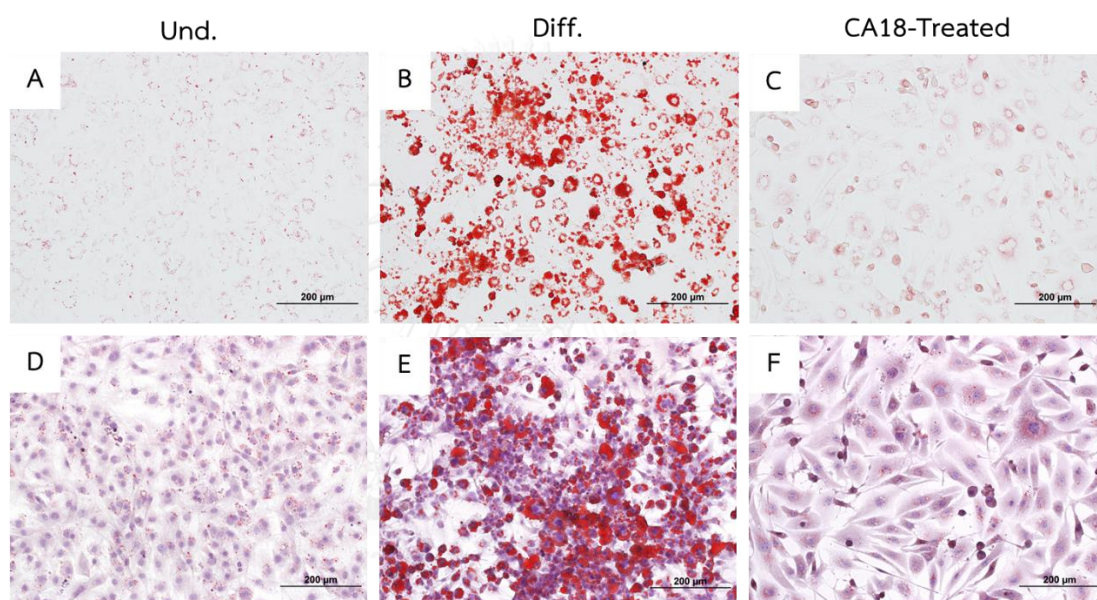
Figure 15 Effect of crude aqueous phase extract from I-EHB-18 isolates (50 μg/ml) on adipocyte differentiation. (A-C): ORO staining and (D-F): ORO (red) and hematoxylin (pink purple) staining

A, D: undifferentiated 3T3-L1 as positive control

B, E: differentiated 3T3-L1 as negative control

C, F: differentiated 3T3-L1 treated with CA18
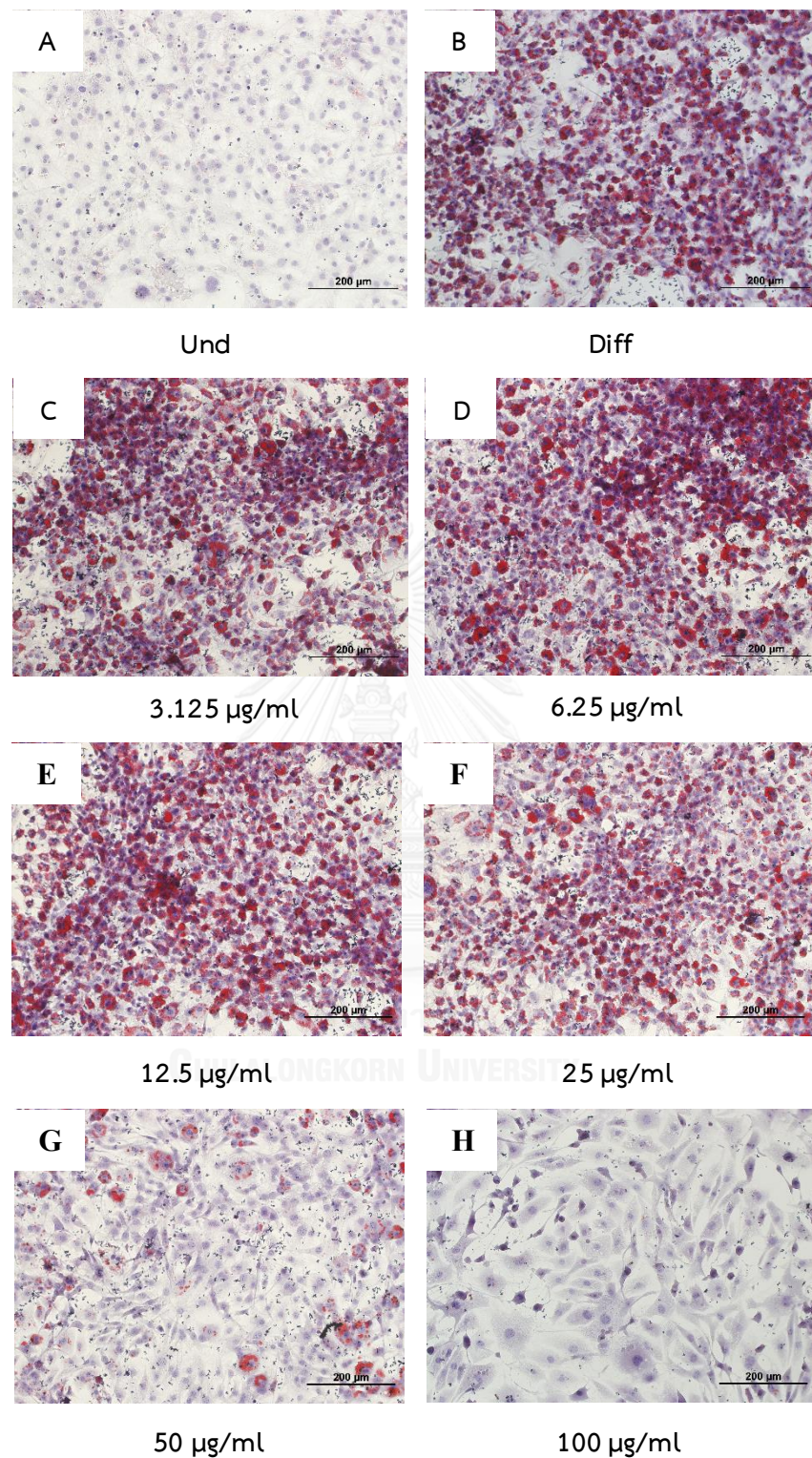
Figure 16 Effect of crude aqueous phase extract from I-EHB-18 isolates on adipocyte differentiation in a dose-dependent manner. A: undifferentiated 3T3-L1 cells, B: differentiated 3T3-L1 cells, (C-H): differentiated 3T3-L1 cells treated with CA18

4.7 Effects of crude aqueous phase extract on expression of master regulators of adipocyte differentiation, PPARγ

As the crude aqueous extract from I-EHB-18 stain inhibited the lipid accumulation that occurred upon differentiation from preadipocytes to adipocytes, the extract may interrupt signaling of the adipocyte differentiation. To examine the mechanism of action, the expression of PPARγ which is a master regulator of adipocyte differentiation was examined using Western blot analysis. 3T3-L1 cells were treated with and without the crude extract at 50 µg/ml for 4 and 5 days in the process of stimuli for adipocyte differentiation. The results demonstrated that the crude extract downregulated PPARγ protein levels in 3T3-L1 cells differentiation (Figure 17).
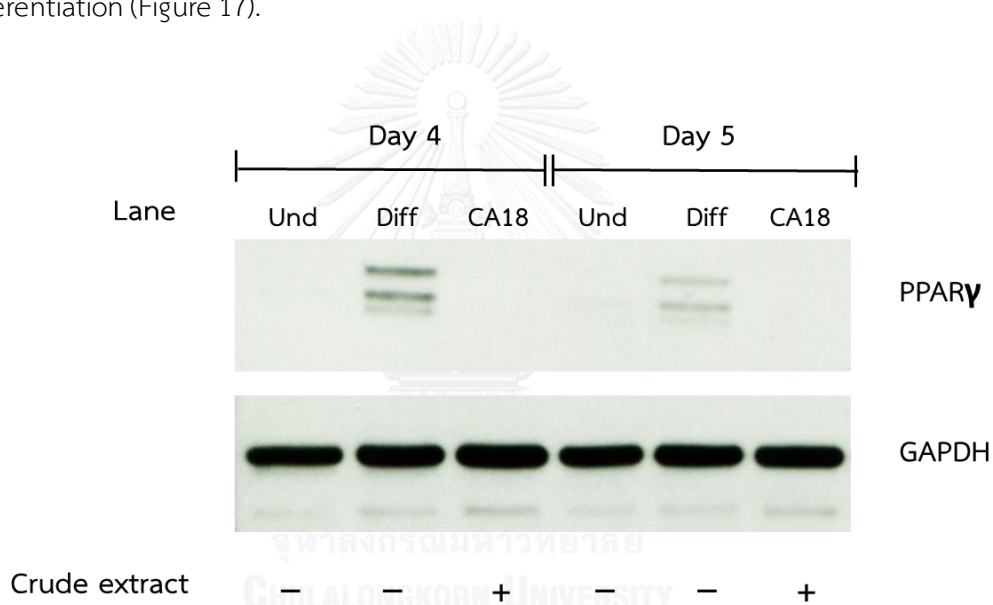
Figure 17 Western blot analysis for PPARγ. At day 4 (Lane 1-3); undifferentiated cells, differentiated cells, and differentiated cells in the presence of crude extract (50 µg/ml) treatment and at day 5 (Lane 4-6); undifferentiated cells, differentiated cells, and differentiated cells in the presence of crude extract (50 µg/ml) treatment, respectively. GAPDH was used as internal loading control

4.8 Timing of anti-adipocyte differentiation activity of crude extract from I-EHB-18

To investigate the timing of the activity of crude extract from I-EHB-18 isolate on adipocyte differentiation, 3T3-L1 cells line were treated with 50 µg/ml crude extract on day 2, 4 and 6 of adipocyte differentiation process (Figure 18). The result revealed that the crude extract only affected the early state of adipocyte differentiation (day 2) and have no effect on intermediate or terminal state of differentiation (day 4 and 6) (Figure 19).
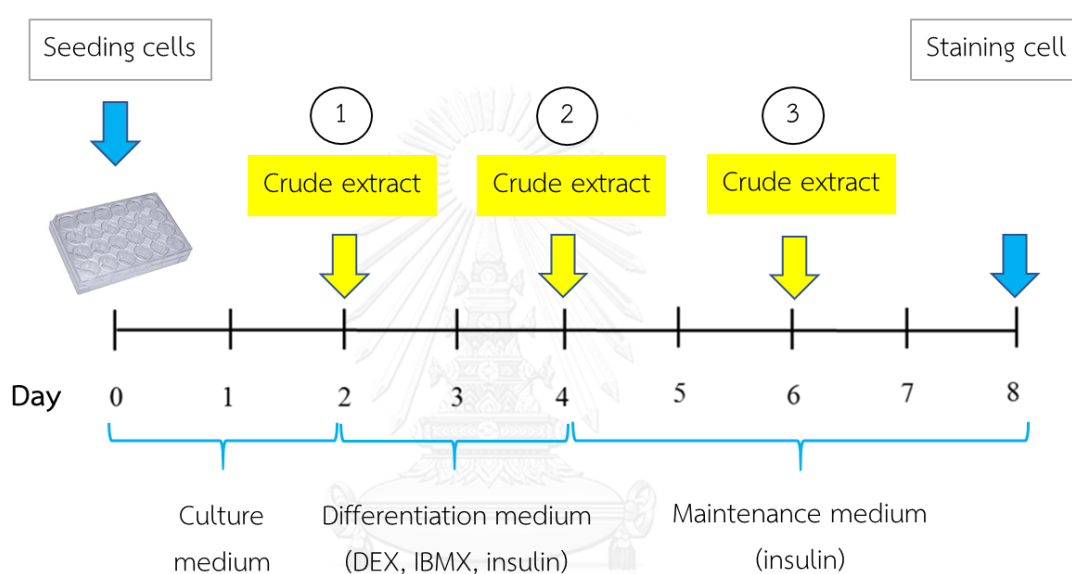
Figure 18 Schematic representation of timing of anti-adipocyte differentiation activity of CA18 procedure
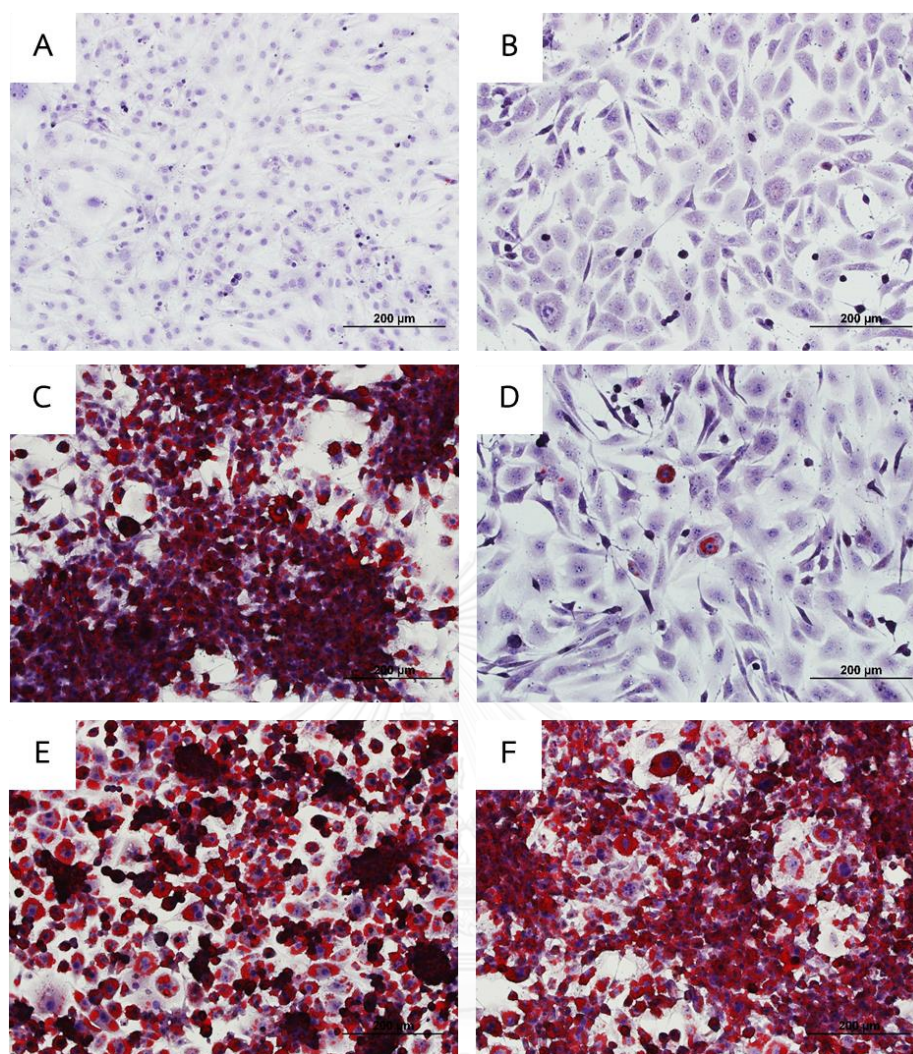
Figure 19 Timing of anti-adipocyte differentiation activity of crude extract from I-EHB-18. A: undifferentiated 3T3-L1 cells, B: CA18 treated without induction, C: differentiated 3T3-L1 cells, (D-F): differentiated 3T3-L1 treated with CA18 on day 2, 4 and 6 of adipocyte differentiation process, respectively

To confirm that this compound suppresses adipocyte differentiation or lipid accumulation at early stage of adipocyte differentiation, the cells were treated with 50 µg/ml of crude extract for 48 hours after induction. Subsequently, the crude compound was removed from the system and replaced with fresh maintenance medium without crude extract (Figure 20). The result demonstrated that this compound suppresses adipocyte differentiation at early stage completely (Figure 21).
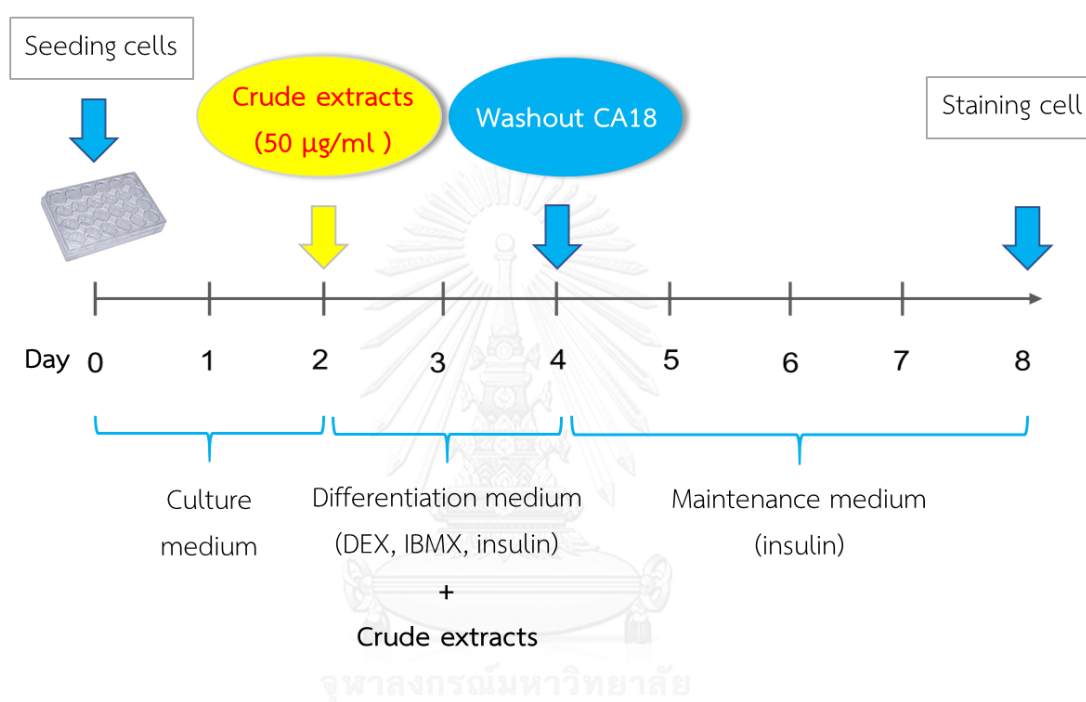
Figure 20 Schematic representation of anti-adipocyte differentiation activity of CA18 procedure
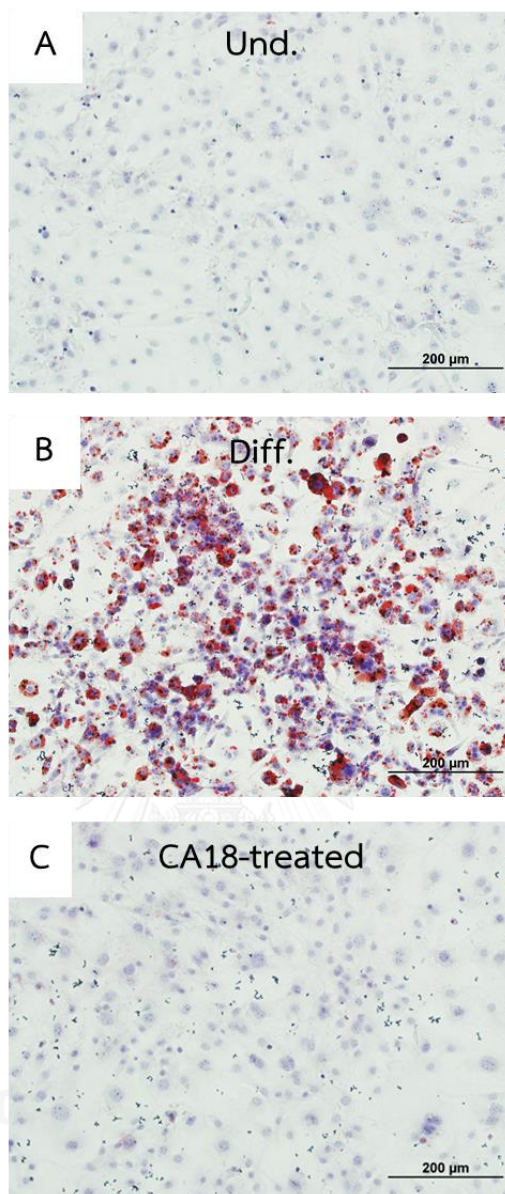
Figure 21 Timing of anti-adipocyte differentiation activity of crude extract from I-EHB-18. Cells were treated with crude extract (50 µg/ml) for 48 hours and then crude extract was eliminated from culture system. A: undifferentiated 3T3-L1 cells, B: differentiated 3T3-L1 cells and C: differentiated 3T3-L1 treated with CA18, respectively

CHAPTER V

DISCUSSION

In this study, *Streptomyces* spp. were isolated from two types of bees, Eastern honey bee (*A. cerana*) and stingless bee (*T. laeviceps*) on the selective medium Humic-Acid Vitamin agar containing soil humic acid as the source of carbon and nitrogen for isolation of actinomycetes (Hayakawa and Nonomura, 1987). The total of 125 isolates of *Streptomyces* were obtained from both external and internal samples of bees. The number of *Streptomyces* found was comparable to Poulsen *et al*. (2011) that isolated more than 200 *Streptomyces* from two solitary wasp species, black and yellow mud daubers (*S. caementarium*) and blue mud daubers (*C. californicum*) (Poulsen *et al*., 2011). Therefore, genus *Streptomyces* are apparently mutualists, found in the food stores, hive materials, or cuticle of Hymenoptera including solitary and social insects (Anderson *et al*., 2013).

The primary screening of antimicrobial activity resulted in five isolates, I-SB-38, I-SB-71, I-EHB-05, I-EHB-06 and I-EHB-18 which showed antagonistic activity against all tested microorganisms; *S. aureus*, *B. subtilis*, *E. coli* and *C. albicans*. The result indicated these isolates could secreted at least one broad spectrum secondary metabolite possessing antibacterial or antifungal activity. The role of *Streptomyces* in symbiotic association with bees may be important for protection of the insect host or its nutritional resources against pathogen attack by producing beneficial bioactive compounds (Kaltenpoth, 2009). However, five isolates were likely to produce interesting secondary metabolites and these isolates were selected for further characterization.

The 16S rDNA sequences of I-SB-38 and I-SB-71 were closely related to *S. bikiniensis* strain 13661V (EU741193.1). *S. bikiniensis* was reported to produce melanin (Vasanthabharathi *et al*., 2011) correlating with our result that the dark-brown pigment was observed from isolate I-SB-38 and I-SB-71. However, 16S rDNA sequence of I-SB-38 isolate show 92.5% similarity to I-SB-71 isolate 16S rDNA sequence by pairwise alignment and the morphologically of I-SB-38 isolate also differ from I-SB-71 isolate. Ahmad *et al*. (2015) reported that *S. bikiniensis* strain Ess_amA-1 isolated from insect *Tapinoma simrothi* can produce bio stabilized selenium nanorods possessing of anticancer activity (Ahmad *et al*., 2015). Furthermore, I-EHB-05, I-EHB-06 and I-EHB-18 isolates were closely related to *S.*

andamanensis sp. nov (LC008305.1) which was recently reported as a novel species of the genus *Streptomyces* (Sripreechasak *et al.*, 2016), besides, there have been no reported bioactive compounds produced by this species. Although, I-EHB-05, I-EHB-06 and I-EHB-18 seem to be closely related but their colonies appearances were slightly different. Furthermore, 16S rDNA sequence of I-EHB-05 isolate also show 99.0% and 97.7% similarity to I-EHB-06 and I-EHB-18 isolate, respectively, while I-EHB-18 show 97.5% similarity to I-EHB-06 16S rDNA sequence by pairwise alignment. I-EHB-18 isolate produced brownish-grey aerial mycelia similar to *S. andamanensis*. Furthermore, the result also suggested that the evolution which *Streptomyces* spp. isolated from the same insect were not rooted together (Kaltenpoth *et al.*, 2014).

To recover the crude bioactive compounds, each of five isolates were cultured in C4 medium. The supernatant of these isolates was extracted with ethyl acetate and tested for antimicrobial activity using the disc diffusion assay. The antimicrobial activity of crude extract compounds lacked antifungal activity which is different from the primary screening result using cross-streak method. The loss of antifungal activity may be a result of the different composition of medium. In the further experiment for the screening of metabolites contain with antimicrobial activity should be adjust culture medium to be suitable for the metabolites production of *Streptomyces*. As the active compounds were aqueous phase, it suggested that these compounds were likely to have high polarity such as aminoglycosides (Thiele-Bruhn, 2003). The further purification and identification by liquid chromatography mass spectrometry (LC-MS) were required.

From the secondary screening of anti-adipocyte differentiation activity, we have investigated the effects of the crude extracts on cell viability of 3T3-L1 cells which commonly used as preadipocytes in studies of adipocyte differentiation. These crude extracts did not affect cell viability of 3T3-L1cell and the IC_{50} of these crude extracts are more than 100 µg/ml. To screen for anti-adipocyte differentiation activity, the 3T3-L1 cell line were treated with crude extracts at 50 µg/ml. We found that CA18 compounds inhibit the differentiation of 3T3-L1 into mature adipocytes. However, long-duration treatment using crude solvent extracted from I-EHB-05, I-EHB-06 and I-EHB-18 isolates resulted in chronic toxicity to the cells. To determine the mechanism of action of CA18, we first investigated its effect on the expression of PPARγ. The result showed that the CA18 crude compound could inhibit the expression of the main regulator PPARγ, consequently, the differentiation process

was inhibited. Furthermore, the timing dependent experiments also strongly suggested that CA18 suppress the early signal of adipocyte differentiation. Therefore, the crude compound might affect the early regulator such as, GATA-3, KLF2 and KLF-3, which were recently reported (Brey *et al*., 2009). Berrelidin isolated from *Streptomyces sp.* TK08330 was reported to inhibit 3T3-L1 cells differentiation by upregulation of GATA-2 and GATA-3 (Matsuo *et al*., 2015b) and cineromycin B isolated from *S. cinerochromogenes* have been reported to inhibit differentiation in 3T3-L1 cells mainly via upregulation of KLF2 and KLF3 (Matsuo *et al*., 2015a). However, the mechanism of action of CA18 needs for the investigation.

The further studied of the early regulator expression of adipocyte differentiation such as C/EBP-β, C/EBP-δ, GATA-2, GATA-3, KLF2, KLF3, KLF7 is required to better understand how the mechanism of action of this compound on differentiation. However, in order to eliminate factors from the impurities with in the crude compound, the recovery of the pure compound would be the first priority to achieve before proceeding any further experiment. This is the first report of bioactive compound possessing anti-adipocyte differentiation activity produced by *Streptomyces* associated Eastern honey bees and stingless bees. Hence, the results may lead to obtain a new compound for developing therapeutic drugs in the future.

CHAPTER VI

CONCLUSION

The total of 125 isolates of *Streptomyces* were isolated from Eastern honey bees (*A. cerana*) and stingless bees (*T. laeviceps*). We found that I-SB-38, I-SB-71, I-EHB-05, I-EHB-06 and I-EHB-18 potentially produce the metabolites that have antimicrobial activity against all tested species of Gram positive bacteria, Gram negative bacteria and yeast. Moreover, the crude compound extracted from I-EHB-18 isolate inhibit adipocyte differentiation of 3T3-L1 cells at the early stage of differentiation. Hence, metabolites produced by *Streptomyces* spp. from Eastern honey bee and stingless bee from the novel sources may lead to new therapeutic agents for the future.

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APPENDIX

APPENDIX A

Mediums for Microorganism Culture

1. Humic acid-Vitamin agar (Masayuki Hayakawa, 1987)

Humic acid (dissolved with HCl overnight)	1 g
K_2HPO_4	0.5 g
Agar	15 g
Cyclohexamide	50 mg
Vitamin B	
- Thiamine-HCl	0.5 mg
- Riboflavin	0.5 mg
- Niacin (nicotinic acid)	0.5 mg
- Pyridoxine-HCl	0.5 mg
- Inositol (myo-Inositol)	0.5 mg
- Ca-pantothenate (D-Pantothenic acid)	0.5 mg
- P-aminobenzoic acid (4-aminobenzoic acid)	0.5 mg
- Biotin	0.25 mg
Deionized water	1 l

Adjust pH to 7.4 and autoclave for 15 minutes at 15 psi pressure 121 °C

2. Starch-Yeast medium

Soluble starch	10 g
Yeast extract	1 g
N-Z amine	1 g
Agar	15 g
Deionized water	1 l

3. Nutrient Agar (NA)

Peptone	5 g
Beef extract	3 g
Agar	15 g
Distilled water	1 l

4. Nutrient broth (NB)

Peptone	5 g
Beef extract	3 g
Deionized water	1 l

5. C4 Medium

Starch	10 g
Yeast extract	4 g
Soybean meal	25 g
NaCl	2 g
Glucose	20 g
20 % Beef extract	1 µl
5 % K_2HPO_4	0.2 µl
Deionized water	1 l

APPENDIX B

Medium for cell culture

1. Culture medium

Calf serum	10 ml
HEPES buffer	1 ml
Penicillin Streptomycin mixtures (Pen-Strep)	1 ml
Sodium Pyruvate	1 ml
DMEM	100 ml

2. Predifferentiation medium

Fetal bovine serum (FBS)	10 ml
HEPES buffer	1 ml
Penicillin Streptomycin mixtures (Pen-Strep)	1 ml
Sodium Pyruvate	1 ml
DMEM	100 ml

3. Differentiation medium

Predifferentiation medium	1200 µl
1 mM Dexamethasone	1.2 µl
0.5 M IBMX	1.2 µl
1 mg/ml insulin	1.2 µl

4. Maintenance medium

Predifferentiation medium	600 µl
1 mg/ml insulin	0.6 µl

APPENDIX C

Buffers and reagents

1. 10x Phosphate buffer saline (PBS)

NaCl	80 g
KCl	2 g
$NaHPO_4$	14.4 g
KH_2PO_4	2.4 g
Deionized water	100 ml
Adjust pH7.6	

2. 50x TAE Buffer

Tris-base	242 g
Acetic acid	57.1 g
5M EDTA solution (pH 8)	100 ml
Deionized water	100 ml

3. 1.5% Agarose Gel

Agarose	0.6 g
1x TAE Buffer	40 ml

4. PCR Master-mix for one reaction

Emerald Amp® GT PCR Master Mix	12.5 µl
10µM 27F primer	0.5 µl
10µM 1492R primer	0.5 µl
Molecular Biology Grade Water	9*/11.5** µl
DNA Template	2.5* µl

*For using DNA Template

** For colony PCR

5. 10% Formalin

Formalin (37%, Merck)	27 ml
10x PBS	10 ml
Deionized water	63 ml

6. 60% Isopropanol

    Isopropanol (100%, Merck)   6 ml

    Deionized water   4 ml

7. Oil Red O Stock

    Oil Red O (Sigma, USA)   0.35 g

    100% Isopropanol   100 ml

    Mix well and filter with 0.25 µm filter and store at room temperature

18. Oil Red O Working Solution

    Oil Red O stock   6 ml

    Deionized water   4 ml

    Mix well and let sit at room temperature for 20 minutes followed by filtering (0.25 µm)

9. Hematoxylin Solution

    Hematoxylin stock   6 ml

    Deionized water   4 ml

10. 10% Separating Gel

    Water   3.836 ml

    40% Acrylamide/Bis   2 ml

    1.5 M Tris-HCL pH 8.8   2 ml

    10% SDS   0.08 ml

    10% APS   0.08 ml

    TEMED   0.004 ml

11. 5% Stacking Gel

    Water   1.204 ml

    40% Acrylamide/Bis   0.25 ml

    1.5 M Tris-HCL pH 8.8   0.504 ml

    10% SDS   0.02 ml

    10% APS   0.02 ml

    TEMED   0.002 ml

12. Buffer A

10 mM DTT	1 ml
0.5 M Tris-Cl pH 7.2	1 ml
1.4 M KCl	1 ml
Deionized water	5 ml

Mix well and add 1 tablet of Protease inhibitor, mix well and store at 4 ℃

13. Buffer B

Buffer A	990 µl
Nonidet p-40	10 µl

Mix well and store at 4 ℃

14. 2x loading dye

100 mM Tris-HCl pH 6.8	1 ml
10% SDS	4 ml
Glycerol	2.02 ml
Deionized water	1.98 ml
β-mercaptoethanol	100 µl

15. 5x Running Buffer

Glycine	94 g
Trisma Base	15.1 g
SDS	5 g
Deionized water	1 l

16. Transfer Buffer

Glycine	2.9 g
Trisma Base	5.08 g
SDS	0.37 g
Deionized water	800 ml
Absolute MetOH (100%)	200 ml

17. PBS-T

10 x PBS	100 ml
Deionized water	900 ml

Tween 20	500 µl

18. Blocking Solution

Skim milk powder	6 g
PBS-T	200 ml

19. Solution A

1 mM Tris pH 8.5 (4°C)	2.5 ml
Coumaric acid	11 µl
Luminol	25 µl

20. Solution B

1 mM Tris pH 8.5 (4°C)	2.5 ml
H_2O_2	1.5 µl

21. 5 mg/ml MTT solution

MTT	50 mg
Sterile 1x PBS	10 ml

The solution was sterilized by using 0.45 µM filter and kept in aliquots at 4°C
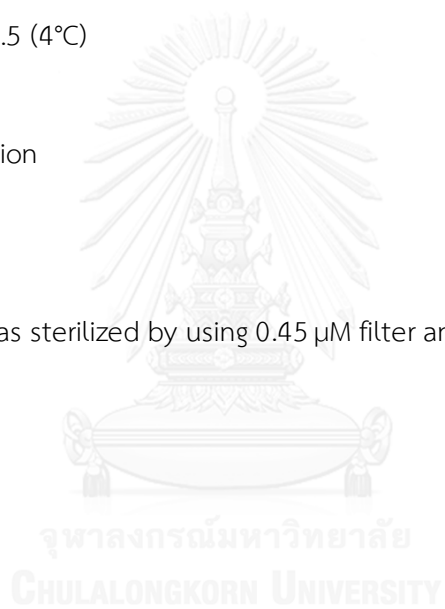
APPENDIX D

Supplements

1. Screening for antimicrobial activity

Table 1.1 Antimicrobial activities of putative *Streptomyces* isolated from Eastern honey bees and stingless bees

Isolates	Inhibition level			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
I-SB-01	+	+	-	+
I-SB-02	-	-	-	-
I-SB-03	-	-	-	+
I-SB-04	-	-	-	+
I-SB-05	-	-	-	+
I-SB-06	-	+	-	-
I-SB-07	-	-	-	-
I-SB-08	-	-	-	-
I-SB-09	-	+	-	-
I-SB-10	-	-	-	-
I-SB-11	-	+	-	-
I-SB-12	-	+	-	-
I-SB-13	-	+	-	-
I-SB-14	-	+	-	-
I-SB-15	-	+	-	-
I-SB-16	-	+	-	-
I-SB-18	-	+	-	-
I-SB-19	-	+	-	-
I-SB-20	-	-	-	-
I-SB-21	+	-	-	-
I-SB-22	-	+	-	-
I-SB-23	-	-	-	-

Table 1.1 Antimicrobial activities of putative *Streptomyces* isolated from Eastern honey bees and stingless bees (cont.)

Isolates	Inhibition level			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
I-SB-24	-	-	-	+
I-SB-25	-	-	-	-
I-SB-26	-	+	-	-
I-SB-27	-	+	-	-
I-SB-28	-	-	-	-
I-SB-29	-	-	-	-
I-SB-30	-	-	-	-
I-SB-31	-	-	-	-
I-SB-32	-	-	-	-
I-SB-33	-	-	-	-
I-SB-34	-	+	-	-
I-SB-36	-	-	-	+
I-SB-37	-	-	-	-
I-SB-38	+	+	+	+
I-SB-39			-	-
I-SB-40	+	+	-	-
I-SB-44	-	-	-	-
I-SB-45	-	-	-	-
I-SB-46	-	-	-	-
I-SB-47	-	-	-	-
I-SB-48	-	-	-	-
I-SB-49	-	-	-	-
I-SB-50	+	-	-	-
I-SB-51	-	-	-	-
I-SB-52	+	+	-	+

Table 1.1 Antimicrobial activities of putative *Streptomyces* isolated from Eastern honey bees and stingless bees (cont.)

Isolates	Inhibition level			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
I-SB-53	+	+	-	-
I-SB-54	-	-	-	-
I-SB-55	-	-	-	-
I-SB-56	-	-	-	-
I-SB-57	-	-	-	-
I-SB-61	+	-	-	-
I-SB-62	+	+	-	-
I-SB-63	+	+	-	-
I-SB-64	+	+	-	-
I-SB-67	-	-	-	-
I-SB-69	-	-	-	-
I-SB-70	-	-	-	-
I-SB-71	+	+	+	+
I-SB-72	-	-	-	-
I-SB-73	-	-	-	-
I-SB-74	+	-	-	-
I-SB-75	+	-	-	-
I-SB-77	+	+	-	-
I-SB-78	+	+	-	-
I-SB-79	-	-	-	-
I-SB-83	+	+	-	-
I-SB-85	-	-	-	-
I-SB-86	-	-	-	-
E-SB-01	-	+	-	-
E-SB-02	-	-	-	-

Table 1.1 Antimicrobial activities of putative *Streptomyces* isolated from Eastern honey bees and stingless bees (cont.)

Isolates	Inhibition level			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
E-SB-03	-	-	-	+
E-SB-05	-	-	-	+
E-SB-06	-	+	-	-
E-EHB-01	+	+	+	-
E-EHB-02	+	+	-	+
E-EHB-03	+	-	-	-
E-EHB-04	-	+	-	-
E-EHB-05	-	+	-	-
E-EHB-06	+	+	-	+
E-EHB-07	+	-	-	-
E-EHB-08	-	-	-	-
E-EHB-09	+	-	-	-
E-EHB-10	-	-	-	-
E-EHB-11	-	-	-	-
E-EHB-12	+	+	-	+
I-EHB-01	-	-	-	-
I-EHB-02	-	-	-	-
I-EHB-03	+	-	-	+
I-EHB-04	-	-	-	-
I-EHB-05	+	+	+	+
I-EHB-06	+	+	+	+
I-EHB-07	+	+	+	+
I-EHB-08	-	-	-	-
I-EHB-09	-	-	-	-
I-EHB-10	+	+	+	+
I-EHB-11	-	-	-	-

Table 1.1 Antimicrobial activities of putative *Streptomyces* isolated from Eastern honey bees and stingless bees (cont.)

Isolates	Inhibition level			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
I-EHB-12	+	+	+	+
I-EHB-13	+	-	-	-
I-EHB-14	-	-	-	-
I-EHB-15	-	+	-	-
I-EHB-16	-	-	-	-
I-EHB-17	-	-	-	+
I-EHB-18	+	+	+	+
I-EHB-19	+	+	+	+
I-EHB-20	+	+	-	-
I-EHB-21	-	-	-	-
I-EHB-22	-	+	-	-
I-EHB-23	-	+	-	-
I-EHB-24	-	-	-	-
I-EHB-25	-	-	-	-
I-EHB-26	-	+	-	-
I-EHB-27	+	+	+	+
I-EHB-28	-	-	-	-
I-EHB-29	-	-	-	-
I-EHB-30	-	+	-	-
I-EHB-31	-	-	-	-
I-EHB-32	-	-	-	-
I-EHB-32	-	-	-	-
I-EHB-33	+	+	-	-
I-EHB-34	+	+	-	-
I-EHB-35	-	-	-	-

Table 1.1 Antimicrobial activities of putative *Streptomyces* isolated from Eastern honey bees and stingless bees (cont.)

Isolates	Inhibition level			
	S. aureus	*B. subtilis*	*E. coli*	*C. albicans*
I-EHB-36	-	-	-	-
I-EHB-37	-	-	-	-

The inhibition levels were interpreted by author's criteria as;

+   exhibit inhibition zone, -   no inhibition zone
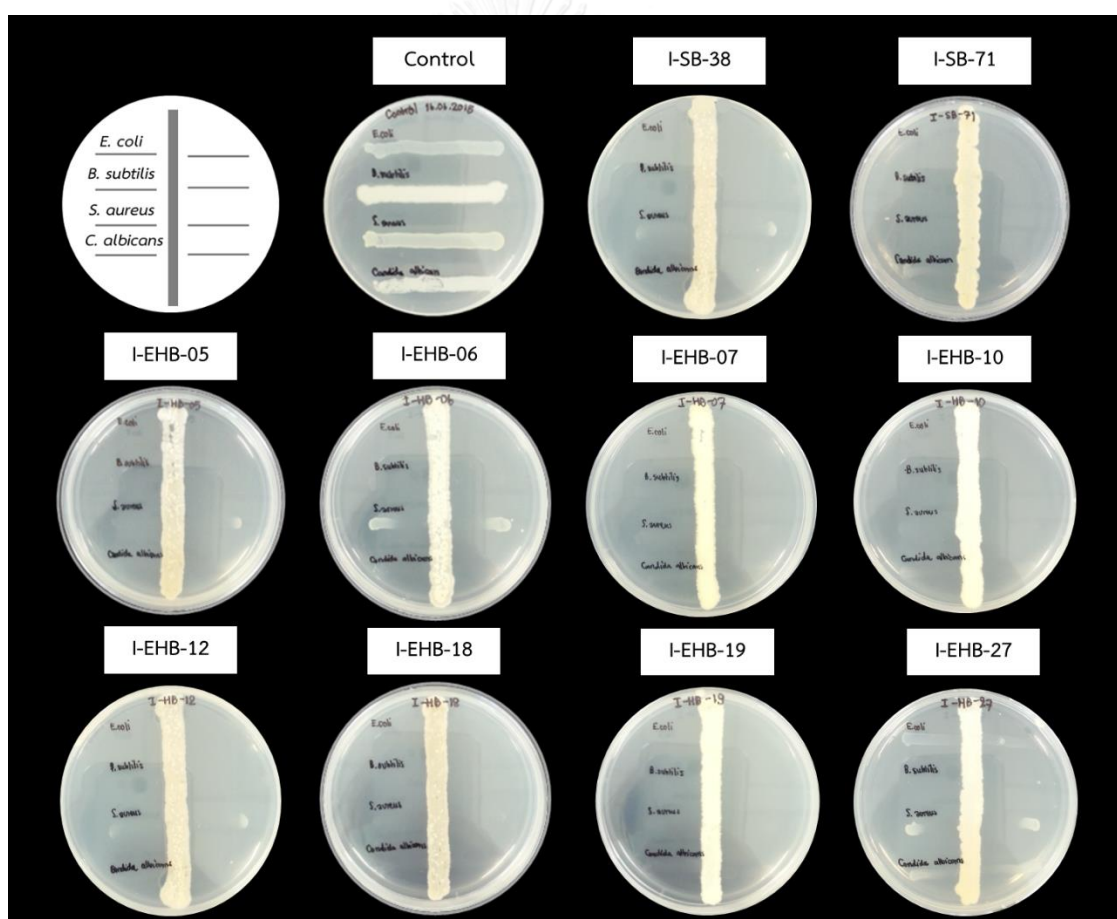
Figure 1.1 Representative of antimicrobial activity of *Streptomyces* spp. using modified cross-streak method

2. Molecular identification of *Streptomyces*

2.1 The 16S rRNA sequences of *Streptomyces* isolates

2.1.1 I-SB-38

```
1     GTTTGATCATGGCTCAGGACGAACGCTGGGCGGCGTGCTTAACACATGCA
101   TGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAGCCCTGGAAA
201   GCTCCGGCGGTGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGT
301   CACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGG
401   ATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGT
501   TAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTC
601   CTGCATCCGATACGGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCC
701   GCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGGAGC
801   TAGGGTGTTGGCGACATTCCACGTNGTCGGTGCCGCAGCTAACGCATTTA
901   ACGGGGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGC
1001  CCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTG
1101  TTGCCAGCATGCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGG
1201  CTTGGGCTGCACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATGCCG
1301  CAACTCGACCCCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTG
1401  AAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGC
```

2.1.1 I-SB-71

```
1     GTGCAATGGGGCTCTACCATGCAGTCGAACGATGAAGCCCTTCGGGGTGG
101   GGACAAGCCCTGGAAACGGGGTCTAATACCGGATACGACTGCGGGAGGCA
201   AGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGCCGGCT
301   GGCCCAAAAATTCCTAAAGGGAAGGCCAACAAGTGGGGAAATATTGAGAC
401   ATGAAAGGGCCCATTCGGAGTTGTAAAACCTTCATTTCAGCCAGGGAAAG
501   TACGTGCCCAGCAGCCAGCGGTAATTAAGTAGGGAGGCAAGCGTTGTTCC
601   TGTGAAAGCCCCGGGGCTTAAACCCCGGGTCTGCATCCGATACGGGCAGG
701   CAGATATCAGGAGGAACACCGGTGGCGAAGGCGGATCTCTGGGCCATTAC
801   TAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTC
901   CAAGGCTAAAACTCAAAGGAATTGACGGGGGCCCGCACAAGCAGCGGAAG
1001  ACATATACCGGGAAAGCATCAGAGATTAGGTGACCCCCTTGTGGTATGTG
1101  GGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCATGC
1201  AAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGCAC
1301  AATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCC
1401  CGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTAAC
```

2.1.3 I-EHB-05

```
1      AGTAGAGTTTGATCATGACTCAGGACGAACGCTGGCGGCGTGCTTAACAC
101    GAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAAC
201    CTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTA
301    ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG
401    GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGA
501    ATACGTAGGGCGCAAGCGTTGTCTCGGAATTATTGGGCGTAAAGAGCTCG
601    TGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCT
701    CGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGA
801    TGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCC
901    GCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAA
1001   TTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGA
1101   GCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACTC
1201   TGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGTG
1301   ACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGGT
1401   GTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTTGAAGG
```

2.1.4 I-EHB-06

```
1      TGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATG
101    TAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAACGGG
201    CGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTG
301    CTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAAT
401    GGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGACGG
501    ACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
601    AGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCCTGGT
701    ATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAAA
801    GTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGTGCCC
901    GCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAATCGCGAAGA
1001   CTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTG
1101   AGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAACT
1201   CTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAGGT
1301   GACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGCGG
1401   GGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGAAGG
```

2.1.5 I-EHB-18

```
1     AGTTTGATCATGGCGACGAACGCTGGGCGGCGTGCTTAACACATGCAAGT
101   CGTGGGCAATCTGCCCTGCACTCTGGGACAAAGCCCTGGAAACGGGGTCT
201   GGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAGTGGCTC
301   GACTGAGACACGACCCAGACTCCTACGGCAGGCAGCAGTGGGGAATATT
401   TGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTG
501   GGTAATTACGTAGGGCGCAAGCGTTGTCCGGAAATATTGGGCGTAAGAGC
601   GTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATT
701   AGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAGCGTGGGGAG
801   AGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAGT
901   GGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAACGCGAA
1001  CCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCG
1101  CCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGTCAA
1201  GGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGTGAG
1301  TCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGCTGC
1401  TCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTTCG
```

2.2 Pairwise alignment of *Streptomyces* isolates

    2.2.1 I-SB-38 and I-SB-71

```
I-SB-38      1 --------GTTTGATCATGGCTCAGGAC------GAACGCTGGGCGGCGT       36
               .|||     |.|..||.||.|       ||||.|.|.|.||||.
I-SB-71      1 TCAACTTAATTT----AAGAGTCCGGCCCTTCCGGAACCCAGTGGGGCGG       46

I-SB-38     37 GC-TTAACACATGCAAGTCAGAAACGATAGAAGCCCCTTCGGGGGTGGA-       84
               || |||.|| ||||||.||| ..||||||| |||..||||||| ||||||||
I-SB-71     47 GCATTATCA-ATGCATGTC-TTAACGAT-GAACGCCCTTC-GGGGTGGAT      92

I-SB-38     85 TTAGTGGCGAACGGGGTGAGTAACACGTGGGCAATCTGCCC---TTCACT      131
               ||||.|||||||| |.||||||||.|||.||||||||||..||   |.||||
I-SB-71     93 TTAGAGGCGAAC-GAGTGAGTAAAACGAGGGCAATCTAACCCATTACACT      141

I-SB-38    132 CTGGG-ACAAGCCCTGG-AAACGGGGTCTAATACCGGAT-ACGACT-GCG      177
               ||||| ||||.|||.|| |||||||||||||||||||| |.|||| .||
I-SB-71    142 CTGGGTACAACCCCGGGAAAACGGGGTCTAATACCGGATAATGACTACCG      191

I-SB-38    178 GGAGGCATCT--CCTGTGGTGGAAAGCT-CCGGCGGTGAA--GGATGAG-      221
               ||||||||||  |||||.||||||||||| ||||||||||||   |||.|||
I-SB-71    192 GGAGGCATCTTCCCTGGGGTGGAAAGCTCCCGGCGGTGAAAGGGAAGAGC      241

I-SB-38    222 CCCGCGGCCTA-TCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACG      270
               ||||||||||| |||||||||||.||||||||||||||||||||||||||||
I-SB-71    242 CCCGCGGCCTATTCAGCTTGTTGGGGGGGTAATGGCCTACCAAGGCGACG      291

I-SB-38    271 ACGGGTAGCCGGCCTGAGA--GGGCGACCGGCCACAC--TGGGAC-TGAG      315
               |||||||||||||||||||| |.||||||||||||||  |||||| ||||
I-SB-71    292 ACGGGTAGCCGGCCTGAGAAGGAGCGACCGGCCACACTTTGGGACTTGAG      341

I-SB-38    316 ACACGG-CCCAGACTCC-TACGGGAGGCAGCAGTGGGGAATATTGCACAA      363
               ||||||| |||||||.||| ||||||||||||||||||||||||||||||||
I-SB-71    342 ACACGGTCCCAGAATCCTTACGGGAGGCAGCAGTGGGGAATATTGCACAA      391

I-SB-38    364 TGGGCAAAAGCCTGATGCAGTCGACGCCGCGTGAGGGATGACGGCCTTCG      413
               ||||||.||||||||||||||||..|||||||||||.|||.||||||||||
I-SB-71    392 TGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAAGACGGCCTTCG      441

I-SB-38    414 GGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCA      463
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    442 GGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCA      491

I-SB-38    464 GAAGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGC      513
               |||||||||.||||||||||||||||||||||||||||||||||||||||
I-SB-71    492 GAAGAAGCCCCGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGGC      541

I-SB-38    514 GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGT      563
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    542 GCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAGGCGGCTTGT      591

I-SB-38    564 CACGTCGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATAC      613
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    592 CACGTCGGGTGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATCCGATAC      641

I-SB-38    614 GGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTG      663
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    642 GGGCAGGCTAGAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTG      691

I-SB-38    664 AAATGCGCAGATATCA-GGAGGAACACCGGTGGCGAAGGCGGATCTCTGG      712
               |||||||||||||||| |||||||||||||||||||||||||||||||||
I-SB-71    692 AAATGCGCAGATATCAGGGGAGGAACACCGGTGGCGAAGGCGGATCTCTGG      741
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I-SB-38    713 GCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGGAGCGAACAGGATTAT    762
               |||||||||||||||||||||||||||||| |||||||||||||||||||
I-SB-71    742 GCCATTACTGACGCTGAGGAGCGAAAGCGT-GGGGAGCGAACAGGATTA-    789

I-SB-38    763 GATACCCT-GGTAGTCCACCCCCGTAATACGTTGGGAACTAGGGT-GTTG    810
               ||||||||| |||||||||| |·||||||| |||||·|||||||||·||| ||||
I-SB-71    790 GATACCCTGGGTAGTCCA-CGCCGTAA-ACGTGGGGAACTAAGGTGGTTG    837

I-SB-38    811 GCGACATTCCACGTNGTCGGTGCCGCAGCTAACGCATTTAAGTTCCCCGC    860
               |||||||||||||| |||||·||||||||||||||| |||||||||||||
I-SB-71    838 GCGACATTCCACGTCGTCGGGGCCGCAGCTAACGCA-TTAAGTTCCCCGC    886

I-SB-38    861 CTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC    910
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    887 CTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGGGGGCCC    936

I-SB-38    911 GCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTT    960
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    937 GCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTT    986

I-SB-38    961 ACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGT   1010
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71    987 ACCAAGGCTTGACATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGT   1036

I-SB-38   1011 GGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG   1060
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1037 GGTCGGTATACAGGTGGTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATG   1086

I-SB-38   1061 TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCAT   1110
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1087 TTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCTGTGTTGCCAGCAT   1136

I-SB-38   1111 GCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGA   1160
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1137 GCCCTTCGGGGTGATGGGGACTCACAGGAGACCGCCGGGGTCAACTCGGA   1186

I-SB-38   1161 GGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGC   1210
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1187 GGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTCTTGGGCTGC   1236

I-SB-38   1211 ACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATGCCGCGAGGCGGAG   1260
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1237 ACACGTGCTACAATGGCCGGTACAAAGAGCTGCGATGCCGCGAGGCGGAG   1286

I-SB-38   1261 CGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACC   1310
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1287 CGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACC   1336

I-SB-38   1311 CCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAA   1360
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1337 CCATGAAGTCGGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAA   1386

I-SB-38   1361 TACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTA   1410
               ||||||||||||||||||||||||||||||||||||||||||||||||||
I-SB-71   1387 TACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGAAAGTCGGTA   1436

I-SB-38   1411 ACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTT-GAAGGGG   1459
               |||||||||||||||||||||||||||||||||||||||||| |||||||
I-SB-71   1437 ACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTTCGAAGGGG   1486

I-SB-38   1460 --------------    1459

I-SB-71   1487 ACGGGGGGGGGCCC    1500
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2.2.2 I-EHB-05 and I-EHB-06

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I-EHB-05      1 AGTAGAGTTTGATCATGACTCAGGACGAACGCTGGCGGCGTGCTTAACAC      50
                .||||||||||||||.||||||||||||||||||||||||||||||||||
I-EHB-06      1 ---TGAGTTTGATCATGGCTCAGGACGAACGCTGGCGGCGTGCTTAACAC      47

I-EHB-05     51 ATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGT     100
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06     48 ATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGT      97

I-EHB-05    101 GAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAAC     150
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06     98 GAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAGCCCTGGAAAC     147

I-EHB-05    151 GGGGTCTAATACCGGATACGAGTCTCCAAGGCATCTTGGAGACTGTAAAG     200
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    148 GGGGTCTAATACCGGATACGAGTCTCCAAGGCATCTTGGAGACTGTAAAG     197

I-EHB-05    201 CTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTA     250
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    198 CTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTA     247

I-EHB-05    251 GTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC     300
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    248 GTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCC     297

I-EHB-05    301 ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG     350
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    298 ACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG     347

I-EHB-05    351 AATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT     400
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    348 AATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGAT     397

I-EHB-05    401 GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGA     450
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    398 GACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCGAGAGTGA     447

I-EHB-05    451 CGGTACCTGCAGAA-GAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT     499
                ||||||||||||| |||||||||||||||||||||||||||||||||||||
I-EHB-06    448 CGGTACCTGCAGAATGAAGCGCCGGCTAACTACGTGCCAGCAGCCGCGGT     497

I-EHB-05    500 AATACGTAGGGCGCAAGCGTTGTCTCGGAATTATTGGGCGTAAAGAGCTC     549
                |||||||||||||||||||||||| |||||||||||||||||||||||||
I-EHB-06    498 AATACGTAGGGCGCAAGCGTTGTC-CGGAATTATTGGGCGTAAAGAGCTC     546

I-EHB-05    550 GTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGT     599
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    547 GTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTTAACCCCGGGT     596

I-EHB-05    600 CTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCC     649
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    597 CTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGATCGGAATTCC     646

I-EHB-05    650 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG     699
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    647 TGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCGGTGGCGAAG     696

I-EHB-05    700 GCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG-AAAGCGTGGGGAGC     748
                |||||||||||||||||||||||||||||||||||| ||||||||||||||
I-EHB-06    697 GCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAAGCGTGGGGAGC     746
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I-EHB-05    700 GCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG-AAAGCGTGGGGAGC    748
                |||||||||||||||||||||||||||||||||| |||||||||||||||
I-EHB-06    697 GCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAAGCGTGGGGAGC    746

I-EHB-05    749 G-AACAGGGATTAGAT-ACCCTGGTAGTCCACGCCGTAAACGGTGGGCAC    796
                | ||||| |||||||| |||||||||||||||||||||||||||||||||
I-EHB-06    747 GAAACA-GGATTAGATAACCCTGGTAGTCCACGCCGTAAACGGTGGGCAC    795

I-EHB-05    797 TAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAG    846
                |||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    796 TAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTAACGCATTAAG    845

I-EHB-05    847 TGCCCCGCCTGGGGAGTACGGCCGC-AAGGCTAAAACTCAAAGGAATTGA    895
                ||||||||||||||||||||||||| ||||||||||||||||||||||||
I-EHB-06    846 TGCCCCGCCTGGGGAGTACGGCCGCAAAGGCTAAAACTCAAAGGAATTGA    895

I-EHB-05    896 CGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAA-CGC    944
                |||||||||||||||||||||||||||||||||||||||||||||| |||
I-EHB-06    896 CGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCGACGCAATCGC    945

I-EHB-05    945 GAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCTGGAGACAGGG    994
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    946 GAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCTGGAGACAGGG    995

I-EHB-05    995 TCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTG   1044
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    996 TCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGTCAGCTCGTG   1045

I-EHB-05   1045 TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTG   1094
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1046 TCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTCCCGTG   1095

I-EHB-05   1095 TTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGT   1144
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1096 TTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGACCGCCGGGGT   1145

I-EHB-05   1145 CAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTC   1194
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1146 CAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCCCCTTATGTC   1195

I-EHB-05   1195 TTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGT   1244
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1196 TTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTGCGATACCGT   1245

I-EHB-05   1245 GAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGC   1294
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1246 GAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGC   1295

I-EHB-05   1295 AACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGC   1344
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1296 AACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGATCAGCATTGC   1345

I-EHB-05   1345 TGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGA   1394
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1346 TGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACGTCACGA   1395

I-EHB-05   1395 AAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCT   1444
                |||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1396 AAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGC-   1444

I-EHB-05   1445 TGAAGGG   1451
                ||||||.
I-EHB-06   1445 TGAAGGC   1451
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2.2.3 I-EHB-05 and I-EHB-18

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I-EHB-05      1 AGTAGAGTTTGATCATGACTCAGGACGAACGCT-GGCGGCGTGCTTAACA      49
                |||||||||||||·|     |||||||||| ||||||||||||||||||
I-EHB-18      1 -----AGTTTGATCATGGC----GACGAACGCTGGGCGGCGTGCTTAACA      41

I-EHB-05     50 CATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGG      99
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18     42 CATGCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGG      91

I-EHB-05    100 TGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGAC-AAGCCCTGGAA     148
                ||||||||||||||||||||||||||||||||||||| ||||||||||||
I-EHB-18     92 TGAGTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAAGCCCTGGAA     141

I-EHB-05    149 ACGGGGTCTAATACCGGATACGAGTCTCCAAGGCATCTTGGAGACTGTAA     198
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    142 ACGGGGTCTAATACCGGATACGAGTCTCCAAGGCATCTTGGAGACTGTAA     191

I-EHB-05    199 AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGG     248
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    192 AGCTCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGG     241

I-EHB-05    249 TAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGG     298
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    242 TAGTGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGG     291

I-EHB-05    299 CCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGG     348
                ||||||||||||||||||||||||·||||||||||||||·||||||||||
I-EHB-18    292 CCACACTGGGACTGAGACACGACCCAGACTCCTACGGCAGGCAGCAGTGG     341

I-EHB-05    349 GG-AATATT-------GCACAATGGGCGAAAGCCTGATGCAGCGACGCCG     390
                || ||||||      ·|||||||||||||||||||||||||||||||||
I-EHB-18    342 GGAAATATTTTTTTTGACACAATGGGCGAAAGCCTGATGCAGCGACGCCG     391

I-EHB-05    391 CGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAA     440
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    392 CGTGAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAA     441

I-EHB-05    441 GCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAAC-TACGTGCC-A     488
                |||||||||||||||||||||||||||||||||||||| ||||||| |
I-EHB-18    442 GCGAGAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACNTACGTGCCNA     491

I-EHB-05    489 GC-AGCCGCGGTAA-TACGTAGGGCGCAAGCGTTGTCTCGGAATTATTGG     536
                || ||||||||||| |||||||||||||||||||||| |||||·||||||
I-EHB-18    492 GCNAGCCGCGGTAATTACGTAGGGCGCAAGCGTTGTC-CGGAAATATTGG     540

I-EHB-05    537 GCGTAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGG     586
                ||||| ||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    541 GCGT-AAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGG     589

I-EHB-05    587 CTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGG     636
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    590 CTTAACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGG     639

I-EHB-05    637 AGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC     686
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    640 AGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAAC     689

I-EHB-05    687 ACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAA     736
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    690 ACCGGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAA     739
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I-EHB-05    737 AGCGTGGGGAGCGAACAGGGATTAGATACCCTGGTAGTCCACGCCGT-AA    785
                |||||||||||||||| ||||||||||||||||||||||||||||| ||
I-EHB-18    740 AGCGTGGGGAGCGAACA-GGATTAGATACCCTGGTAGTCCACGCCGTAAA    788

I-EHB-05    786 ACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCT    835
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    789 ACGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCT    838

I-EHB-05    836 AACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCA    885
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    839 AACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCA    888

I-EHB-05    886 AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCG    935
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    889 AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCG    938

I-EHB-05    936 ACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCTG    985
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    939 ACGCAACGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCTG    988

I-EHB-05    986 GAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGT   1035
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18    989 GAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCGT   1038

I-EHB-05   1036 CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT   1085
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1039 CAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCT   1088

I-EHB-05   1086 TGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGAC   1135
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1089 TGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGAC   1138

I-EHB-05   1136 CGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCC   1185
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1139 CGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGCC   1188

I-EHB-05   1186 CCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTG   1235
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1189 CCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCTG   1238

I-EHB-05   1236 CGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATT   1285
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1239 CGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATT   1288

I-EHB-05   1286 GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT   1335
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1289 GGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGAT   1338

I-EHB-05   1336 CAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC   1385
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1339 CAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTC   1388

I-EHB-05   1386 ACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGG   1435
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-18   1389 ACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGG   1438

I-EHB-05   1436 GAGGGAGCTT-GAAGGG-    1451
                |||||||||| ||||||
I-EHB-18   1439 GAGGGAGCTTCGAAGGGA    1456
```

2.2.4 I-EHB-06 and I-EHB-18

```
I-EHB-18      1 --AGTTTGATCATGGC----GACGAACGCTGGGCGGCGTGCTTAACACAT       44
                ||||||||||||||    |||||||||| |||||||||||||||||||||
I-EHB-06      1 TGAGTTTGATCATGGCTCAGGACGAACGCT-GGCGGCGTGCTTAACACAT       49

I-EHB-18     45 GCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGA       94
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06     50 GCAAGTCGAACGATGAACCACTTCGGTGGGGATTAGTGGCGAACGGGTGA       99

I-EHB-18     95 GTAACACGTGGGCAATCTGCCCTGCACTCTGGGACAAAGCCCTGGAAACG      144
                |||||||||||||||||||||||||||||||||||| |||||||||||||
I-EHB-06    100 GTAACACGTGGGCAATCTGCCCTGCACTCTGGGAC-AAGCCCTGGAAACG      148

I-EHB-18    145 GGGTCTAATACCGGATACGAGTCTCCAAGGCATCTTGGAGACTGTAAAGC      194
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    149 GGGTCTAATACCGGATACGAGTCTCCAAGGCATCTTGGAGACTGTAAAGC      198

I-EHB-18    195 TCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAG      244
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    199 TCCGGCGGTGCAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGAGGTAG      248

I-EHB-18    245 TGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCA      294
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    249 TGGCTCACCAAGGCGACGACGGGTAGCCGGCCTGAGAGGGCGACCGGCCA      298

I-EHB-18    295 CACTGGGACTGAGACACGACCCAGACTCCTACGGCAGGCAGCAGTGGGGA      344
                |||||||||||||||||||||.||||||||||||||||.|||||||||||
I-EHB-06    299 CACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGG-      347

I-EHB-18    345 AATATTTTTTTGACACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT       394
                ||||||      .|||||||||||||||||||||||||||||||||||||
I-EHB-06    348 AATATT-------GCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGT       390

I-EHB-18    395 GAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCG      444
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    391 GAGGGATGACGGCCTTCGGGTTGTAAACCTCTTTCAGCAGGGAAGAAGCG      440

I-EHB-18    445 AGAGTGACGGTACCTGCAGAA-GAAGCGCCGGCTAACNTACGTGCCNAGC      493
                ||||||||||||||||||||| |||||||||||||| ||||||| |||
I-EHB-06    441 AGAGTGACGGTACCTGCAGAATGAAGCGCCGGCTAAC-TACGTGCC-AGC      488

I-EHB-18    494 NAGCCGCGGTAATTACGTAGGGCGCAAGCGTTGTCCGGAAATATTGGGCG      543
                ||||||||||| ||||||||||||||||||||||||||.|||||||||||
I-EHB-06    489 -AGCCGCGGTAA-TACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCG      536

I-EHB-18    544 T-AAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTT      592
                | ||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    537 TAAAGAGCTCGTAGGCGGCTTGTCACGTCGGTTGTGAAAGCCCGGGGCTT      586

I-EHB-18    593 AACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGA      642
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    587 AACCCCGGGTCTGCAGTCGATACGGGCAGGCTAGAGTTCGGTAGGGGAGA      636

I-EHB-18    643 TCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC      692
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    637 TCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACC      686

I-EHB-18    693 GGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCG-AAAG      741
                |||||||||||||||||||||||||||||||||||||||||||| ||||
I-EHB-06    687 GGTGGCGAAGGCGGATCTCTGGGCCGATACTGACGCTGAGGAGCGAAAAG      736
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I-EHB-18    742 CGTGGGGAGCG-AACAGGATTAGAT-ACCCTGGTAGTCCACGCCGTAAAA    789
                |||||||||||| |||||||||||||| ||||||||||||||||||||| |||
I-EHB-06    737 CGTGGGGAGCGAAACAGGATTAGATAACCCTGGTAGTCCACGCCGT-AAA    785

I-EHB-18    790 CGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTA    839
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    786 CGGTGGGCACTAGGTGTGGGCGACATTCCACGTCGTCCGTGCCGCAGCTA    835

I-EHB-18    840 ACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGC-AAGGCTAAAACTCA    888
                |||||||||||||||||||||||||||||||||| |||||||||||||||
I-EHB-06    836 ACGCATTAAGTGCCCCGCCTGGGGAGTACGGCCGCAAAGGCTAAAACTCA    885

I-EHB-18    889 AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCG    938
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    886 AAGGAATTGACGGGGGCCCGCACAAGCGGCGGAGCATGTGGCTTAATTCG    935

I-EHB-18    939 ACGCAA-CGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCT    987
                |||||| ||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    936 ACGCAATCGCGAAGAACCTTACCAAGGCTTGACATACACCGGAAAACTCT    985

I-EHB-18    988 GGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCG   1037
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06    986 GGAGACAGGGTCCCCCTTGTGGTCGGTGTACAGGTGGTGCATGGCTGTCG   1035

I-EHB-18   1038 TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC   1087
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1036 TCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCC   1085

I-EHB-18   1088 TTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGA   1137
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1086 TTGTCCCGTGTTGCCAGCAGGCCCTTGTGGTGCTGGGGACTCACGGGAGA   1135

I-EHB-18   1138 CCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGC   1187
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1136 CCGCCGGGGTCAACTCGGAGGAAGGTGGGGACGACGTCAAGTCATCATGC   1185

I-EHB-18   1188 CCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCT   1237
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1186 CCCTTATGTCTTGGGCTGCACACGTGCTACAATGGCCGGTACAATGAGCT   1235

I-EHB-18   1238 GCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGAT   1287
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1236 GCGATACCGTGAGGTGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGAT   1285

I-EHB-18   1288 TGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA   1337
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1286 TGGGGTCTGCAACTCGACCCCATGAAGTCGGAGTCGCTAGTAATCGCAGA   1335

I-EHB-18   1338 TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT   1387
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1336 TCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGT   1385

I-EHB-18   1388 CACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTG   1437
                ||||||||||||||||||||||||||||||||||||||||||||||||||
I-EHB-06   1386 CACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTG   1435

I-EHB-18   1438 GGAGGGAGCTTCGAAGGGA     1456
                |||||||||| |||||.
I-EHB-06   1436 GGAGGGAGCT--GAAGGC-     1451
```

Table 2.1 GenBank database for phylogenetic tree construction (16S ribosomal gene, partial sequence)

GenBank database	Accession
Streptomyces sahachiroi strain NBRC 13928	AB184548.1
Streptomyces cinerochromogenes strain MC10130	AB968639.1
Streptomyces andamanensis sp. Nov 16S	LC008305.1
Streptomyces sp. TK08330 16S	AB973399.1
Streptomyces krungchingensis sp. Nov	LC008304.1
Streptomyces bikiniensis strain 13661V	EU741193.1
Streptomyces venezuelae strain N2S8	EU221352.1
Streptomyces tanashiensis strain CB00881	HF935088.1
Streptomyces bikiniensis strain Ess_amA-1	KF588366.1
Streptomyces azureus strain NBRC 12744	NR112511.1
Streptomyces sp. e122	GQ351307.1
Streptomyces coeruleofuscus strain NRRL B-5417	DQ026668.1
Streptomyces coelicolor strain NBRC 12854	AB184196.1
Streptomyces megasporus	Z68100.1
Streptomyces caelestis strain AW9-9C	JX204833.1
Streptomyces nashvillensis strain NBRC 13064	NR041106.1
Streptomyces venezuelae strain N2S8	EU221352.1
Streptomyces pseudogriseolus strain NRRL B-3288	NR043835.1
Streptomyces castaneus strain NBRC 13670	AB184453.2
Streptomyces rubiginosohelvolus strain CSSP731	NR115443.1
Streptomyces violaceorectus strain NBRC 13102	NR041114.1
Streptomyces sp. e14	GQ351299.1
Streptomyces sp. e47	GQ351300.1
Streptomyces sp. e59	GQ351301.1
Streptomyces sp. e69	GQ351302.1
Streptomyces sp. e75	GQ351303.1
Streptomyces sp. e83	GQ351304.1

Table 2.1 GenBank database for phylogenetic tree construction (16S ribosomal gene, partial sequence) (cont.)

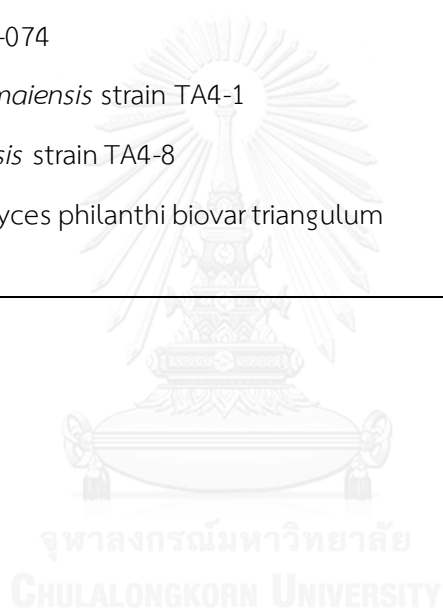| GenBank Database	Accession
Streptomyces sp. e110	GQ351305.1
Streptomyces sp. e113	GQ351306.1
Streptomyces sp. e122	GQ351307.1
Streptomyces sp. MD7	GQ351308.1
Streptomyces sp. MD6	GQ351309.1
Streptomyces sp. Ao10	FJ490543.1
Streptomyces sp. SPB-074	EU798707.1
Streptomyces chiangmaiensis strain TA4-1	NR113180.1
Streptomyces lannensis strain TA4-8	NR113181.1
Candidatus Streptomyces philanthi biovar triangulum	DQ375802.2
Escherichia coli	J01859.1

Table 2.2 GenBank database of insect-associated *Streptomyces*

Genbank database	Accession	Host	Reference
Streptomyces sp. e14	GQ351299.1	solitary wasp	Poulsen *et al.*, 2011
Streptomyces sp. e47	GQ351300.1	solitary wasp	Poulsen *et al.*, 2012
Streptomyces sp. e59	GQ351301.1	solitary wasp	Poulsen *et al.*, 2013
Streptomyces sp. e69	GQ351302.1	solitary wasp	Poulsen *et al.*, 2014
Streptomyces sp. e75	GQ351303.1	solitary wasp	Poulsen *et al.*, 2015
Streptomyces sp. e83	GQ351304.1	solitary wasp	Poulsen *et al.*, 2016
Streptomyces sp. e110	GQ351305.1	solitary wasp	Poulsen *et al.*, 2017
Streptomyces sp. e113	GQ351306.1	solitary wasp	Poulsen *et al.*, 2018
Streptomyces sp. e122	GQ351307.1	solitary wasp	Poulsen *et al.*, 2019
Streptomyces sp. MD7[th]	GQ351308.1	solitary wasp	Poulsen *et al.*, 2020
Streptomyces sp. MD6[th]	GQ351309.1	solitary wasp	Poulsen *et al.*, 2021
Streptomyces sp. Ao10	FJ490543.1	leaf-cutting ant	Haeder *et al.*, 2009
Streptomyces sp. SPB-074	EU798707.1	southern pine beetle	Oh *et al.*, 2009
S. chiangmaiensis strain TA4-1	NR113180.1	stingless bee	Promnuan *et al.*, 2013
S. lannensis strain TA4-8	NR113181.1	stingless bee	Promnuan *et al.*, 2014
Candidatus Streptomyces philanthi biovar triangulum	DQ375802.2	female European beewolves	Kaltenpoth *et al.*, 2006

3. Effects of crude extracts on cell viability of pre-adipocyte like cell line 3T3-L1
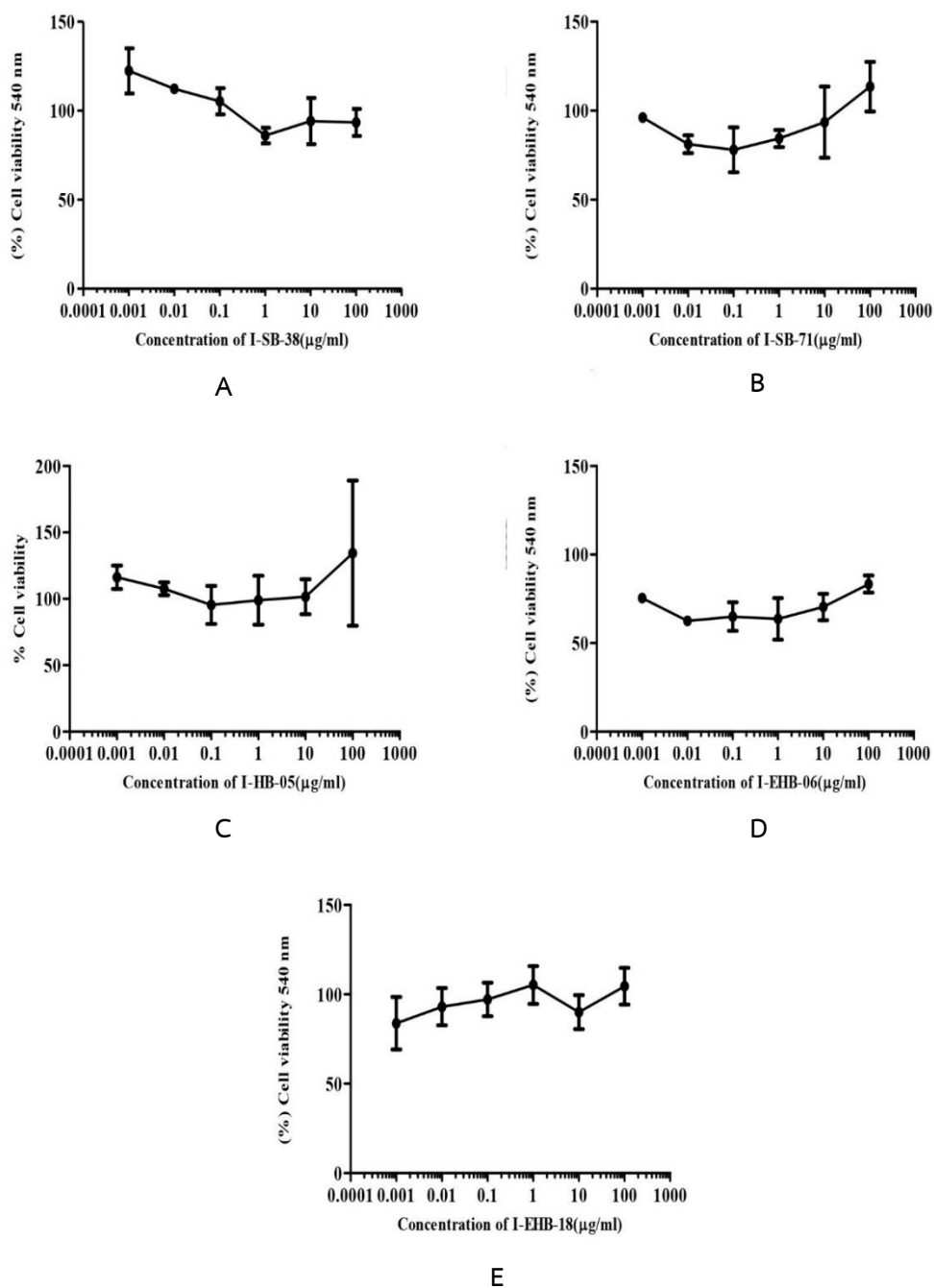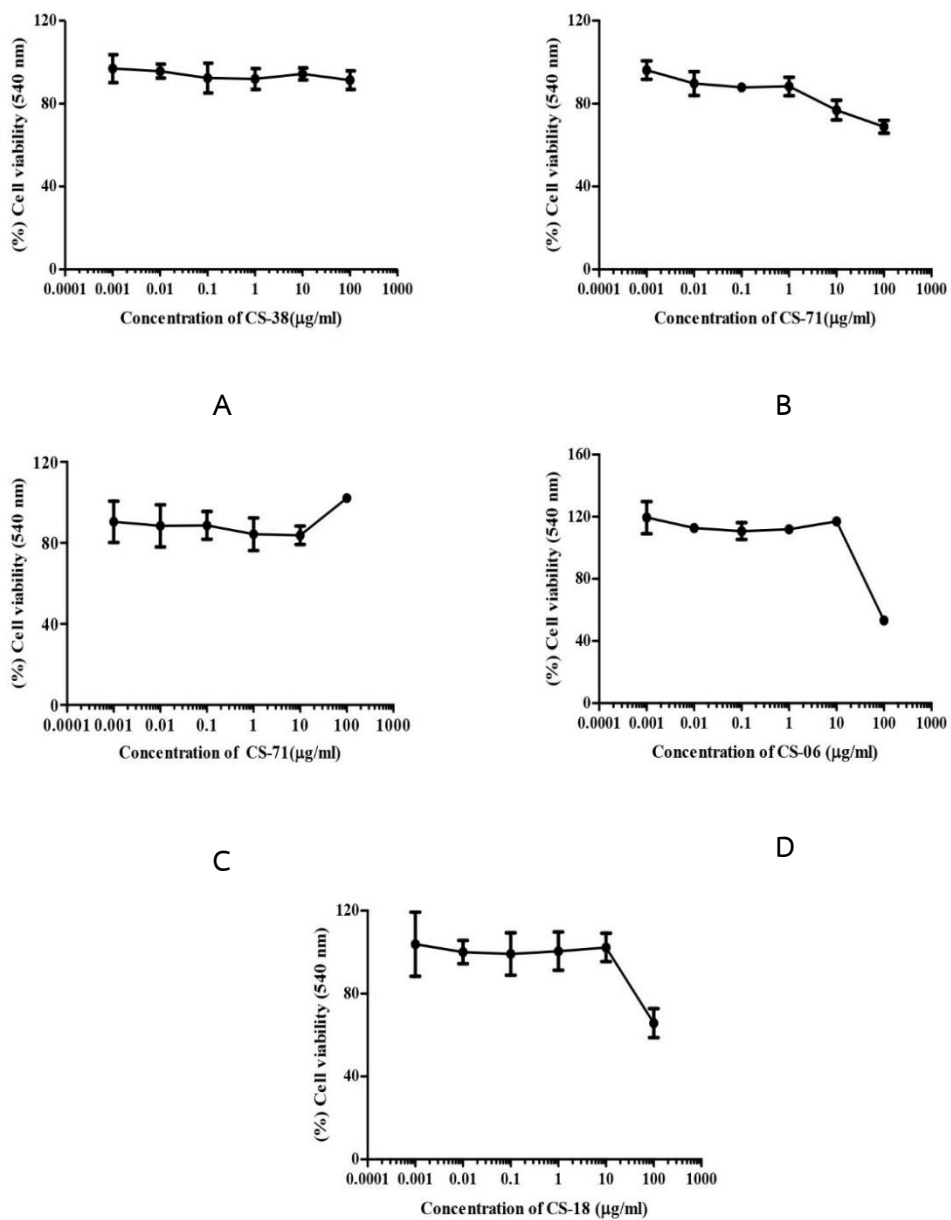

A

B

C

D

E

Figure 3.1 Effect of crude extracts from aqueous phase on cell viability of 3T3-L1 cell by MTT assay. A: I-SB-38, B: I-SB-71, C: I-EHB-05, D: I-EHB-06 and E: I-EHB-18, respectively

Figure 3.2 Effect of crude extracts from solvent phase on cell viability of 3T3-L1 cell by MTT assay. A: I-SB-38, B: I-SB-71, C: I-EHB-05, D: I-EHB-06 and E: I-EHB-18, respectively

VITA

I got a Bachelor' degree from the Department of Fishery Products, Faculty of Fisheries, Kasetsart University in 2013. In 2014, I enrolled in Master degree of Microbiology and Microbial Technology, Chulalongkorn University.

I presented my thesis in the topic of "The bioactive compounds produced by Streptomyces sp. isolated from Eastern honey bee (Apis cerana) and stingless bee (Tetragonula laeviceps)" by poster presentation in the 28th Annual Meeting of the Thai Society for Biotechnology and International Conference at The Empress Hotel Chiang Mai.