ฤทธิ์ต้านแบคทีเรียและองค์ประกอบทางเคมีของสารสกัดพรอพอลิสผึ้งพันธุ์ *Apis mellifera* จากจังหวัดน่าน ประเทศไทย

นางสาวภัทรพร บุญซ้าย

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIBACTERIAL ACTIVITY AND CHEMICAL COMPOSITIONS OF *Apis mellifera* PROPOLIS EXTRACTS FROM NAN PROVINCE, THAILAND

Miss Pattaraporn Boonsai

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Zoology
Department of Biology
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Thesis Title	ANTIBACTERIAL ACTIVITY AND CHEMICAL COMPOSITIONS OF *Apis mellifera* PROPOLIS EXTRACTS FROM NAN PROVINCE, THAILAND
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ภัทรพร บุญช้าย: ฤทธิ์ต้านแบคทีเรียและองค์ประกอบทางเคมีของสารสกัดพรอพอลิสผึ้งพันธุ์ *Apis mellifera* จากจังหวัดน่าน ประเทศไทย. (ANTIBACTERIAL ACTIVITY AND CHEMICAL COMPOSITIONS OF *Apis mellifera* PROPOLIS EXTRACTS FROM NAN PROVINCE, THAILAND) อ. ที่ปรึกษาวิทยานิพนธ์หลัก: รศ. ดร. จันทร์เพ็ญ จันทร์เจ้า, อ. ที่ปรึกษาวิทยานิพนธ์ร่วม: ผศ. ดร. ปรีชา ภูวไพรศิริศาล, 111 หน้า.

พรอพอลิสมีลักษณะเหนียวและมีสีน้ำตาลเข้ม ได้มาจากยางของพืช ตุ่มของต้นไม้ น้ำต้อยและส่วนต่าง ๆ ของพืชที่เก็บโดยผึ้ง ผสมเข้ากับไขและใช้เพื่อสร้างและซ่อมแซมรัง พรอพอลิสมีฤทธิ์ทางชีวภาพหลายอย่าง ในงานวิจัยนี้ ศึกษาพรอพอลิสจากผึ้งพันธุ์ *Apis mellifera* จากจังหวัดน่านเพื่อทดสอบหาฤทธิ์ต้านแบคทีเรียต่อเชื้อ *Staphylococcus aureus* (แบคทีเรียแกรมบวก) *Paenibacillus larvae larvae* (แบคทีเรียแกรมบวก) และ *Escherichia coli* (แบคทีเรียแกรมลบ) พรอพอลิสจะสกัดด้วยเมทานอล ไดคลอโรมีเทนและเฮกเซน ก่อนการทดสอบฤทธิ์ต้านแบคทีเรียจากสารสกัดอย่างหยาบทั้งสามชนิด ทำการยืนยันแบคทีเรียที่เลือกศึกษาทั้งสามชนิดว่าเป็นชนิดที่ถูกต้องโดยใช้สองวิธีคือการย้อมสีแกรมและการวิเคราะห์ลำดับเบสบางส่วนของยีน 16 เอสอาร์ดีเอ็นเอ หลังจากนั้นทดสอบฤทธิ์ต้านแบคทีเรียของสารสกัดอย่างหยาบเหล่านี้ด้วย agar well diffusion assay และ microbroth dilution assay ใช้ streptomycin ใช้เป็น positive control สารสกัดอย่างหยาบของพรอพอลิสด้วยเมทานอล (CMC) แสดงฤทธิ์ต้านแบคทีเรียด้วยค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งได้หรือ MIC ที่ 5 มก./มล. และมีประสิทธิภาพต่อการยับยั้งทั้ง *S. aureus* และ *E. coli* นอกจากนั้น CMC มีประสิทธิภาพต่อการยับยั้ง *P. larvae larvae* ด้วยค่า MIC ที่ 6.25 มก./มล. ดังนั้นจึงนำ CMC ไปสกัดบริสุทธิ์ต่อไปโดยใช้ quick column chromatography และได้สามแฟรกชันที่มีฤทธิ์ยับยั้งการเติบโตของทั้ง *S. aureus* และ *E. coli* ด้วยค่า MIC ที่ 6.25 และ 31.25 มก./มล. ตามลำดับ หลังจากนั้นนำแฟรกชันที่มีฤทธิ์มาทำการสกัดให้บริสุทธิ์มากขึ้นด้วย adsorption chromatography แฟรกชัน A1A ที่บริสุทธิ์สามารถแสดงค่าของความเข้มข้นที่ยับยั้งการเพิ่มจำนวนของแบคทีเรียที่ร้อยละ 50 (IC_{50}) ที่ 0.175 ไมโครกรัม/มล. สำหรับ *E. coli* และที่ 0.683 ไมโครกรัม/มล. สำหรับ *P. larvae larvae* หลังจากแฟรกชัน A1A ถูกวิเคราะห์ต่อไปเพื่อหาโครงสร้างทางเคมีโดยใช้ nuclear magnetic resonance พบว่าสารออกฤทธิ์อยู่ในกลุ่ม cardanol สังเกตการเปลี่ยนแปลงทางสัณฐานวิทยาและการหา time kill ด้วย cardanol ที่มีต่อ *E. coli* โดยกล้องจุลทรรศน์อิเล็กตรอนชนิดส่องกราดและชนิดส่องผ่าน สังเกตเห็นฤทธิ์ต้าน *E. coli* ในช่วงต้นของการเลี้ยงร่วมกับ cardanol ซึ่งก็คือระยะก่อน 4 ชั่วโมง สำหรับเซลล์ที่เลี้ยงร่วมกับสารข้างต้น เห็นรูปร่างที่ผิดปกติ โดยเฉพาะในเซลล์ที่กำลังแบ่งตัว เซลล์มีความเสียหายและตายได้อย่างเด่นชัด

ภาควิชา.....ชีววิทยา.....ลายมือชื่อนิสิต.....

สาขาวิชา.....สัตววิทยา.....ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์หลัก.....

ปีการศึกษา.....2555.....ลายมือชื่อ อ. ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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PATTARAPORN BOONSAI: ANTIBACTERIAL ACTIVITY AND CHEMICAL COMPOSITIONS OF *Apis mellifera* PROPOLIS EXTRACTS FROM NAN PROVINCE, THAILAND. ADVISOR: ASSOC. PROF. CHANPEN CHANCHAO, Ph.D., CO-ADVISOR: ASST. PROF. PREECHA PHUWAPRAISIRISAN, Ph.D., 111 pp.

Propolis is sticky and dark brown. It is derived from plant resins, tree buds, sap flows, and other botanical sources collected by honeybees. It is mixed with wax and used to construct and repair the nest. It possesses many bioactivities. In this research, propolis from *Apis mellifera* from Nan province was tested for the antibacterial activity against *Staphylococcus aureus* (Gram + bacteria), *Paenibacillus larvae larvae* (Gram + bacteria), and *Escherichia coli* (Gram – bacteria). Before all of three crude extracts were tested for antibacterial activity, the correct types of three selected bacteria were confirmed by two methods which were Gram staining and partial sequence of *16S rDNA* analysis. Propolis was extracted by methanol, dichloromethane, and hexane. Thereafter, the antibacterial activity of these crude extracts was determined by agar well diffusion and microbroth dilution assay. In addition, streptomycin was used as a positive control. Crude methanol extract of propolis (CME) showed the best antibacterial activity with the minimum inhibition concentration or MIC value of 5 mg/ml and was effective to inhibit both *S. aureus* and *E. coli*. In addition, CME was effective to inhibit *P. larvae larvae* with MIC value of 6.25 mg/ml. Thus, CME was used for further purification by quick column chromatography. Only three active fractions were able to inhibit the growth of both *S. aureus* and *E. coli* with the MIC values of 6.25 and 31.25 µg/ml, respectively. Later, the active fraction was further purified by adsorption chromatography. The purified fraction A1A could present the IC_{50} values of 0.175 µg/ml for *E. coli* and of 0.683 µg/ml for *P. larvae larvae*. After fraction A1A was further analysed for chemical structure by using Nuclear Magnetic Resonance, it was in a cardanol group. The morphology change and time-killed assay by cardanol on *E. coli* was observed by scanning and transmission electron microscopy. The anti-*E. coli* activity was observed in the early period of treatment which was before 4 h. For treated cells, unusual shape, especially in dividing cells, damaged and dead cells could be distinguished.

Department: Biology Student's Signature .....

Field of Study: Zoology Advisor's Signature .....

Academic Year: 2012 Co-advisor's Signature .....

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LIST OF ABBREVIATIONS

BHI agar	Brain heart infusion agar
bp	Base pair
^{13}C NMR	Carbon-13 nuclear magnetic resonance
δ	Chemical shift
CDE	Crude dichloromethane extract of propolis
CHE	Crude hexane extract of propolis
CME	Crude methanol extract of propolis
DNA	Deoxyribonucleic acid
DMSO	Dimethyl sulfoxide
EtOH	Ethanol
EDTA	Ethylene diamine tetra-acetic acid
INT	*p*-iodonitrotetrazolium violet
IC_{50}	Concentration required for 50% inhibition *in vitro*
°C	Degree Celsius
g	Gram
LB	Lysogeny broth
MIC	Minimal inhibitory concentration
MBC	Minimum bactericidal concentration
NMR	Nuclear magnetic resonance
1D NMR	One dimensional nuclear magnetic resonance
2D NMR	Two dimensional nuclear magnetic resonance
SEM	Scanning electron microscope

TMS	Tetramethylsilane
TLC	Thin layer chromatography
TEM	Transmission electron microscope
TBE	Tris-boric-ethylene diamine tetraacetic acid
MeOH	Methanol
CH_2Cl_2	Methylene chloride or dichloromethane
NaCl	Sodium chloride
h	Hour
min	Minute
µl	Microlitre
µg	Microgram
mg	Milligram
mM	Millimolar
ml	Millilitre
ppm	Part per million
%	Percentage
RT	Room temperature
rpm	Revolutions per minute
v/v	Volume by volume
w/v	Weight by volume

CHAPTER I

INTRODUCTION

Propolis has long been used worldwide in traditional and alternative medicine. For example, since ancient times, Greeks have used propolis for treating infected and swollen wounds. In addition, Assyrians have used it to treat tumors and Egyptians have used it for the anti-putrefaction on mummification. In the 17th century, it was recorded that propolis was applied in pharmaceutical products and drug used in UK. Until now, propolis in the pure form and in the mixed form with other natural products has been used or applied in cosmetics, daily used products, and supplementary constituents in healthy foods. Thus, that has been an interest in searching for active compounds and their bioactivities (Ghisalberti, 1979; Castaldo and Capasso, 2002; Bankova, 2005a; Sforcin, 2007).

Propolis is sticky and dark brown. It is a natural product derived from plant resins, tree buds, sap flows, and other botanical sources collected by bees. Since propolis is from plant resin, it is possible to find the same compounds found directly in plant resin. Alternatively, the modified or derivative compounds can be found since forager bees can alter the chemical structure of compounds by their enzymes secreted from their saliva glands. In general, propolis is used to construct and repair the hive, mainly to fill cracks in the bee hive, and to defend against pathogenic microorganisms. Propolis was analysed to contain resin at 50%, beeswax at 30%, oil at 10%, pollen 5%, and other compounds at 5% (Katircioglu and Mercan, 2006).

Main chemical compounds such as aromatic acids, aromatic ester, phenolic acids, flavonoid, ketone, fatty acid, aromatic alcohol were analysed. Also, they could perform many interesting bioactivities such as anti - inflammatory (Massignani *et al*., 2009), antioxidative (Gregoris and Stevanato, 2010), antiproliferative (Umthong *et al.,* 2009), antidiabetic (Kang *et al*., 2010), antiviral (Schnitzler *et al*., 2010), antifungal (Quiroga *et al*., 2006), and antibacterial (Kim and Chung, 2011) activities. Moreover, flavonoids, phenolic compounds, and terpenes in propolis could provide the antimicrobial activity (Hidayathulla *et al.,* 2011). Park *et al*. (1998) showed that the ethanol extract of propolis in Brazil could inhibit both glucosyltransferase activity and the growth of *Streptococcus mutans*. In 2011, Hendi *et al*. reported that crude ethanolic extract of Al-Museiab propolis provided the better antimicrobial activity against *Staphylococcus aureus* than other bacteria. In contrast, it was not effective to *Candida albicans*. Yaghoubi *et al*. (2007) reported the antimicrobial activity of ethanol extract of Iranian propolis against *S. aureus*, *S. epidermidis*, *Bacillus cereus*, and *C. albicans* whereas no activity was observed against *Salmonella enteritidis*, *Escherichia coli*, *Klebsiella pneumonia,* and *Proteus vulgaris*.

The properties of propolis are likely to depend on geographical conditions since it indicates plant species. Kujumgiev *et al*. (1999) reported that the chemical compositions of propolis collected from different geographic regions where were the temperate zone including Asia, Europe, North Africa, and North America were different in either types or concentrations.

Like regions, the properties of propolis depend on bee species, hive locations, the management, extraction methods, storage conditions, and seasons (Moreira *et al*., 2008).

In this research, propolis from *Apis mellifera* from Nan province was tested for the antibacterial activity against *Escherichia coli* O157: H7, *Staphylococcus aureus* (ATCC 20651), and *Paenibacillus larvae* (PL 44). Also, its active chemical compounds were analysed. These compounds are capable to be new antibacterial agents which may be developed to use in the future. Furthermore, this may promote the interest and the importance in bee farming of the country which eventually will increase the income of bee farmers.

CHAPTER II

LITERATURE REVIEW

2.1 Biology of *Apis mellifera*

Apis mellifera or Western honeybee is imported to Thailand for bee farming because its foraging behavior is excellent. It is native to the continents of Europe and Africa. Among *Apis* spp., it can produce the highest quantity of honey but it performs the least aggressive bahavior. In addition, it does not swarm easily. Nowadays, it can be well managed in an apiary around the country (Wongsiri, 1989). Other than honey production, *A. mellifera* have widely been used as efficient pollinators for economic crops such as strawberry, melon, apple (Williams, 2000; Yoshiyama and Kimura, 2011).

A hive of *A. mellifera* looks similar to a hive of *A. cerana* or Eastern bee. They build their multiple comb nests in holes, boxes, and sealed areas (Figure 2.1) (Oldroyd and Wongsiri, 2006). This protects them from a bee hunter and predators. Both bees often make the nest entrance clear and smooth. The cavity surrounding area is coated by a thin layer of propolis (Seeley and Morse, 1976).

Figure 2.1 A colony of *A. mellifera* in a hive box.

A nest consists of many hexagonal cells made of wax. Wax is secreted from wax glands locating in a bee's abdomen. Interestingly, a bee can use its mouth to make a thin sheet of wax and use it to build a hexagonal cell. Considering a hexagonal shape of cells within a hive, the size of all cells are the same. It is believed that the hexagonal shape can provide the most use of the limited area. A nest is created by bees to serve many purposes like home, food storage. Within a nest, honey and larvae are kept on the top part of a comb. Then, it is followed by the storage area of pollen, worker-brood cells, and drone-brood cells, respectively. The peanut-shaped queen cells are normally built at the lowestarea of the comb when a new queen is needed.

A honeybee has a complete metamorphosis. Its life cycle takes 24 days. At the beginning, an egg stage, it takes about 3 days. After hatching, a little worm-like larva crawls out. At this stage (for1-3 days), a larva will be fed with pollen and honey by a nurse bee (7 days old). Soon, it spins a web wrapping itself inside and, then, becomes a pupa (for 16-24 days). After that, a full grown bee climbs out of the cell (Oldroyd and Wongsiri, 2006).

The taxonomy of *A. melllifera* is presented as below:

Taxonomy identification of *A. mellifera* (Wongsiri, 1989)

Kingdom Animalia

Phylum Arthropoda

Class Insecta

Order Hymenoptera

Superfamily Apoidea

Family Apidae

Subfamily Apinae

Genus *Apis*

Species *Apis mellifera*

Bees are known to be eusocial insects because they livein a hive as a community or a family.Within a hive, there are 3 castes of honeybees which are a queen, workers, and drones (Oldroyd and Wongsiri, 2006; Ruttner, 1988; Winston, 1987) (Figure 2.2).

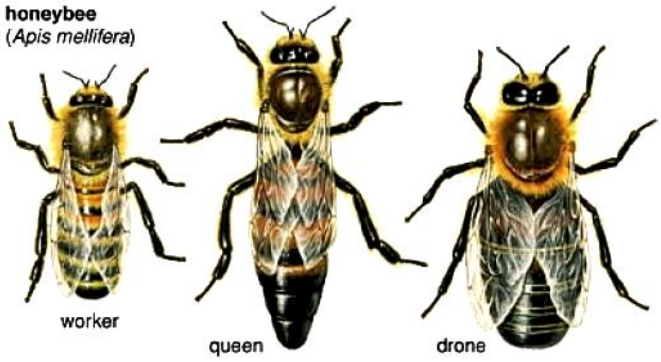


Figure 2.2 Three castes of *A. mellifera* (http://spartanburgimagery.org/).

Three castes of honeybees can be explained in details as below:

A queen: It is the only fertile female in a hive. It develops from a fertilized egg so it is diploid (2n) with 32 chromosomes. A queen is the largest in size within a hive but its wings are very short. For mating, it will fly out of the hive to Drone Aggregation Area (DCA). A queen will mate with many drones and save sperms in a spermatheca. Thus, it can lay up to 2,000 eggs per day (Rueppell *et al*., 2007). Normally, there is only a single queen in a hive. Also, it produces a queen

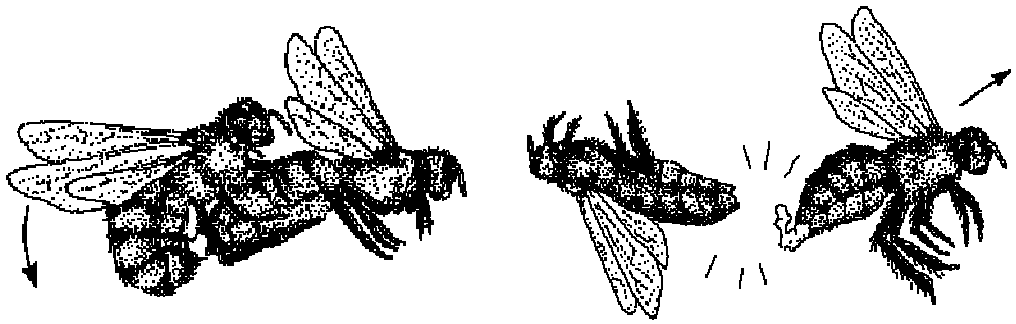
pheromone that controls all behaviors of workers and drones. It has a life span of 2-3 years. If a queen dies, a new queen cell will be made.

Workers: They are the smallest in size and the most abundant in a hive. They are sterile females and diploid (Barron and Oldroyd, 2001). They do all tasks in a hive. It is interesting that their duties depend on age. When they are at 7 days old, they will feed larvae, so called “nurse bee”. After that, as guard bees (12-21 days old), they will protect an invader or enemy within a hive area. Later, at the age of 21 days old and s on, they will be foragers flying out of a hive to search for food (pollen and nectar from flowers). In overall, they have a shortlife about 6-8 weeks.

In 2000, Nieh and Tautz reported a comb vibration of waggle dancing as a communication for distance and direction of food. When a worker bee found the location of food, after returning, she would inform the other bees within a hive by waggle dancing. Since there were ten thousands of bees in a hive, only a waggle dance and sound was not good enough to win the buzz in the hive. Thus, honeybees surrounding the waggle dancer would help by also performing the waggle dancing. This behavior would help to indicate the location of the waggle dancer. By doing this, they also vibrated a comb which led to the communication of bees widely.

Drones: They are haploid males. It has 16 chromosomes (Oldroyd and Wongsiri, 2006). A drone looks fatter and shorter than a queen and has no sting (Seeley, 1985; Winston, 1987). Distinguishingly, it has very large compound eyes. A drone does not do any task except mating. In order to increase the genetic variation

of queen's descendants, the crowd of drones (about 10 days old) from many hives will be formed at the Drone Congregation Area (DCA) where is far away from their own hives (Figure 2.3). After mating, they will die shortly. In case, a drone does not have chance to mate a queen before early winter, workers will remove it out of the hive. Eventually, it will die.



(A) (B)

Figure 2.3 Mating behavior. A drone mounts a queen, inserts its endophallus, and ejaculates its semen (A). After fertilization, surrounding drones will remove the previous drone's endophallus and perform the mating (B). The emasculated drones die very quickly because their abdomens will be burst (http://www.texasdrone.com/).

2.2 Propolis

Honey, propolis, bee venom, bee pollen, royal jelly, and wax are economic bee products. Propolis is sticky and dark brown. It is derived from plant resins, tree buds, sap flows, and other botanical sources collected by honeybees. It is mixed with wax and used to construct and repair the nest. As in Figure 2.4, it is mainly used to fill out cracks in the bee hive and to defend pathogenic microorganisms (Marcucci *et al*., 2001).

Propolis was analysed to contain 50% resin, 30% wax, 10% oil, 5% pollen, and 5% other compounds (Katircioglu and Mercan, 2006). It was reported to consist of many chemical compounds such as aromatic acids, aromatic ester, phenolic acids, flavonoid in many forms (flavonoles, flavones, flavonones, dihydroflavonoles, and chalcones), terpenes, beta-steroids, aromatic aldehydes and alcohols, sesquiterpenes, stibene, terpene, ketone, fatty acid, aromatic alcohol (Aga *et al*., 1994; Russo *et al*., 2002). Moreover, in 2002, Castaldo *et al*. reported that propolis also consisted of vitamin B.

Figure 2.4 Propolis, a very sticky resin, is used by bees to glue things in a hive (http://curbstonevalley.com/).

2.3 Bioactivity of propolis

2.3.1 Anti-inflammatory activity

Inflammation is the body's response against pathogens or other factors such as a substance or treatment that reduces inflammationeven though no infection. If bacteria are not removed, the wound will not be healed. This will cause various diseases and life threatening.

Tan-no *et al*. (2006) reported nitric oxide production in mouse paw edema induced by carrageenin could be inhibited by ethanol extract of Brazilian propolis. The best anti-inflammatory effect of propolis [1: 1000 and 1: 100 (w/w), p.o.] could be obtained when it was compared to diclofenac, a non-steroidal anti-inflammatory drug at the dose of 12.5 and 50 mg/kg (p.o.) and L-N^G-nitro arginine methyl ester or

L-NAME, a nitric oxide synthase inhibitor, at the dose of 10 and 100 mg/kg (s.c.). The better medication for using this extract was 10 min application before carrageenan injection.

Later, in 2008, Paulino *et al*. reported that artepillin C, the major compound in Brazilian propolis, could relieve the inflammatory in paw edema of male Swiss mice which were induced by carrageenan (300 μg/paw). Later, carrageenan-induced peritonitis and prostaglandin E_2 determination was observed. It was presented that artepillin C could decrease the number of neutrophils during peritonitis [IC_{50}: 0.9 (0.5-1.4) mg/kg]. Also, it could decrease prostaglandin E_2 by 29 ± 3% and 58 ± 5% at 1 and 10 mg/kg, respectively, with a mean ID_{50} of 8.5 (8.0-8.7) mg/kg.

In addition, they continued to test artepillin C *in vitro* by measuring the nitric oxide production from RAW 264.7 cells and NF-κB activity in human embryonic kidney lineage (HEK) 293 cells. The result showed that artepillin C could reduce the nitric oxide production with a mean ID_{50} of 8.5 (7.8–9.2) μM and reduce the NF-κB activity with a mean IC_{50} of 26 (22–30) μg/ml, respectively.

2.3.2 Anti-oxidative activity

The metabolism of cell is for taking the necessary nutrients and oxygen into the cells but getting rid of all toxic materials, including an oxidant, out of the cell. The oxidation reaction can cause cell deterioration and destruction. Normally, a living body is able to prevent and reduce an oxidant by creating a substance called an

anti-oxidant. Besides, this oxidant can be supplied from food or drug (Kohen and Nyska, 2002).

In 2010, Gregoris and Stevanato reported the antioxidant activity of propolis from four sampling sites in Venetian. All of them were from different orography and habitats. The result showed the antioxidant activity was in the range of 62-75% by the ethanol extract of those propolis extracts at the concentration of 1.2 mg/l. Those Venetian propolis showed high anti-oxidant activity because it could inhibit and control the lipid peroxidation of cell membrane.

In addition, Laskar *et al.*(2010) reported the antioxidant activity of water extract and ethanol extract of Indian propolis (WEP and EEP, respectively) using Ferric reducing antioxidant power, DPPH, and cyclic voltammetry assays. The results showed that WEP had the stronger antioxidant activity than EEP because WEP had more polyphenol content than EEP.

2.3.3 Antiproliferation activity

Cancer is a disease which cells divide abnormally and uncontrollably. It can spread to other parts of a living body which is called “metastasis” via blood and lymphatic system. Thus, that makes cancer become the leading cause to death and difficult for a treatment. Due to side effects of chemotherapy and drug-resistance of cancer, new anticancer agent is still needed (Anand *et al.*, 2008).

Umthong *et al*. (2009) reported the activity against the proliferation of colon cancer cells (SW620) by water crude extract (WEP) and methanol extract (MEP) of *Trigona laeviceps* propolis. The concentration of MEP had a reciprocal relationship to the survival percentage of cancer cells. However, the survival percentage of cancer cells treated with WEP at 50 and 150 μg/ml was lower than that with MEP at the same concentrations. In contrast, the survival percentage of cancer cells of the concentration of WEP at 100 μg/ml was the highest.

Kouidhi *et al*. (2010) reported the antiproliferation of cancer cells by EEP from Tunisia. Cancer cell lines of the intestinal epithelial cell line (HT-29), the respiratory epithelial cell line (A549), the human epithelial cell line (Hep-2), the murine leukemic monocyte macrophage cell line (raw 264.7), and the normal African green monkey kidney epithelial cell line (Vero) were used. Also, the human fibroblast-like foetal lung cell line (MRC-5) as normal cell was used as control. By MTT assay, the IC_{50} value of EEP against HT-29, A549, Hep-2, raw264.7, and Vero were in the range of 15.7 ± 3.4 to 200 ± 22.2 mg/ml, respectively.

2.3.4 Anti-diabetic activity

Diabetes is one of widely found diseases in human. The number of diabetic patients trend to increase nowadays. It is known to be abody disorder caused by lacking insulin. That leads to high blood sugar levels in a body or hyperglycemic condition (Kang *et al*., 2010).

Abo-Salem *et al*. (2009) discovered that propolis extracts data concentration of 100, 200, and 300 mg/kg could prevent oxidative stress or cell damage which was implicating in Alzheimer's disease. In additon, it could delay the occurrence of renal complications in diabetic patients.

Later, Kang *et al.* (2010) reported that propolis extract could perform the anti-oxidative activity and reduce the symptoms of diabetes. The propolis extract could reduce the expression and activity glucose-6-phosphatase (G6Pase) in HepG2 cells incubated in high glucose media. Also, it could inhibit the activity of glycogen synthase kinase (GSK3), α and β, by inhibiting the serine and tyrosine phosphorylation, specifically Y279 for GSK3α and Y216 for GSK3β. Since anti-diabetic activity of propolis extract was not depended on hydrogen peroxide and N-acetylcysteine, they suggested that the extract inhibited the expression of G6Pase by inhibiting the autophosphorylation of Y279 and Y216 of GSK3α and GSK3β, respectively.

2.3.5 Antiviral activity

Virus is one of microorganisms that can become an infectious agent in humans, animals, plants and other cellular life. It is believed to a living particle which needs a host for survival. Its genetic material can be RNA or DNA. A disease caused by virus is harder to treat, comparing to other pathogens like bacteria or

protists because the mutation rate of virus is very high. Therefore, it is important to search for the better prevention and treatment (Breitbart and Rohwer, 2005).

Schnitzler (2010) found that EEP from *A. mellifera* from Moravia in the Czech Republic could inhibit the growth of herpes simplex virus type 1 (HSV-1). The IC_{50} value of WEP and EEP was 0.0004% and 0.000035%, respectively. Galangin and chrysin were found to be active ingredients in the extract.

2.3.6 Antifungal activity

Fungi are one of pathogenic microorganisms mostly found in contaminated food and damp/moist areas. It can survive in harsh condition and produce mycotoxins which can harm our body badly (Marin *et al*., 1999; Janardhana *et al*., 1998). Since fungi are eukaryote containing cell wall, it is harder to be killed, comparing to bacteria or protozoa (Bata and Lasztity, 1999).

Quiroga *et al.* (2006) reported galangin, an active substance in *A. mellifera* propolis, could inhibit the growth of *Phomopsis* spp., *Fusarium* sp., *Trichoderma* spp., and *Penicillium notatum* better than ketoconazole, recent used fungicide drug. However, the antifungal activity of galangin against the growth of *Schizophyllum commune* and *Pycnoporus sanguineus* was worse than ketoconazole.

In 2011, Yang *et al*. reported the antifungal activity of propolis harvested in Hebei province, China. Propolis was extracted by bioassay-guided isolation and

tested on *Penicillium italicum* that could cause a disease in fruits. Ethyl acetate, *n*-butanol, water, and petroleum ether were used as solvents. It was shown that crude ethyl acetate extract of propolis or CEA (200 mg/l) could completely inhibit the growth of *P. italicum*. In addition, crude petroleum ether (PEP) and *n*-butanol (BEP) extracts of propolis could provide the antifungal activity at 35% and 25%, respectively. In contrast, WEP had the weak antifungal activity against *P. italicum*.

2.3.7 Antibacterial activity

Raghukumar *et al.* (2010) reported the antigrowth of methicillin-resistant *Staphylococcus aureus* (MRSA) by EEP of "Pacific propolis" originating from the Solomon Islands. Fifteen MRSA clinical isolates were *in vitro* screened by using an agar dilution assay. Subsequent purification of this crude extract afforded 23 fractions. Later, by the spectroscopic data (1D-, 2D-Nuclear Magnetic Resonance or NMR, and Mass spectroscopy or MS),the purified and active fractions were analyzed to be prenylflavanones aspropolin C, propolin D, propolin G, and propolin H. Minimum Inhibition Concentration (MIC) against MRSA of propolin C and D was 8-32 and 8-16 mg/l, respectively.

Furthermore, Kouidhi *et al*. (2010) reported ethanol extract of propolis (EEP) from Tunisia at a concentration of 100 mg/ml could inhibit the growth of oral pathogenic bacteria like streptococci and enterococci by removing biofilm.

In addition, Kim and Chung (2011) revealed the antibacterial activity of EEP from Korea against the growth of foodborne pathogens. *Bacillus cereus* was reported to be the most sensitive with minimum inhibitory concentration (MIC) of 0.036 mg/ml. Moreover, they could observe the morphology change of propolis-treated *B. cereus* by transmission electron microscopy (TEM). Also, it was found that cell membrane of treated *B. cereus* was damaged.

2.4 Minimal Inhibitory Concentration (MIC)

It is defined as the lowest concentration of substance that can inhibit growth of a microorganism. Alternatively, this value presents the susceptibility of microorganisms to the interest. The lower MIC value indicates the better susceptibility of the interest.

2.5 Minimum Bactericidal Concentration (MBC)

It is defined as the lowest concentration of substance that can kill total bacteria.

2.6 Phenolic compounds

Phenolic compounds can be briefly defined as organic chemical groups that include one or more aromatic hydrocarbon rings bonded to a hydroxyl group (Vermerris and Nicholson, 2008). In the case of more than one aromatic ring, the compound becomes a polyphenol. The simplest phenolic compound is phenol, for which the group is named. It includes a hydroxyl group bonded to a benzene aromatic ring as shown in Figure 2.5. Phenolic compounds are weak acids due to the presence of the aromatic ring (Vermerris and Nicholson, 2008). Phenolic compounds are typically plant-derived and included in esters and glycosides (Vermerris and Nicholson, 2008). Phenolic acids may be liquid or solid. Most of them are colourless and insoluble even though they may be oxidized (Vermerris and Nicholson, 2008).

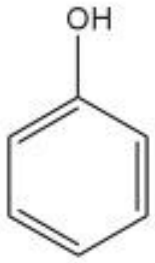


Figure 2.5 The structure of phenol which is served as a basis for other phenolic compounds**.**

There are a number of different ways to characterize and classify phenolic compounds (Vermerris and Nicholson, 2008). Chemical structure can be used to identify 20 different groups of phenols. Alternatively, a simpler classification which

is based on distribution, can be used to divide phenolic compounds into 3 groups which are widely distributed, less widely distributed, and only present in polymers (Vermerris and Nicholson, 2008). Most phenolic compounds found in plants are flavonoids (over than half), coumarins, or cinnamic acids (Balasundram *et al*., 2006; Shahidi and Ho, 2005). A standard classification of phenolic compounds described by Harborn and Simmons (1964) is presented in Figure 2.6 (Vermerris and Nicholson, 2008). Twenty different groups are characterized by a unique arrangement of aromatic hydrocarbon and hydroxyl groups.

Structure	Class
C_6	simple phenolics
C_6 - C_1	phenolic acids and related compounds
C_6 - C_2	acetophenones and phenylacetic acids
C_6 - C_3	cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols
C_6 - C_3	coumarins, isocoumarins, and chromones
C_{15}	chalcones, aurones, dihydrochalcones
C_{15}	flavans
C_{15}	flavones
C_{15}	flavanones
C_{15}	flavanonols
C_{15}	anthocyanidins
C_{15}	anthocyanins
C_{30}	biflavonyls
C_6-C_1-C_6, C_6-C_2-C_6	benzophenones, xanthones, stilbenes
C_6, C_{10}, C_{14}	quinones
C_{18}	betacyanins
Lignans, neolignans	dimers or oligomers
Lignin	polymers
Tannins	oligomers or polymers
Phlobaphenes	polymers

Figure 2.6 Classes of phenolic compounds.

The structure and nomenclature of the simple phenolic compounds is shown in Figure 2.7. It shows the arrangements of substitution patterns between the benzene group and the aromatic groups which may be substituted in a variety of patterns such as 1,2, 1,3, and 1,4 for two functional groups and 1,3,5 or 1,2,6 for three functional groups (Vermerris and Nicholson, 2008). A full review of the chemical structures of all phenol groups is out of the scope of this document but it can be found in standard and special textbooks.

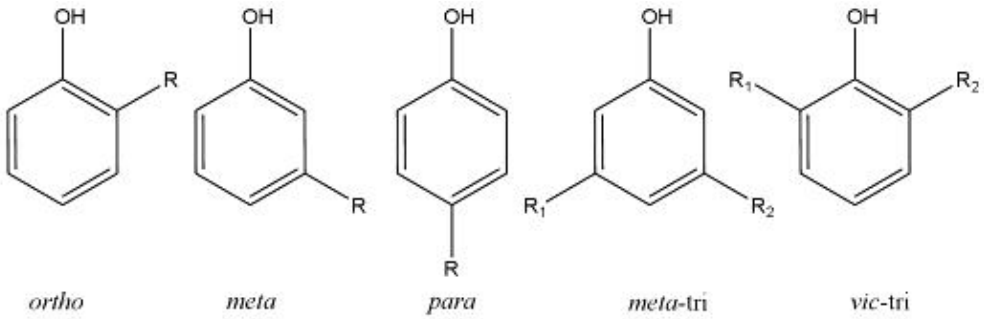


Figure 2.7 The structure of simple phenols. The substitution patterns and nomenclature patterns are indicated.

One of the key characteristics of the phenolic compound is that it is primarily plant-derived (Shahidi and Ho, 2005). They can be found in fruits, seeds and nuts, grains, and many other plant products which are commonly consumed as food, scent and flavoring, and nutritional products (Shahidiand and Ho, 2005). Some common phenolic compounds that naturally occur in food products include capsaicin (contributing the "hot" taste of chilli peppers) and tannin (contributing the oaky taste of tea) (Shahidi and Ho, 2005). Phenols may also be found in crude biomass such as wood and foliage plants, which are used to produce industrial esters, disinfects, and other commercial products (Shahidi and Ho, 2005). These properties make phenolic compounds potentially commercially valuable for food products.

In addition to flavor and scent, phenolic compounds in food offer a number of different nutritional and health benefits. For example, flavonoids have antioxidant effects which terminate free radicals within the bloodstream and reduce the potential for vascular damage resulting from these free radicals (Balasundram *et al*., 2006). Phenolic compounds may have a variety of other medical uses such as analgesic (pain-killing), anti-inflammatory, anti-asthmatic, antibacterial, anti-carcinogenic, and other effects (Jang *et al*., 2010; Huang *et al*., 2009). These characteristics make phenolic compounds - whether derived from the diet or from other sources - offer significant health benefits.

The source of phenolic compounds explored in this research is propolis. It is a by-product of honey production used by bees as a hive construction material (Wolfe, 2009). It is created by an amalgamation of sap, pollen, and other substances

which the bees accumulate during their pollen-gathering activities, and which is then removed by the bees in their return to the hive (Wolfe, 2009). It is a complex compound including a large range of phenolic compounds (mostly flavonoids) derived from the underlying plant material (Wolfe, 2009). It is reputed to have various health properties including anti-inflammatory and antibacterial properties and is used in alcohol suspension as a natural remedy (Wolfe, 2009). Although its use in natural remedies does suggest further potential for commercial drug development, this has not been explored in detail. Thus, at present, there are no current commercial drugs based on propolis (Sforcin and Bankova, 2011). One of the complicating factors in this development is that, as research from Greece and East Cyprus, the precise phenolic compounds found in propolis differs depending on its origin (Kalogeropoulos *et al*., 2009).

A phenolic compound of interest in this research is cardanol which is derived from the cashew nut shell (in the form of cashew nut shell liquid or CNSL) (Tullo, 2008). Specifically, it is a major component in the anacardic acid that makes up the majority of the CNSL (Chiellini *et al*., 2003). Previously, cardanol has mostly been used for industrial purposes including in resins and surfactants (Tullo, 2008). One of the most commonplace uses of cardanol in its current industrial formulation is as the base for a polymer, when combined with hydrogen peroxide (Chiellini *et al*., 2003). Figure 2.8 presents the phenolic structure of cardanol, showing that the largest component is tri-unsaturated cardanol (Chiellini *et al*., 2003).

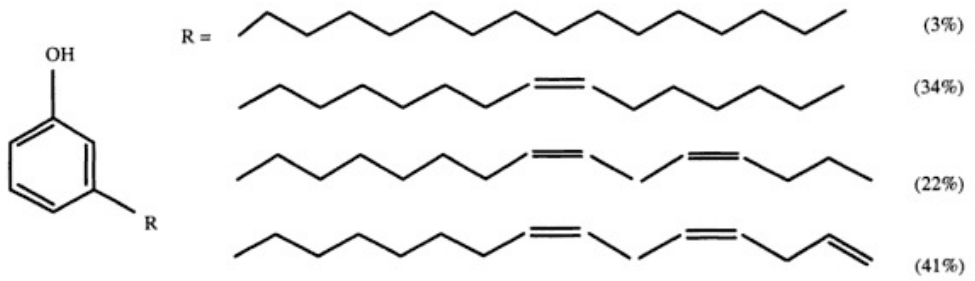


Figure 2.8 Chemical structures of cardanol.

Although cardanol has been used industrially, it is currently under-used compared to the amount of CNSL produced as a cashew nut processing by-product (Chiellini *et al*., 2003). This means that there is a demand to identify new uses for cardanol. Some of which has included resination and used as a coating material, oxidation, and creation of gelatingnanofibers (Chiellini *et al*., 2003). There have also been some examinations of cardanol as an antibacterial agent. One study used anacardic acid (the carrier acid that contains mostly cardanol) against methicillin-resistant *Staphylococcus aureus* (MRSA) which is increasingly a problem for hospitals (Kubo *et al*., 2003). This study found an enhancing effect against MRSA resulting in a fractional inhibitory concentration index of 0.281 (Kubo *et al*., 2003). A second study suggested that cardanol could have a broader antimicrobial effect against gram-positive bacteria (Celis *et al*., 2011). These studies suggest that, like other phenolic compounds that have been tested more thoroughly, cardanol also has antibacterial effects.

2.7 Spectroscopy technique

The main investigative technique of interest in this research is spectroscopy which can be briefly defined as the use of spectrographic information (or wavelength and radiation intensity identification) to identify various compounds (Hollas, 2004). Spectroscopy is commonly used as an experimental method of compound identification, since each compound has its own unique radiation intensity (Hollas, 2004). There are a variety of spectroscopy techniques that can be used depending on available.

The specific technique used in this research is nuclear magnetic resonance (NMR) spectroscopy. NMR spectroscopy uses the magnetic properties of the atomic nuclei, rather than visible light or other spectra, to identify the components involved in a given diagnostic situation (Lambert and Mazzola, 2004). In order to use NMR spectroscopy, the molecule is excited using energy, and then the vibration, rotation, and reversal of the molecule's magnetic field is observed in order to identify the molecule in question (Lambert and Mazzola, 2004). NMR spectroscopy is commonly used in identification of organic compounds (such as phenolic compounds), with between one and four dimensions used to identify molecules of different sizes (Lambert and Mazzola, 2004). This technique is particularly useful for identifying organic compounds because it is both flexible (allowing for small or large samples, using in liquid or solid situations, and so on) and highly accurate (Lambert and Mazzola, 2004). It can be used with proteins, carbohydrates, acids, and

other organic molecules, allowing for rapid identification of the compounds (Lambert and Mazzola, 2004).

Principle of NMR

The physical principle of NMR spectroscopy is a relatively straightforward one. When at rest and not under the influence of a strong magnetic field, the nuclei of the atoms in the compound are randomly oriented (Lambert and Mazzola, 2004). When a strong magnetic field is applied to the compound, the nuclei orient themselves into a particular position and vibrate at a specific frequency, depending on the molecular structure and atomic components of the compound (Lambert and Mazzola, 2004). The strength of this reaction depends on the strength of the magnetic field applied as well as the nuclear magnetic moment of the molecule (Lambert and Mazzola, 2004). A chemical shift then occurs across two distinct spin states (spin up and spin down), allowing for identification of isotopes and compounds through the average resonance and peaks observed during the spin process (Lambert and Mazzola, 2004).

2.8 *p*-iodonitrotetrazolium violet (INT) reaction

A reaction is indicative of the growth of bacteria or fungi. It is based on the transfer of electrons from NADH, a product of the threonine dehydrogenase (TDH) catalyzed reaction, to the tetrazolium dye (*p*-iodonitrotetrazolium violet, INT). TDH from bacteria or fungi catalyses the NAD-dependent oxidation of threonine to form 2-amino-3-ketobutyrate and NADH. If bacteria or fungi have the active growth, an electron is transferred from NADH to INT resulting in a formazan dye which is violet red as described in Figure 2.9.

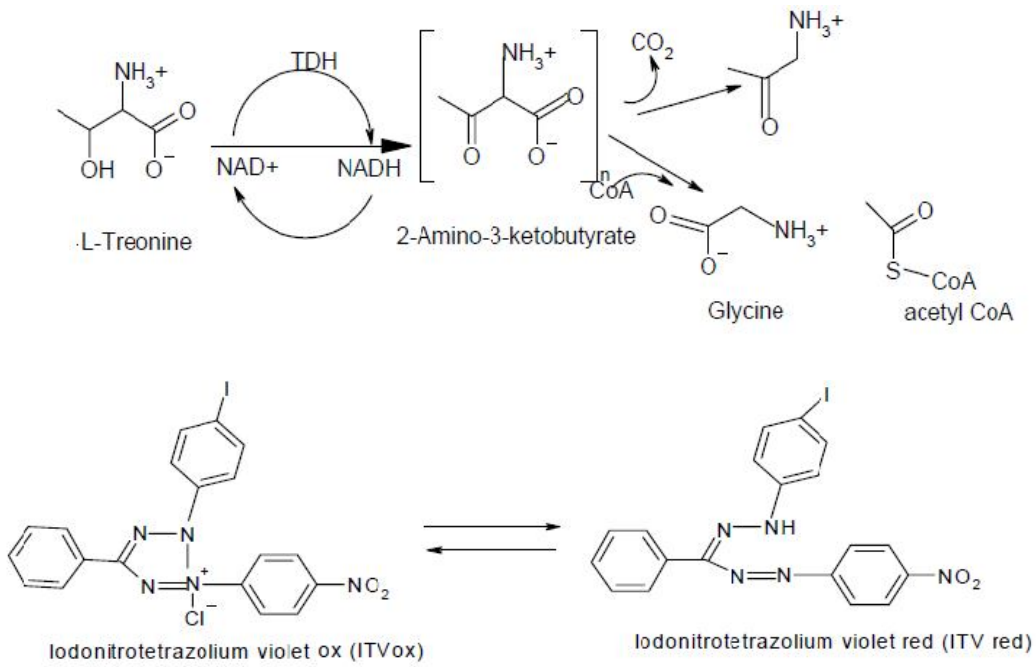


Figure 2.9 INT, a coupling reagent for the colorimetric assay (reaction pathway for the assay of TDH).

2.9 Streptomycin

Streptomycin is an antibiotic drug used to treat infections. It was produced by bacteria, *Streptomyces griseus* and was discovered in 1943 by American biochemists, Selman Waksman, Albert Schatz, and Elizabeth Bugie. It can inhibit the growth of microorganisms by disturbing the synthesis of certain vital proteins. It was the second man-made antibiotics after penicillin but it was the first drug used to treat tuberculosis (Singh and Mitchison, 1954). Later, it was used to treat a variety of diseases. The structure of streptomycin is shown in Figure 2.10.

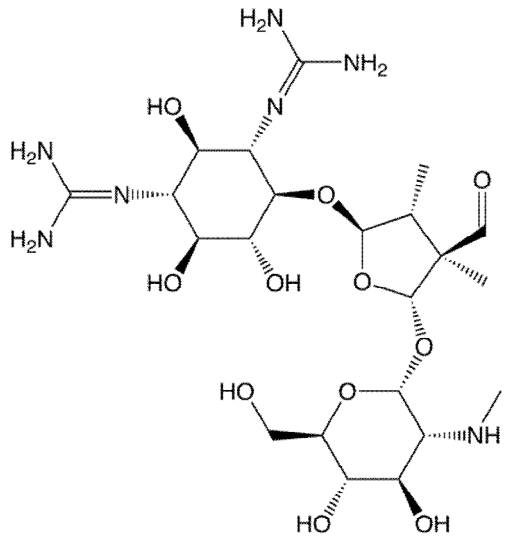


Figure 2.10 Streptomycin (en.wikipedia.org).

2.10 Bacteria

Bacteria are unicellular and prokaryotic. They can be found everywhere on the earth like soil, water, air, living organisms including human. Bacteria are in the range of 0.3 to 2 microns in diameter. Their shape is typically divided into 3 types of coccus, bacillus, and spiral.

In general, normal flora bacteria are found in human body such as upper respiratory tract, skin, gut, and urinary tract, throughout the life time. When a host is healthy, they are harmless. However, it is possible that some can cause the abnormality to the immune system. Besides, a few are beneficial to a host. For example, some types of bacteria in the intestine can make vitamins which can help the host body digest food and absorb nutrients better. Thus, it is possible that normal flora bacteria are opportunistic. For pathogenic bacteria, they can cause many infectious diseases such as tuberculosis, cholera, diarrhea, cystitis, gonorrhea, syphilis, pneumonia, sore throat, and so on. At present, the antibiotic-resistant bacteria keep increasing (Fish, 2002). Meantime, it was reported that some bacteria such as *Lactobacillus* and some strains of *Bifidobacterium* have an antagonistic effect on pathogenic bacteria causing diarrhea, ulcer, colitis, urinary tract and reproductive organ infections, and various allergies, etc (Goderska and Czarnecki, 2007).

The usefulness of bacteria is obviously seen in industry. They have long been used for the production of lactic acid, amino acids, vinegar, soy sauce, dairy

products, antibiotics, and so on. Furthermore, they have been used for the work on genetic engineering and biotechnology including bioremediation.

2.10.1 *Escherichia coli*

E. coli is a Gram-negative, rod-shaped, and facultative anaerobe (Figure 2.11). It is in family Enterobacteriaceae and belongs to coliform group. Normally, it is found in feces of human and warm-blooded animals. It is used as indicators of food and water hygiene. Almost *E. coli* strains are harmless. Thus, it can benefit it hosts through better digestion and vitamin production. However, some serotypes can cause serious food poisoning in human.

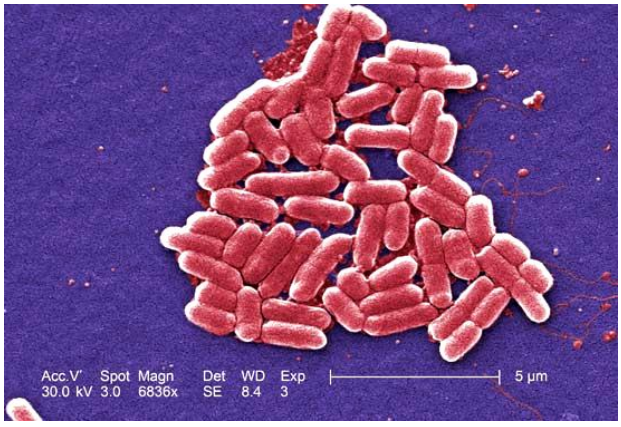


Figure 2.11 Scanning Electron Micrograph of *E. coli* with the magnification of 6,836x (http://www.britannica.com).

2.10.2 *Staphylococcus aureus*

S.aureus is a gram positive coccus (Figure 2.12). Its cell wall contains peptidoglycan and teichoic acid. It is frequently found as normal flora on skin and nasal passages. It can produce enterotoxin causing serious food poisoning and minor skin infection in human. In addition, it is resistant to high temperatures (up to 50 °C), high salt concentrations, and dry condition.

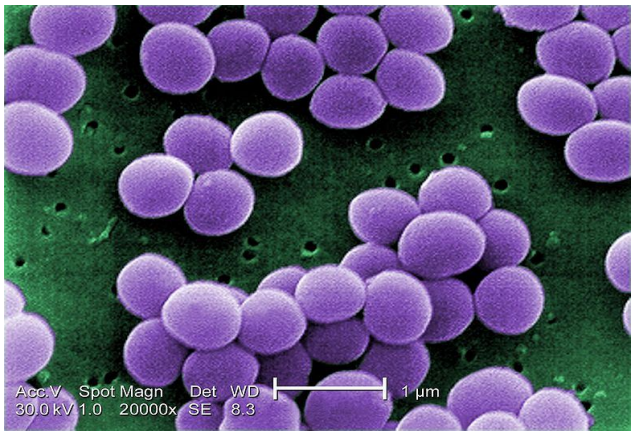


Figure 2.12 Scanning electron micrograph of *S. aureus* with the magnification of 20,000x and false color added (en.wikipedia.org).

2.10.3 *Paenibacillus larvae larvae*

P. larvae larvae is a gram positive and rod shaped bacterium (Figure 2.13). The rod varies in length from about 2.5-5 µm and approximately 0.5 µm in width. It is spore forming. A spore looks oval and its size is roughly 0.6 x 1.3 μm^2. It can be resistant to heat and chemicals for a long time. *P. larvae larvae* can cause American foulbrood disease (AFB), a key larval pathogen of the honey bee, *A. mellifera* which causes the economic lose to bee industry.

Figure 2.13 Light micrograph of *P. larvae larvae* with the original magnification of 1,000x (Rieg *et al.*, 2010).

From the data mentioned above, they led us the interest to search for a new antibacterial agent from propolis of *A. mellifera* in Thailand which hopefully would be fruitful for the health of people themselves and bees in the future. Thus, the purposes of this research would be as follow:

1. To determine the antibacterial activity of *A. mellifera* propolis from Nan province against *S. aureus*, *E. coli,* and *P. larvae larvae*

2. To analyze the chemical structure of active compounds in propolis

CHAPTER III

MATERIALS AND METHODS

3.1 Equipments

- Rotary evaporator, Buchi Rotavapor R-114, Switzerland
- Shaking incubator, Bioer Technology
- Lamina flow, Renowm Technical Co., Ltd.
- Autoclave, Conbraco Industry Inc., USA
- Centrifuge, Hettich, Germany
- Microplate reader, Titertek Multiskan® MCC/340
- Microwave oven, Sharp Carousel R7456, Thailand
- Vacuum column chromatography, NK Laboratory, Schott Duran, Germany
- Round bottom (50, 500, and 1,000 ml in size), NK Laboratory, Schott Duran, Germany
- Varian Mercury^{+} 400 NMR spectrometer
- Mass spectrometer, VG Trio 2000
- Beaker, Pyrex®, Germany

- Filter paper, qualitative circle of 110 mm, Whatman International, Ltd., England
- Flask (50, 250, 500, and 1,000 ml in size), Schott Duran, Germany
- Microtube (1.5 ml in size), Sarstedt, Germany
- Centrifuge tube (15 and 50 ml in size), Sarstedt, Germany
- Tissue culture plate 96 well, DSI, Thermo Fisher Scientific, Denmark
- Petridish, Sterilin, UK
- Ultraviolet light, Electronic Money Detector, Thailand
- Automatic micropipettes (P10, P20, P100, P200, and P1,000), Gilson, France
- Pipette tips (200 and 1,000 µl in size), BioScience, Inc., USA
- Pipette tips (10 µl in size), Axygen Scientific, Inc., USA
- Centrifuge and vortex mixer, model: Centrifuge FVL-2400, Biosan, Latvia
- Electrophoresis chamber set, model: Mupid, Advance Co., Ltd., Japan
- Critical point dryer: Balzers model CPD 020, Liechtenstein
- Sputter coater, Balzers model SCD 040, Liechtenstein

- Scanning electron microscope: JEOL, model JSM-5410LV, Japan
- Premiere® brand 9105 microscope slides

  (Single frosted glass, 75 x 25 x 1 mm^3 in size)

3.2 Chemicals

- Absolute ethanol (C_2H_6O, M = 46.07 g/mol), Merck KGaA Darstadt, Germany
- Hexane ($C_6H_{14}C_6H_{12}$), TSL Chemical, Thailand
- Methylene chloride (CH_2Cl_2), TSL Chemical, Thailand
- Methanol (CH_3OH), TSL Chemical, Thailand
- Silica gel 60 for column chromatography, 0.063-0.200 mm in Ø (O_2Si, M = 60.08 g/mol), Merck KGaA, Germany
- Silica gel 60 G for thin layer chromatography (TLC) (M= 60.08 g/mol), Merck KGaA, Germany
- TLC silica gel 60 F_{254}, Merck KGaA, Germany
- *p*-iodonitrotetrazolium violet, 2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyl-2*H*-tetrazolium chloride (INT), Fluka, Austria

- Dimethyl sulfoxide, Sigma-Aldrich Laborchemikalien GmbH, USA
- Tween 80
- QIAamp ®spin mini kit, cat. # 27104, Qiagen, USA
- QIAquick® PCR purification kit ,cat.# 28104, QIAgen, USA
- Agarose, low EEO, Research Organics Inc., USA
- λ *Hin*dIII marker, Takara, Japan
- 100 bp DNA ladder marker, 5Prime, Germany
- Ethidium bromide ($C_{21}H_{20}BrN_3$, M.W. = 934.32), Bio Basic Inc., USA

3.3 Propolis sampling

Propolis of *Apis mellifera* was collected by scraping at edges, frames, and entrances of bee hives from a bee farm in Pua district, Nan province, Thailand in January, 2011 (Figure 3.1). Propolis was wraped by aluminum foil and kept in the dark at -20 °C until used.

(A)

(B)

(C)

Figure 3.1 Collection of *Apis mellifera* propolis. Some bee hives in an apiary were shown (A). Location of propolis within a bee hive were presented by yellow arrows (B and C).

3.4 Crude extraction

The protocol used in this experiment was modified from Kang *et al*. (2003). Propolis (90 g) was cut to small pieces and was dissolved in 400 ml of 80% MeOH and was shaken at 100 rpm, 15 °C for 18 h. Later, it was centrifuged at 7,000 rpm, 20 °C for 15 min. The supernatant was kept and evaporated by a rotary evaporator (Buchi Rotavapor R-114). The obtained crude MeOH extract of propolis (CME) was kept in the dark at -20 °C until used. Next, the pellet after CME was dissolved by CH_2Cl_2 in the volume of 400 ml and shaken at 100 rpm, 15 °C for 18 h. Later, it was centrifuged at 7,000 rpm, 20 °C for 15 min. The supernatant was kept and evaporated to dryness. The obtained crude CH_2Cl_2 extract of propolis (CDE) was also kept in the dark at -20 °C. Subsequently, the pellet after CDE was then dissolved by hexane in a volume of 400 ml. It was shaken at 100 rpm, 15 °C for 18 h. Then, it was centrifuged at 7,000 rpm, 20 °C for 15 min. The supernatant underwent completely evaporated. The obtained crude hexane extract of propolis (CHE) was also kept in the dark at -20 °C in the dark until used.

The antibacterial activity of CME, CDE, and CHE was assayed by agar well diffusion and microbroth dilution assays as described in 3.6.5 and 3.6.6.

3.5 Chromatography

3.5.1 Quick column chromatography

The protocol was modified from Teerasripreecha *et al*. (2012). A column was packed by silica gel 60 G (0.063-0.2 mm) into the sintered glass (250 ml). A vacuum pump was used for the tightly pack of the silica gel 60 G. The active crude extract (from the experiment in 3.4) was mixed with silica gel 60 until it was not sticky. Then, it was put onto the packed gel. A piece of filter paper (Whatman, qualitative circle of 110 mm in Ø, cat. # 1003110) was laid on top of the gel. Final volume of 1.5 l (500 ml each for 3x) of 100% (v/v) hexane, 25% (v/v) CH_2Cl_2-hexane, 50% (v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, 100% (v/v) CH_2Cl_2, 4% (v/v) MeOH-CH_2Cl_2, and 30% (v/v) MeOH-CH_2Cl_2 was separately loaded into the column for 3 times, respectively. The antibacterial activity of each fraction was, then, assayed by agar well diffusion and microbroth dilution assays as described in 3.6.5 and 3.6.6. The chemical profile of fractions was checked by Thin Layer Chromatography (TLC) as described in 3.5.3. In addition, the active fraction was used for further purification.

3.5.2 Adsorption chromatography

The protocol was modified from Teerasripreecha *et al*. (2012). Active pooled fractions (from the experiment in 3.5.1) would be further purified by adsorption chromatography. The column (250 ml in volume) was packed with silica gel 60

soaking in hexane (200 ml) in the proper height. After the fraction was evaporated and mixed with silica gel 60 (5-7 g) at RT until it was dry. Later, it was laid on top of the column. The left over solvent was removed in order that the sample was better absorbed into the silica gel. Additional silica gel was placed on top in order to make the layer containing the sample smooth. Next, cotton was laid on top and, again, the left over solvent was removed. Five hundred ml of each 100% (v/v) hexane, 50% (v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, and 100% (v/v) MeOH was loaded into the column, respectively. All eluted fractions (2.5 ml/fraction) were collected and the chemical profile of each fraction was determined by TLC as described in 3.5.3. Due to the similar pattern of chemical compounds on the TLC plate, fractions were pooled and evaporated. The antibacterial activity was assayed by agar well diffusion and microbroth dilution assays as described in 3.6.5 and 3.6.6.

3.5.3 Thin layer chromatography (TLC)

The protocol was modified from Teerasripreecha *et al*. (2012). A TLC plate (a silica coated plate) was cut into the size of 5 × 5 cm^2. The first line was drawn above the bottom line at 0.5 cm by a light pencil and the second line was drawn below the top line at 0.5 cm by a dark pencil. Each fraction was small spotted on the lower drawn line of a TLC plate by using a small capillary tube. If the sample was too sticky, it could be diluted by most soluble organic solvent. A sample-spotted TLC plate was air dried. Then, it was placed into a glass chamber containing the solvent system as mobile phase. The solvent system of 100% (v/v) hexane, 50%

(v/v) CH_2Cl_2-hexane, 75% (v/v) CH_2Cl_2-hexane, 100% (v/v) CH_2Cl_2, and 2% (v/v) MeOH-CH_2Cl_2 were used as the mobile phase. The level of solvent system had to be lower than the spotted sample. After the solvent front migrated to the upper drawn line, the TLC plate was pulled out of the chamber and was left at RT until dry. The pattern of migrated compounds on the TLC plate was visualized under the ultraviolet light.

3.6 Antibacterial activity assay

3.6.1 Selected pathogens

Representatives of Gram positive bacteria were *Staphylococcus aureus* (ATCC 20651) and *Paenibacillus larvae larvae* (PL 44) while a representative of Gram negative bacteria was *Escherichia coli* O157: H7. *S. aureus* and *E. coli* were obtained from Department of Medical Sciences, Ministry of Public Health, Thailand while *P. larvae larvae* was obtained from Honeybee Research Group, National Institute of Livestock and Grassland Sciences, Tsukuba, Japan. Three pathogens were confirmed by Gram staining and partial sequence analysis.

3.6.2. Culture of microorganisms

In order to obtained colonies, small amount of *S. aureus* and *E. coli* glycerol stock was picked up by a needle loop and streaked on Luria-Bertani (LB) agar [1 % (w/v) bacto-tryptone, 0.5 % (w/v) bacto-yeast extract, 1 % (w/v) NaCl, and 4.5 %

(w/v) nutrient agar]. The agar plate was incubated at 37 °C for overnight under aerobic condition. In contrast, from the glycerol stock of *P. larvae larvae,* it was grown on brain heart infusion agar (BHI agar) at 35 °C for 48 h under an anaerobic condition.

For bacterial culture, selected single colonies of *S. aureus* and *E. coli* were inoculated into 5 ml LB [1 % (w/v) bacto-tryptone, 0.5 % (w/v) bacto-yeast extract, and 1 % (w/v) NaCl]. It was shaken at 130 rpm, 37 °C for 4 - 8 h. The turbidity of the culture was adjusted with LB to match the turbidity of 0.5 McFarland (OD 0.08 - 0.1 at 625 nm). In contrast, selected single colonies of *P. larvae larvae* were inoculated into BHI broth (5 ml) and incubated at 35 °C for overnight under an anaerobic condition. The turbidity of the bacterial culture was adjusted with BHI broth to match the turbidity of 0.5 McFarland (OD 0.08-0.1 at 625 nm).

3.6.3 Gram staining

It was followed by Gram (1884). Briefly, a loop full of H_2O was spotted on a glass slide and picked up of a targeted colony from an agar plate. Then, it was smeared in a H_2O drop. After the slide was dry by heat, the sample was stained by crystal violet for 1 min and rinsed by H_2O. It was further soaked by iodine solution for 1 min and rinsed by H_2O again. It was decolorized by 95% EtOH. After being dipped into H_2O, it was stained by safranin O for 20-30 sec and rinsed by H_2O. After air dry, the shape and Gram of bacteria was observed under a light microscope.

3.6.4 Partial sequence analysis of *16S rDNA*

DNA extraction (the first method)

E. coli was verified by colony PCR. A small amount of colonies were dissolved in 20 µl of TE buffer, pH 7.5 (10 mM Tris-HCl and 1 mM EDTA) and mixed. The cell suspension was incubated at – 20 °C for 30 min, 95 °C for 5 min, and mixed by vortex. This step was repeated 3 x. Finally, the extracted DNA was kept at -20 ºC until used.

DNA extraction (the second method)

For *S. aureus* and *P. larvae larvae*, the DNA extraction was performed by using QIAMP mini kit (Qiagen, cat. # 27104). Briefly, the overnight culture (1 ml) was centrifuged at 8,000 rpm, RT for 3 min. After the supernatant was removed, the cell pellet was resuspended in 250 µl of buffer P1. It was mixed by 250 µl of buffer P2. After that, 350 µl of buffer N3 was added and mixed. The sample was centrifuged at 13,000 rpm, RT for 10 min. Next, the supernatants was kept and transferred into QIAprep spin column by pipetting. It was centrifuged at 13,000 rpm, RT for 30-60 sec. The flow through (FT) was discarded. Then, 500 µl of buffer PB was added to wash the spin column by being centrifuged at 13,000 rpm, RT for 30-60 sec. The FT was discarded. The buffer PE (750 µl) was repeatedly added to wash the QIAprep spin column again. It was later centrifuged at 13,000 rpm, RT for 30-60 sec and the FT was discarded. In order to confirm the complete removal of wash

buffer, the column was re-centrifuged at 13,000 rpm, RT for another 1 min. After that, the QIAprep column was placed into a clean 1.5 ml microcentrifuge tube. To elute DNA, 30 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added. After the QIAprep spin column was incubated at RT for 1 min, it was centrifuged at 13,000 rpm, RT for 1 min. The concentration (formula 1) and purity (formula 2) of extracted DNA was determined by measuring the absorbance at 260 and 280 nm as below. The quality of extracted DNA was observed by agarose gel electrophoresis. Then, it was kept at -20 ºC until used.

Concentration of DNA (µg/ml) = (Abs260)(dilution factor)(50)*……...(1)

The purity of DNA = Abs260**/Abs280*** ..……...(2)

Whereas: * Extinction coefficient for double-stranded DNA

** the absorbance at 260 nm

*** the absorbance at 280 nm

Agarose gel electrophoresis

An agarose gel [1.2% (w/v)] was prepared. Agarose (0.36 g) was added to 30 ml of 1x TBE buffer (50 mM Tris aminomethane at pH 8.3, 0.65 mM EDTA, and 50 mM Boric acid) and melt. While it was warm, it was poured onto a gel tray with a comb and was left until solidified. DNA (0.5 µg) was mixed with 1x loading dye (Fermentas) before being loaded. λ *Hin*dIII (1.25 µg) and/or 100 bp DNA ladder (0.6

μg) were used as standard DNA markers. The electrophoresis was performed in 1x TBE as running buffer at 80 V for 30 min. After electrophoresed, the gel was stained by 10 μg/ml of ethidium bromide (EtBr) for 10 min and destained in d-H_2O for 30 min. DNA was visualized under ultraviolet light.

PCR amplification

A PCR reaction (20 μl) was composed of d-H_2O (10.5 μl), 1 U of Ex *Taq* DNA polymerase (0.5 μl), 10x buffer (2 μl), 25 mM $MgSO_4$ (2 μl), 10 mM dNTP (2 μl), 10 μM eu27.F (1 μl), 10 μM eu1495.R (1 μl) and DNA template (1 μl). Primers were designed to amplify *16S rDNA* according to Yoshiyama and Kimura (2009). Sequence of forward primer (eu27.F) was 5'-GAG AGT TTG ATC CTG GCT CAG-3' and sequence of reverse primer (eu1495.R) was 5'-CTA CGC CTA CCT TGT TAC GA-3'. The PCR program was composed of initial denaturation at 95 °C for 60 sec, followed by 35 cycles of 95 °C for 60 sec, 55 °C for 60 sec, and 72 °C for 60 sec. Finally, the last extension at 72 °C for 10 min was done. After electrophoresis, the PCR product was purified by using QIAquick® PCR purification kit (Cat. # 28104, Qiagen) and direct sequenced by BigDye Terminator Cycle Sequencing Kit methods using a DNA sequencer (ABI 3730; Applied Biosystems). After that, the obtained nucleotide sequence was blasted to search for the homology of recorded sequences in GenBank.

Purification of PCR product

It was followed by a protocol of QIAquick® PCR purification kit (Cat. # 28104, Qiagen). First, PCR product was mixed by buffer PB (5x volume). A QIAquick spin column was placed in a 2 ml collection tube. It was centrifuged at 13,000 rpm, RT for 30-60 sec. The FT was discarded. A QIAquick column was washed by Buffer PE (750 µl). It was also centrifuged at 13,000 rpm, RT for 30-60 sec. After FT was discarded, the column was further centrifuged at 13,000 rpm, RT for additional 1 min. Next, 20 µl of buffer EB (10 mM Tris-Cl, pH 8.5) was added and incubated at RT for 1 min. Then, it was centrifuged at 13,000 rpm, RT for 1 min. The eluted DNA was kept at -20 ºC.

DNA sequencing

A sequencing reaction was composed of 2 µl of d-H_2O, 5 µl of 5x sequencing buffer (ABI 3730; Applied Biosystems), 10 µl of DNA template (300 ng), 2 µl of Big-Dye enzyme (ABI 3730; Applied Biosystems), and 1 µl of 3.3 µM of primer (either forward and reverse primer for *16S rDNA*). Then, it was amplified under the condition at 96 °C for 60 sec and followed by 25 cycles of 96 °C for 12 sec, 50 °C for 7 sec, 60 °C for 3 min 45 sec. At last, the condition was 60 °C for 30 sec. In order to clean the PCR product, 20 µl of sample was mixed with 2 µl of 3M NaOAc, pH 5.2. Then, it was added by 50 µl of 95% EtOH (2.5x volume) and gently mixed by vortex. After being incubated at RT for 1 min, it was centrifuged at 10,000 rpm, RT

for 15 min. The supernatant was discarded. The pellet was washed by 100 µl of 70% EtOH and spun at 10,000 rpm, RT for 5 min. The supernatant was discarded again and air dry. After that, it was resuspended in 20 µl of Hi-Di formamide and loaded onto the 3730 DNA analyzer for capillary electrophoresis.

3.6.5 Agar well diffusion assay

It was followed by Boorn *et al*. (2010). Briefly, the bacterial culture (10^6 CFU/ml) was spreaded onto an LB agar plate (*S. aureus* and *E. coli*) or BHI agar plate (*P. larvae larvae*) and left at RT until dry. A well in an agar plate was made by a sterile cork borer (9 mm). A tested sample was prepared to be in various concentrations by dissolving in 10% (v/v) DMSO in sterile water which containing 5% (v/v) Tween 80. The tested sample at any desired concentration (100 µl) was transferred into a well. Streptomycin was used as a positive control and 10% (v/v) DMSO containing 5% (v/v) Tween 80 was used as a negative control. Then, an agar plate spreaded with *S. aureus* and *E. coli* was incubated at 37 °C for 12 h and an agar plate spreaded with *P. larvae larvae* was incubated at 35 °C for 24 h under an anaerobic condition. After that the diameter of no growth area (inhibition zone) was measured. Triplication of experiments was done. The data was recorded in term of mean ± S.D.

3.6.6 Microbroth dilution assay

It was followed by Ollar *et al*. (1991). A colony of targeted bacteria was inoculated into LB or BHI broth. The culture was prepared as mentioned earlier in (3.6.2). The culture together with the tested sample at any desired concentration (100 µl) was transferred into a well of a 96-well plate. The culture only was used as a negative control and the culture treated with streptomycin was used as a positive control. Next, the 96-well plate was shaken softly at 80 rpm, 37 °C, for 18 - 24 h or at 80 rpm, 35 °C for 18 - 24 h under an anaerobic condition. After that, 5 µl of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5 phenyl-2H-tetrazolium chloride (INT) at the concentration of 4 mg/ml was added into each well. The lowest concentration of tested sample that made the culture not red is defined as the Minimal Inhibitory Concentration (MIC). Later, 5 µl of the redless culture was spreaded onto an LB agar plate or BHI agar plate. The plate was incubated at the optimum condition of the pathogen as mentioned earlier. The lowest concentration of tested sample that killed colonies totally is defined as Minimal Bactericidal Concentration (MBC). Triplication of experiments was done. The data was recorded in term of mean ± S.D.

3.6.7 Inhibition concentration at 50% (IC_{50})

The absorbance at 600 nm of the culture treated with various concentrations of tested sample (treated groups) was measured. The percentage of cell viability of any treated group was calculated by the following formula.

The percentage of cell viability = $\frac{\text{(Abs of treated group)* x 100}}{\text{(Abs of untreated group)**}}$

* Abs of treated group refers to the absorbance at 600 nm of the culture treated with any tested sample at various concentrations.

** Abs of untreated group refers to the absorbance at 600 nm of the culture only.

Later, a graph was plotted with an X axis representing various concentrations of the tested sample and a Y axis representing the percentage of cell viability. The percentage of cell viability of negative control (untreated group) was set to be 100%. After that, a line was drawn from the point of 50% on Y axis until it reached the curve line. Next, the line was drawn down to an X axis. The reached point on an X axis indicated the IC_{50} value. The data was reported by mean ± S.D. Later, the IC_{50} values were statistically analysed by SPSS statistics (version 17.0).

3.7 Chemical structure analysis

In this research, Nuclear Magnetic Resonance (NMR) was used. The sample was prepared by evaporating the purified active fraction from 3.5.2. Then, 2-3 mg of sample was dissolved in 500 µl of deuterated solvent (deuterated chloroform, $CDCl_3$) and transferred into an NMR tube. Next, the sample was analysed and

interpreted to search for functional groups by a Varian Mercury^{+} 400 NMR spectrometer operated at 400 MHz for ^{1}H and 2D NMR (COSY, HSQC, HMBC) and 100 MHz for ^{13}C nuclei at Department of Chemistry, Faculty of Science, Chulalongkorn University. The chemical shift in δ (ppm) was used to describe signals in the remaining protons in deuterated solvents and TMS was used as an internal standard.

3.8 Time - kill curve

It was followed by Henriques *et al*. (2010). Selected single colonies of *E. coli* O157: H7 were inoculated into a flask containing LB (100 ml) and shaken at 130 rpm, 37 °C for overnight. After that, 200 µl of overnight culture was transferred into a new flask containing LB (100 ml). Six flasks were prepared and divided into 2 groups. As a negative control, the former group had 3 flasks which each flask contained only the culture. The latter group had 3 flasks which each flask contained the culture and the most active fraction at the final concentration of 10x of MIC value. Then, all six flasks were shaken at 130 rpm, 37 °C for 8 h. Every hour, the turbidity was measured at 600 nm.

3.9 Morphology changes

3.9.1 By Scanning electron microscope (SEM)

After the culture of *E. coli* O157: H7 with the most active fraction at the final concentration of 10x of MIC value was shaken at 130 rpm, 37 ℃ for 4 h, it was sent to Scientific and Technological Research Equipment Centre (STREC) of Chulalongkorn University for SEM analysis. The morphology of treated *E. coli* O157: H7 was observed under a SEM (JEOL, model JSM-5410LV).

3.9.2 By Transmission electron microscope (TEM)

The sample was similarly prepared as in (3.9.1). Later, it was sent to Central Laboratory and Greenhouse Complex, Kasetsart University, Kampangsaen campus for TEM analysis.

CHAPTER IV

RESULTS

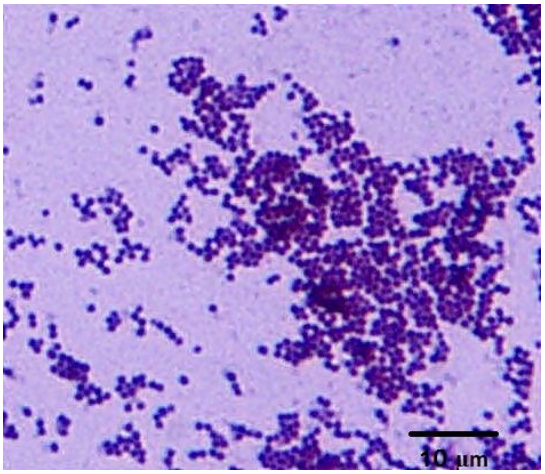
4.1 Crude solvent extract of propolis from *Apis mellifera*

After propolis was partitioned by MeOH, CH_2Cl_2, and hexane, all supernatants were evaporated as mentioned in 3.3. Then, crude MeOH extract (CME), crude CH_2Cl_2 extract (CDE), and crude hexane extract (CHE) could be obtained at the weight of 21.72, 36.31, and 22.5 g, respectively. Considering the characteristics, all crude extracts were sticky.

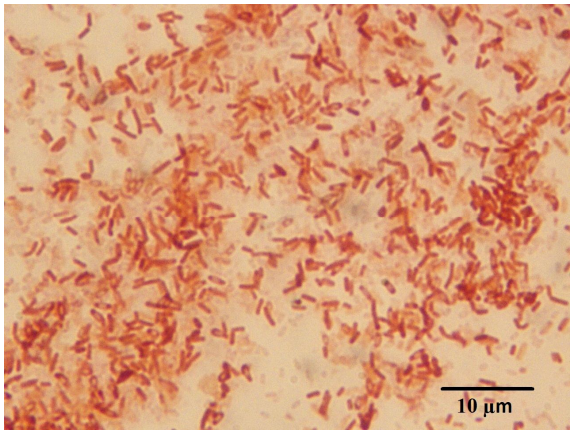
Before all of three crude extracts were tested for antibacterial activity, the correct types of three selected bacteria were confirmed by two methods which were Gram staining and partial sequence of *16S rDNA* analysis.

4.2 Gram staining

Under the light microscope using the oil immersion lens, the violet color of crystal violet could dye the expected *S. aureus*. Thus, it was confirmed for the Gram positive bacteria (Figure 4.2A). In addition, the red color of safranin O could dye the expected *E. coli.* Thus, it was confirmed for the Gram negative bacteria (Figure 4.2B). For this step, Gram staining of *P. larvae larvae* was not done due to the facility inconvenience in Japan.



(A)



(B)

Figure 4.1 Gram stain of selected pathogens. *S. aureus* with the purple color and the cocci shape (A) and *E. coli* with the red color and the rod shape (B) could be observed.

4.3 Partial sequence of *16S rDNA* analysis

After the chromosomal DNA of *S. aureus*, *E. coli*, and *P. larvae larvae* cultures was isolated, it was checked by agarose gel electrophoresis. Under ultraviolet light, the sharp band at high molecular weight was observed which indicated the good condition of the extracted chromosomal DNA. After that, PCR amplifications of partial *16S rDNA* were performed under the optimal condition. The expected PCR product of 1,400 bp was obtained from *S. aureus* (Figure 4.3).

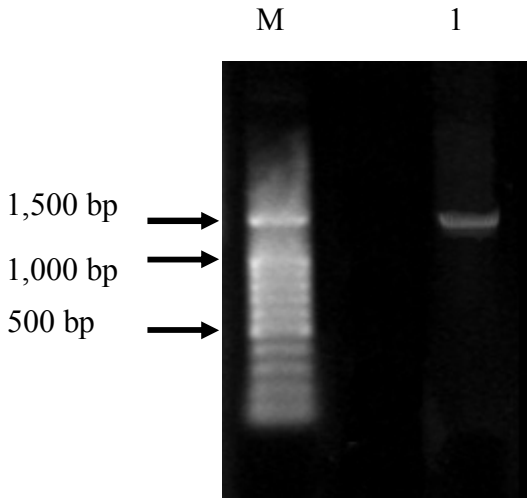


Figure 4.2 Agarose gel electrophoresis (1.2%) of PCR product amplified by primers of *16S rDNA*. Chromosomal DNA of *S. aureus* was used as template. Lane M contained 100 bp DNA ladder as standard marker. Lane 1 represented the PCR product.

Later, the PCR product was purified, sequenced, and searched for similarity. The blasted sequence was confirmed to be the partial sequence of *16S rDNA* of *S. aureus* (Figure 4.4 and Table 4.1).

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80
GAAAGACGGT CTTGCTGTCA CTTATAGATG GATCCGCGCT GCATTAGCTA GTTGGTAAGG AAACGGCTTA CCAAGGCAAC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         90        100        110        120        130        140        150        160
GATGCATATC CGACCTGAGA GGGTGATCGG CCACACTGGA ACTGAGACAG GATCAACACT CAAACGGGAG GCAGCAGTAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         170        180        190        200        210        220        230        240
GGAATCTTCC GCAATGGGCG AAAGCCTGAC GGAGCAACGC CGCGTGAGTG ATGAAGGTCT TCGGATCGTA AAACTCTGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         250        260        270        280        290        300        310        320
ATTAGGGGAA GAACATATGT GTAAGTAACT GTGCACATCT TGGACGGTAC CTAATCAGAA AGCCCCGGCT AACTACGTGC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         330        340        350        360        370        380        390        400
CCAGCAGCCG CGGTAATACG TAGGTGGCAA GCGTTATCCG GAATTATTGG GCGTAAAGCG CGCGTAGGCG GTTTTTTAAG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         410        420        430        440        450        460        470        480
TCTGATGTGA AAAGCCCCAC GGCTCAACCG TGGAGGGTCA TTGGAAACTG GAAAACTTGA GTGCAGAAGA GGGAAAGTGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         490        500        510        520        530        540        550        560
AATTCCATGT GTAGCGGTGA AATGCGCAGA GATATGGAGG AACACCAGTG GCGAAGGCGA CTTTCTGGTC TGTAACTGAC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         570        580        590        600        610        620        630        640
GCTGATGTGC GAAAGCGTGG GGATCAAACA GGATTAGATA CCCTGGTAGT CCACGCCGTA AACGATGAGT GCTAAGTGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         650        660        670        680        690        700        710        720
AGGGGGTTTC CGCCCCTTAG TGCTGCAGCT AACGCATTAA GCACTCCGCC TGGGGAGTAC GACCGCAAGG TTGAAACTCA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         730        740        750        760        770        780        790        800
AAGGAATTGA CGGGGACCCG CACAAGCGGT GGAGCATGTG GTTTAATTCG AAGCAACGCG AAGAACCTTA CCAAATCTTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         810        820        830        840        850        860        870        880
ACATCCTTTG ACAACTCTAG AGATAGAGCC TTCCCCTTCG GGGGACAAAG TGACAGGTGG TGCATGGTTG TCGTCAGCTC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         890        900        910        920        930        940        950        960
GTGTCGTGAG ATGTTGGGTT AAGTCCCGCA ACGAGCGCAA CCCTTAAGCT TAGTTGCCAT CATTAAGTTG GGCACTCTAA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         970        980        990       1000       1010       1020       1030       1040
GTTGACTGCC GGTGACAAAC CGGAGGAAGG TGGGGATGAC GTCAAATCAT CATGCCCCTT ATGATTTGGG CTACACACGT

....|....| ....|....| ....|....| ..
         1050       1060       1070
GCTACAATGG ACAATACAAA GGGCATGAGA GT
```

Figure 4.3 The partial sequence of *16S rDNA* from *S. aureus.*

Table 4.1 Sequence alignment for *S. aureus* confirmation.

Accession	Description	Maximal identity
HE681097.1	*S. aureus* subsp. *aureus,* HO 5096 0412, complete genome	97%
CP003045.1	*S. aureus* subsp. *aureus*, 71193, complete genome	97%
JQ247719.1	*S. aureus* strain SR-05-03, *16S rDNA* gene, partial sequence	97%
CP003033.1	*S. aureus* subsp. *aureus,* VC40, complete genome	97%
JN652892.1	Uncultured *Staphylococcus* sp., clone C14, *16S rDNA* gene, partial sequence	97%

Furthermore, the expected PCR product of 1,400 bp was obtained from *E. coli* (Figure 4.5).

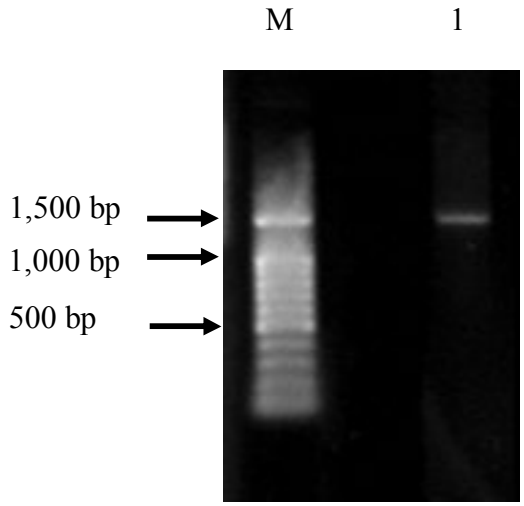


Figure 4.4 Agarose gel electrophoresis (1.2%) of PCR product amplified by primers of *16S rDNA*. Chromosomal DNA of *E. coli* was used as template. Lane M contained 100 bp DNA ladder as standard marker. Lane 1 represented the PCR product.

After the PCR product was purified by using a set of ready-PCR purification kit (QIAgen, Germany), DNA sequencing was performed. After searching for the similarity to the recorded sequences stored in GenBank, the obtained sequence was matched to the *16S rDNA* sequence of *E. coli* (Figure 4.6 and Table 4.2).

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80
CGGGGCTTTC GGCGGTGAGC GCAGCTACCA TGCAGTCGAC GGTAACAGGA GCAGCTTGCT GCTTTGCTGA CGAGTGGCGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          90        100        110        120        130        140        150        160
ACGGGTGAGT AATGTCTGGG AAACTGCCTG ATGGAGGGGG ATAACTACTG GAAACGGTAG CTAATACCGC ATAACGTCGC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          170        180        190        200        210        220        230        240
AAGACCAAAG AGGGGGACCT TCGGGCCTCT TGCCATCGGA TGTGCCCAGA TGGGATTAGC TAGTAGGTGG GGTAAAGGCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          250        260        270        280        290        300        310        320
CACCTAGGCG ACGATCCCTA GCTGGTCTGA GAGGATGACC AGCCACACTG GAACTGAGAC ACGGTCCAGA CTCCTACGGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          330        340        350        360        370        380        390        400
AGGCAGCAGT GGGGAATATT GCACAATGGG CGCAAGCCTG ATGCAGCCAT GCCGCGTGTA TGAAGAAGGC CTTCGGGTTG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          410        420        430        440        450        460        470        480
TAAAGTACTT TCAGCGGGGA GGAAGGGAGT AAAGTTAATA CCTTTGCTCA TTGACGTTAC CCGCAGAAGA AGCACCGGCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          490        500        510        520        530        540        550        560
AACTTCCGTG CCAGCAGCCG CGGTAATACG GAGGGGTTGC AAGCGTTAAT CGGAATTACT GGGCGTAAAG CGCACGCAGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          570        580        590        600        610        620        630        640
CGGTTTGTTA AGTCAGATGT GAAATCCCCG GGCTCAACCT GGGAACTGCA TCTGATACTG GCAAGCTTGA GTCTCGTAGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          650        660        670        680        690        700        710        720
GGGGGGTAGA ATTCCAGGTG TAGCGGTGAA ATGCGTAGAG ATCTGGAGGA ATACCGGTGG CGAAGGCGGC CCCCTGGACG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          730        740        750        760        770        780        790        800
AAGACTGACG CTCAGGTGCG AAAGCGTGGG GAGCAAACAG GATTAGATAC CCTGGTAGTC CACGCCGTAA ACGATGTCGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          810        820        830        840        850        860        870        880
CTTGGAGGTT GTGCCCTTGA GGCGTGGCTT CCGGAGCTAA CGCGTTAAGT CGACCGCCTG GGGAGTACGG CCGCAAGGTT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          890        900        910        920        930        940        950        960
AAAACTCAAA TGAATTGACG GGGGCCCGCA CAAGCGGTGG AGCATGTGGT TTAATTCGAT GCAACGCGAA GAACCTTACC

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
          970        980        990       1000       1010       1020       1030       1040
TGGTTTTGAC ATCCACGGAA GTTTTCAGAA GATGAGAATG TGCCTTCGGG AACCGTGAGA CAGGTGCTGC ATGGCTGTCG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         1050       1060       1070       1080       1090       1100       1110       1120
TCAGCTCGTG TTGTGAAATG TTGGGTTAAG TCCCGCAACG AGCGCAACCC TTATCCTTTG TTGCCAGCGG TCCGGCCGGG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         1130       1140       1150       1160       1170       1180       1190       1200
AACTCAAAGG AGACTGCCAG TGATAAACTG GAGGAAGGTG GGGATGACGT CAAGTCATCA TGGCCCTTAC GACCAGGGCT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         1210       1220       1230       1240       1250       1260       1270       1280
ACACACGTGC TACAATGGCG CATACAAAGA GAAGCGACCT CGCGAGAGCA AGCGGACCTC ATAAAGTGCG TCGTAGTCCG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         1290       1300       1310       1320       1330       1340       1350       1360
GATTGGAGTC TGCAACTCGA CTCCATGAAG TCGGAATCGC TAGTAATCGT GGATCAGAAT GCCACGGTGA ATACGTTCCC
```

Figure 4.5 The partial sequence of *16S rDNA* from *E. coli*.

Table 4.2 Sequence alignment for *E. coli* confirmation.

Accession	Description	Maximal identity
JN811622.1	*Escherichia coli* strain HM01, *16S ribosomal DNA gene*, partial sequence	99%

Furthermore, the expected PCR product of about 400 bp was obtained from *P. larvae larvae* (Figure 4.7).

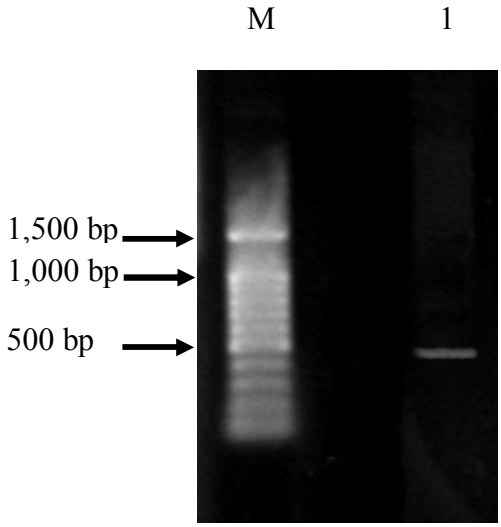


Figure 4.6 Agarose gel electrophoresis (1.2%) of PCR product amplified by primers of *16S rDNA*. Chromosomal DNA of *P. larvae larvae* was used as template. Lane M contained 100 bp DNA ladder as standard marker. Lane 1 represented the PCR product.

After the PCR product was purified by using a set of ready-PCR purification kit (QIAgen, Germany), DNA sequencing was performed. After searching for the similarity to the recorded sequences stored in GenBank, the obtained sequence was matched to the *16S rDNA* sequence of *P. larvae larvae* (Figure 4.8 and Table 4.3).

```
....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         10         20         30         40         50         60         70         80
GGGAAGACGG GGAGCCCCTT TAAAAAACGG TGAGCTAATA GCGGATAGGT GGTGGCTTCG CATGAAGAAG TCATGAAAGA

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         90        100        110        120        130        140        150        160
CGGGGCAACC TGTCACTTAC ACCTGTTTCA AGAGGAGGAT TAGCTAGTTG GTAGGGTAAC GGCTTACCAA CGGGACGATG

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
        170        180        190        200        210        220        230        240
CGTACCGACC TGAGAGGGTG AACGGCCACA CTGGGACTGA GACACGGCCC AGAATACTAG GGGAGGCAGC AGTAGGGAAT

....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....| ....|....|
         250        260        270        280        290        300        310        320
CTTCCGCAAT GGACGAAAGT CCTGACGGAG CAACGCCGCG TGAGTGATGA AGGTTTCCGG ATCGTAAAGC TCTGTTGCCA

....|....| ....|....| ....|....| ....|....| ....|....| ....
         330        340        350        360        370
AAGGAAAAAG TTTTTTTTTG AGTAACTGCC CCGTGGAGTG ACGGTAACTT GAGA
```

Figure 4.7 The partial sequence of *16S rDNA* from *P. larvae larvae*.

Table 4.3 Sequence alignment for *P. larvae larvae* confirmation.

Accession	Description	Maximal identity
AB680856.1	*Paenibacillus larvae* gene for *16S rDNA*, partial sequence, strain: NBRC 15408	86%
FJ538214.1	*Paenibacillus larvae* subsp. *larvae* strain BPSr106, *16S ribosomal DNA* gene, partial sequence	86%
FJ649366.1	*Paenibacillus larvae* strain BMG 259, *16S ribosomal DNA* gene, partial sequence	86%
FJ649365.1	*Paenibacillus larvae* strain BMG 189, *16S ribosomal DNA* gene, partial sequence	86%
FJ649364.1	*Paenibacillus larvae* strain BMG 245, *16S ribosomal DNA* gene, partial sequence	86%

4.4 Screening for antibacterial activity

CME, CDE, and CHE were tested for the antibacterial activity against *S. aureus* (ATCC 20651), *P. larvae larvae* (PL 44), and *E. coli* O157: H7 by agar well diffusion. Also, streptomycin was used as a positive control. The results were recorded as in Table 4.4. It was obvious that only CME at 100 mg/ml showed the antibacterial activity although streptomycin presented the better activity. Among three bacteria, *S. aureus* was the most sensitive to CME with the inhibition zone at 1.73 ± 0.05 cm. Also, it was likely that Gram positive bacteria were more sensitive to CME than Gram negative bacteria. Thus, CME was used for further purification.

Table 4.4. Inhibition zone diameter in cm (Mean ± S.D.) of crude extract of *A. mellifera* propolis by agar well diffusion assay.

Microorganisms / **Sample**	**Inhibition zone diameter (cm)**		
	S. aureus	***E. coli***	***P. larvae larvae***
Streptomycin (200 µg/ml)	2.13 ± 0.05	2.07 ± 0.05	2.07 ± 0.09
Crude propolis (100 mg/ml)			
- CME	1.73 ± 0.05	1.37 ± 0.05	1.53 ± 0.05
- CDE	0	0	0
- CHE	0	0	0

CME was further purified by using quick column chromatography. Seven fractions (fraction Q1-Q7) were collected. The characteristic, weight, and TLC pattern of each fraction was recorded in Table 4.5. Later, all fractions were tested for the antibacterial activity against those selected bacteria by agar well diffusion assay (Table 4.6). There were only two fractions (Q4 and Q5) which could be effective to inhibit the growth of *S. aureus*. Both provided the inhibition zone of 1.50 ± 0.00 and 1.57 ± 0.05 cm. In addition, there were three fractions (Q3-Q5) which could be effective to inhibit the growth of *E. coli* with inhibition zones of 1.47 ± 0.05, 1.17 ± 0.05, and 1.43 ± 0.05 cm, respectively. In contrast, it was apparent that Q1, Q2, Q6, and Q7 at concentration of 100 mg/ml presented no antibacterial activity by agar well diffusion assay.

Table 4.5 Characteristics, weight, and TLC patterns of evaporated fractions from quick column chromatography.

Fractions	Appearance	Weight (g)	TLC pattern
Q1	Yellow and sticky	0.0424	No band
Q2	Yellow and sticky	0.0189	No band
Q3	Yellow and sticky	0.1332	Separated bands
Q4	Yellow and sticky	0.0255	Smear
Q5	Yellow and sticky	0.0336	Smear
Q6	Brown and sticky	0.0253	No band
Q7	Brown and sticky	0.5617	No band

Table 4.6 Inhibition zone diameter in cm (Mean ± S.D.) of fractions from quick column chromatography.

Sample	Inhibition zone diameter (cm)		
	S. aureus	***E. coli***	***P. larvae larvae***
Streptomycin (200 µg)	2.13 ± 0.05	2.07 ± 0.05	2.07 ± 0.09
Quick column chromatography (200 µg)			
- Fraction Q1	0	0	-
- Fraction Q2	0	0	-
- Fraction Q3	0	1.47 ± 0.05	-
- Fraction Q4	1.50 ± 0.00	1.17 ± 0.05	-
- Fraction Q5	1.57 ± 0.05	1.43 ± 0.05	-
- Fraction Q6	0	0	-
- Fraction Q7	0	0	-
Adsorption chromatography (200 µg)			
- Purified fraction A1A	0	1.30 ± 0.08	1.43 ± 0.06

Remark: The inhibition diameter was an average of diameter (triplicate). The diameter of each well was 9 mm and symbol "-" means no test on antimicrobial activity by agar well diffusion assay.

Considering TLC pattern in Table 4.5, fractions Q1, Q2, Q6, and Q7 contained no spot. This observed result was coincided with no activity against the selected bacteria. Due to the thick smear on the TLC plate, fractions Q4 and Q5 were too difficult to be further purified by adsorption chromatography although good activity could be obtained. In contrast, separated bands could be revealed in fraction Q3 resulting in further purified by adsorption chromatography.

After adsorption chromatography, many fractions (over a hundred fractions) were obtained. All collected fractions were evaporated as mentioned in 3.3. The chemical profile was checked by TLC. One dominant spot was obviously discovered in fraction A1A. Its weight of 0.17 g was recorded. It looked sticky and yellow. In addition, it smelled like wax (Figure 4.9).

Figure 4.8 Fraction A1A which was purified by adsorption chromatography.

After purification, the pure compound named A1A was tested for antibacterial activity by agar well diffusion. It effectively inhibited *E. coli* and *P. larvae larvae* with the inhibition zone of 1.30 ± 0.08 and 1.43 ± 0.06 cm, respectively. In contrast, inhibition against *S. aureus* was not observed (Table 4.6).

4.5 Minimum inhibition concentration (MIC) of propolis extracts

MIC of all samples was determined against *S. aureus* and *E. coli* by using microdilution method. After the untreated and treated cultures in a 96-well plate was shaken softly at 80 rpm, 37 °C for 18-24 h, 5 µl of 2-(4-iodophenyl)-3-(4-nitrophenyl)-5 phenyl-2H-tetrazolium chloride (INT) was added to make a final concentration of 4 mg/ml in each well. For interpretation, any sample providing the best antibacterial activity would have the lowest MIC. The MIC of samples from various steps of purification was shown in Table 4.7. The concentration of CME that could change the red culture of both *S. aureus* and *E. coli* to be colorless was 5 mg/ml. Also, CME was effective to inhibit *P. larvae larvae* with the MIC value of 6.25 mg/ml.

For fractions obtained from quick column chromatography, only three active fractions (Q3-Q5) were able to inhibit the growth of both *S. aureus* and *E. coli* with the MIC values of 6.25 and 31.25 µg/ml, respectively. Although the rest of fractions could inhibit the growth of both bacteria, the MIC values could not be observed.

By adsorption chromatography, although many fractions were obtained from fraction Q3, MIC values could not be determined. Thus, the IC_{50} value was calculated instead. It was found that the purified fraction A1A could present the IC_{50} values at 0.175 µg/ml for *E. coli* and at 0.683 µg/ml for *P. larvae larvae* (Table 4.8). Comparing to the IC_{50} value of untreated culture, all obtained IC_{50} values were significantly different at $p \leq 0.05$ (Figures 4.10 and 4.11). In contrast, since MIC of streptomycin could be reported as in Table 4.7, the IC_{50} of streptomycin was not calculated.

Table 4.7 Minimum inhibition concentration of samples against bacterial strains.

Samples	Minimum inhibition concentration (MIC)		
	S. aureus	***E. coli***	***P. larvae larvae***
Streptomycin	12.5 µg/ml	12.5 µg/ml	50 µg/ml
Crude propolis			
- CME	5 mg/ml	5 mg/ml	6.25 mg/ml
- CDE	>500 mg/ml	>500 mg/ml	>500 mg/ml
- CHE	>500 mg/ml	>500 mg/ml	>500 mg/ml
Quick column chromatography			
- Fraction Q1	>100 µg/ml	>100 µg/ml	>100 µg/ml
- Fraction Q2	>100 µg/ml	>100 µg/ml	>100 µg/ml
- Fraction Q3	>100 µg/ml	6.25 µg/ml	>100 µg/ml
- Fraction Q4	31.25 µg/ml	6.25 µg/ml	>100 µg/ml
- Fraction Q5	31.25 µg/ml	6.25 µg/ml	>100 µg/ml
- Fraction Q6	>100 µg/ml	>100 µg/ml	>100 µg/ml
- Fraction Q7	>100 µg/ml	>100 µg/ml	>100 µg/ml
Adsorption chromatography			
- Purified fraction A1A	>50 µg/ml	>50 µg/ml	>50 µg/ml

Table 4.8 The IC_{50} values of fractions after adsorption chromatography.

Sample	IC_{50} values (µg/ml)		
	S. aureus	***E. coli***	***P. larvae larvae***
Purified fraction A1A	ND	0.175	0.683

Remark: ND indicated no data for IC_{50} values since there was no antibacterial activity. All reported data came from the triplication in each experiment.

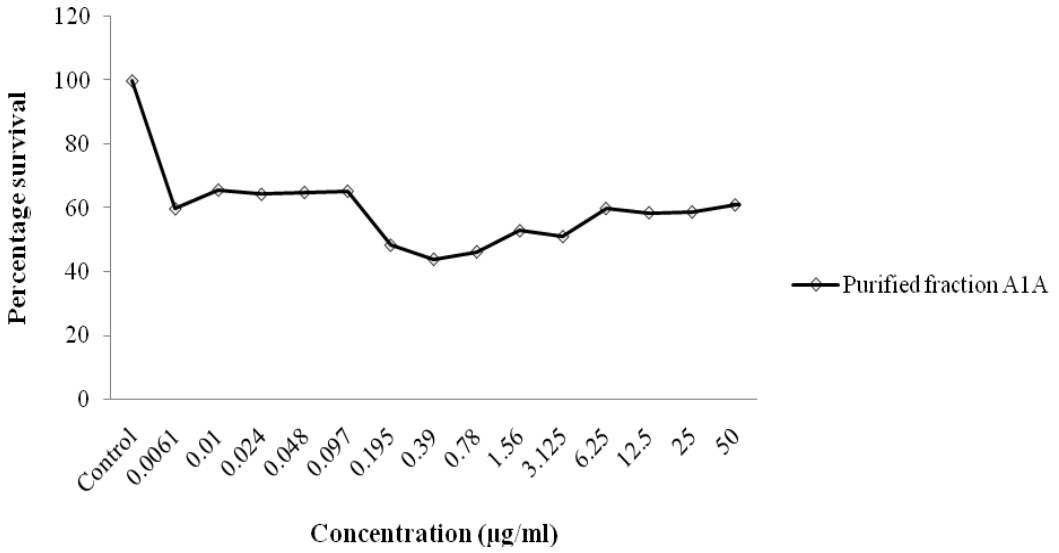


Figure 4.9 Effect of the purified fraction A1A which could inhibit the growth of *E. coli*. The percentage of survival was expressed as mean ± S.D. (µg/ml).

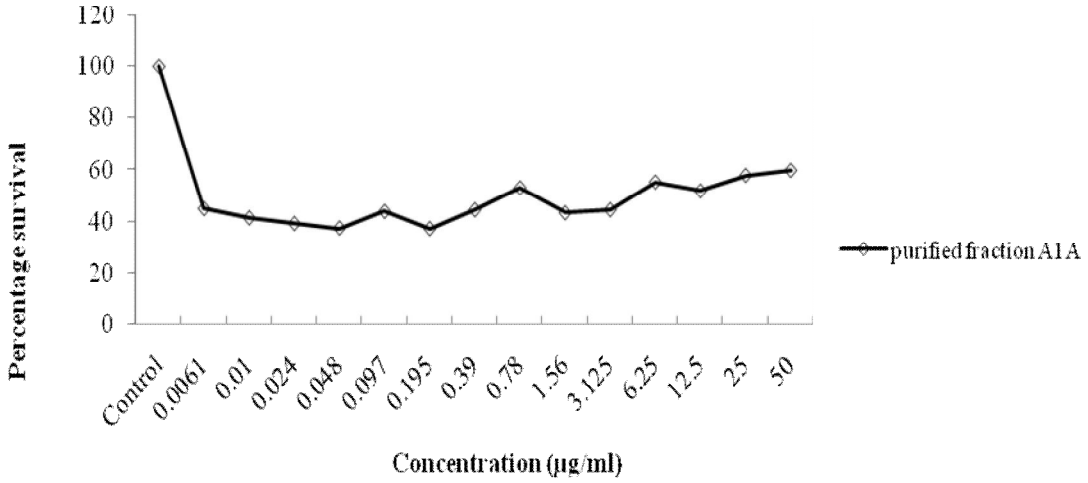


Figure 4.10 Effect of the purified fraction A1A which could inhibit the growth of *P. larvae larvae*. The percentage of survival was expressed as mean ± S.D. (µg/ml).

4.6 Chemical structure analysis

Since fraction A1A was the most active and purified, thus, it was further analysed for chemical structure by using NMR. The data obtained from NMR was as followed: ^{1}H NMR ($CDCl_3$, 400 MHz) δ_H 7.05 (1H, t, J = 7.6 Hz, H-5), 6.67 (1H, d, J = 7.6 Hz, H-6), 6.58 (1H, s, H-2), 6.57 (1H, d, J = 8.0 Hz, H-4), 5.36 (1H, br s, OH), 5.28 (2H, m, olefinic proton), 2.46 (2H, t, J = 7.6 Hz, H-1'), 1.95 (4H, br s), 1.48-1.52 (2H, m), 1.18-1.25 (30H, br s), 0.82 (3H, t, J = 6.8 Hz); ^{13}C NMR ($CDCl_3$, 100 MHz) δ_C 155.4, 145.0, 130.0, 129.9, 129.4, 120.9, 115.3, 112.5, 35.9, 32.0, 31.4, 29.8, 29.7, 29.7, 29.6, 29.6, 29.5, 29.4, 29.3, 27.2, 26.9, 22.4, 14.1; ESIMS *m/z* $[M+H]^+$ in the range of 400-500. Thus, the active compound was in a cardanol group (Figure 4.12).

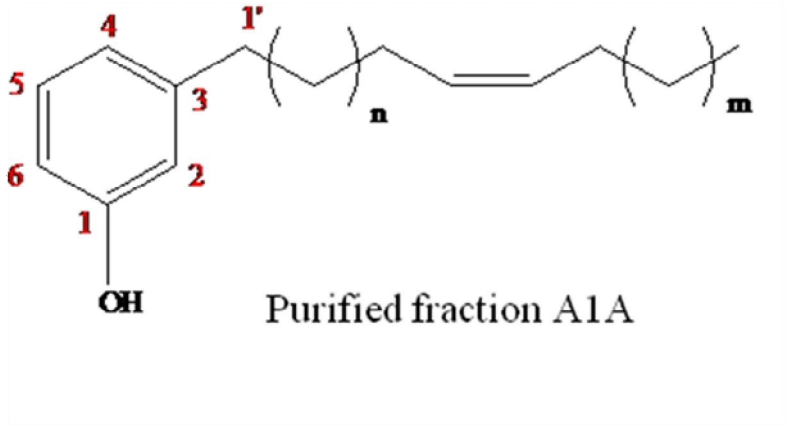


Figure 4.11 The chemical structure of cardanol.

4.7 Effect of cardanol on time - kill curve

According to Table 4.8, *E. coli* was the most sensitive to purified fraction A1A (cardanol) so it was used to assay for time killing. In this experiment, cardanol at ten times of the MIC value was used. Untreated *E. coli* was used as control. Due to Table 4.9, data were presented in terms of the $\log_{10}$ cfu/ml change and were based on the conventional bactericidal activity standard. In over all, the average in log reduction of viable cell count was changed from 5.477 $\log_{10}$ to 7.563 $\log_{10}$ cfu/ml for untreated culture which was noticeably higher than the average values for treated culture. After 1 h of culture, the average in log reduction of treated culture (5.426 $\log_{10}$ cfu/ml) was obviously less than the untreated culture (6 $\log_{10}$ cfu/ml). Furthermore, for the treated culture, during the period of 1-4 h, the average values was changed from 5.523 to 7.039 $\log_{10}$ cfu/ml. Later, during the period of 5-8 h, they were changed from 7.346 to 7.520 $\log_{10}$ cfu/ml. Thus, the obtained result showed that cardol was able to present the bacteriostatic effect on *E. coli* O157: H7 (Table 4.9).

Considering Figure 4.13, it indicated that cardanol could play the anti-*E. coli* activity at the early period of treatment which was before 4 h.

Table 4.9 Period of growth inhibition of *E. coli* by cardanol.

Time (h)	Viable cells (log cfu/ml)	
	Untreated *E. coli*	**Treated *E. coli***
0	5.477	5.477
1	6	5.426
2	6.398	5.523
3	6.8	6.472
4	7.038	7.039
5	7.44	7.346
6	7.508	7.442
7	7.525	7.472
8	7.563	7.52

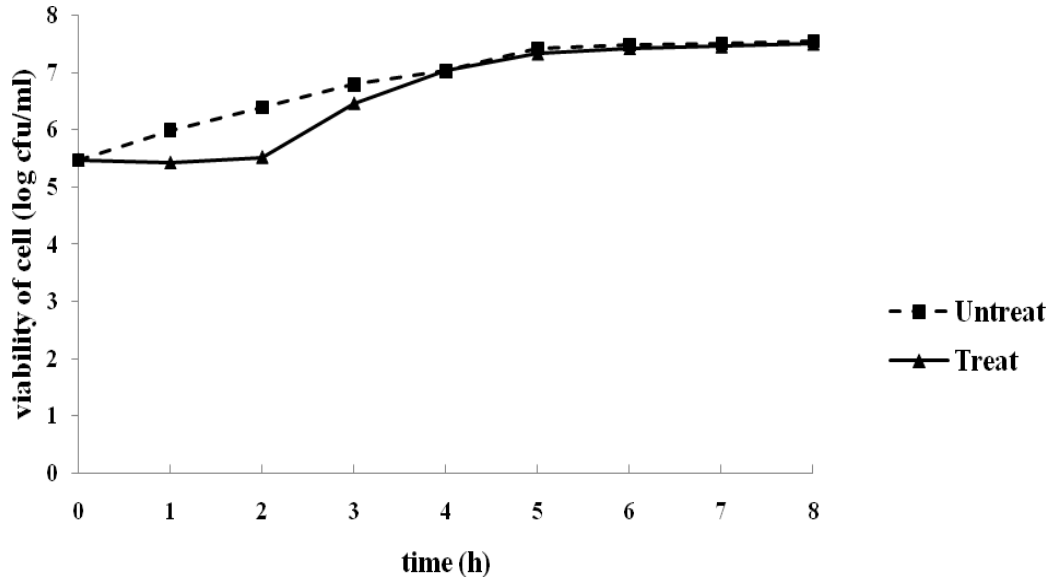


Figure 4.12 The effect of cardanol on the viability of *E. coli.*

4.8 Effect of cardanol on morphology change

Due to Figure 4.13, although the cardanol was able to inhibit the growth of *E. coli* at the early period of incubation only, it was still interesting to find out more parameters those were affected by this compound.

4.8.1 By scanning electron microscope (SEM)

In order to observe the morphology change, at the beginning, untreated and treated cultures were prepared as mentioned in Materials and Methods. After being observed by SEM (model JSM-5410LV, Japan), it was revealed that untreated *E. coli* looked completely long rod in shape with normal growth (Figures 4.14A and 4.14B). However, for treated group, unusual and smaller shape could be noticed. Also, cells preferred to assemble in the culture (Figures 4.14C and 4.14D).

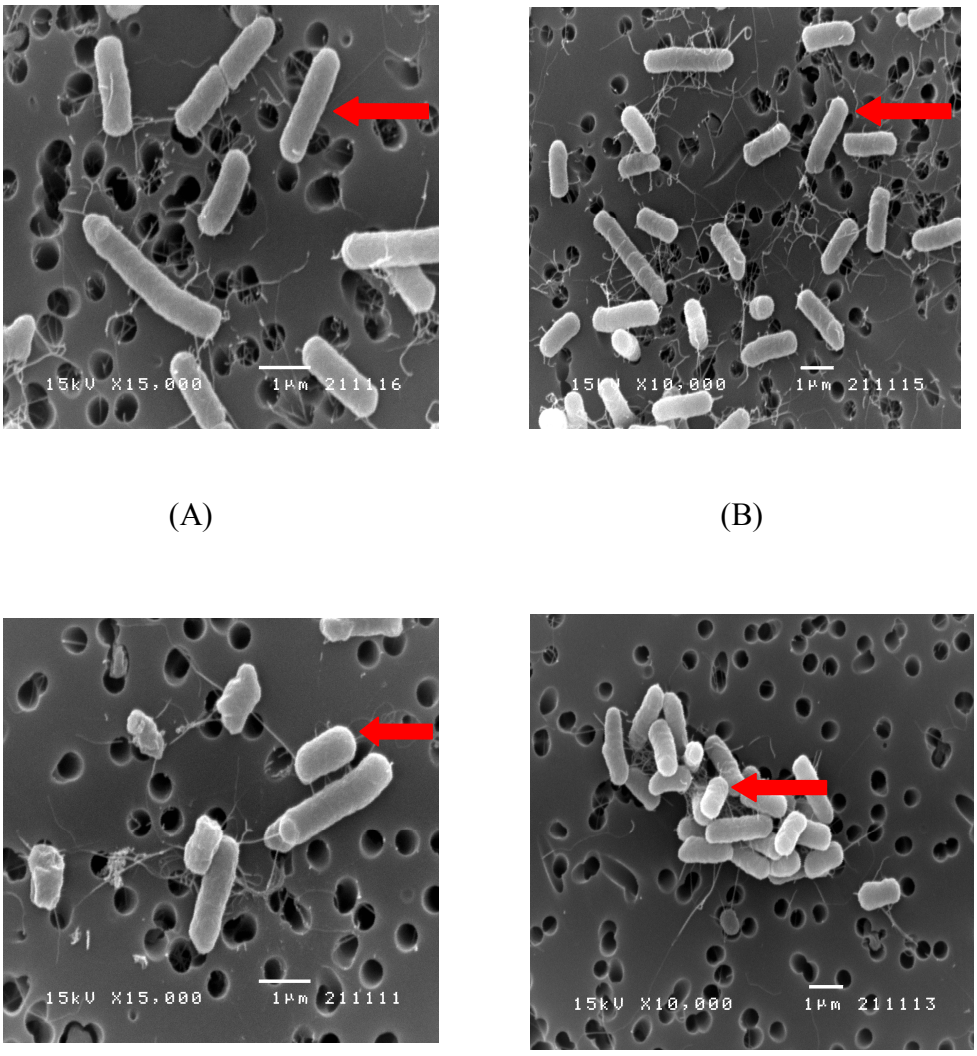
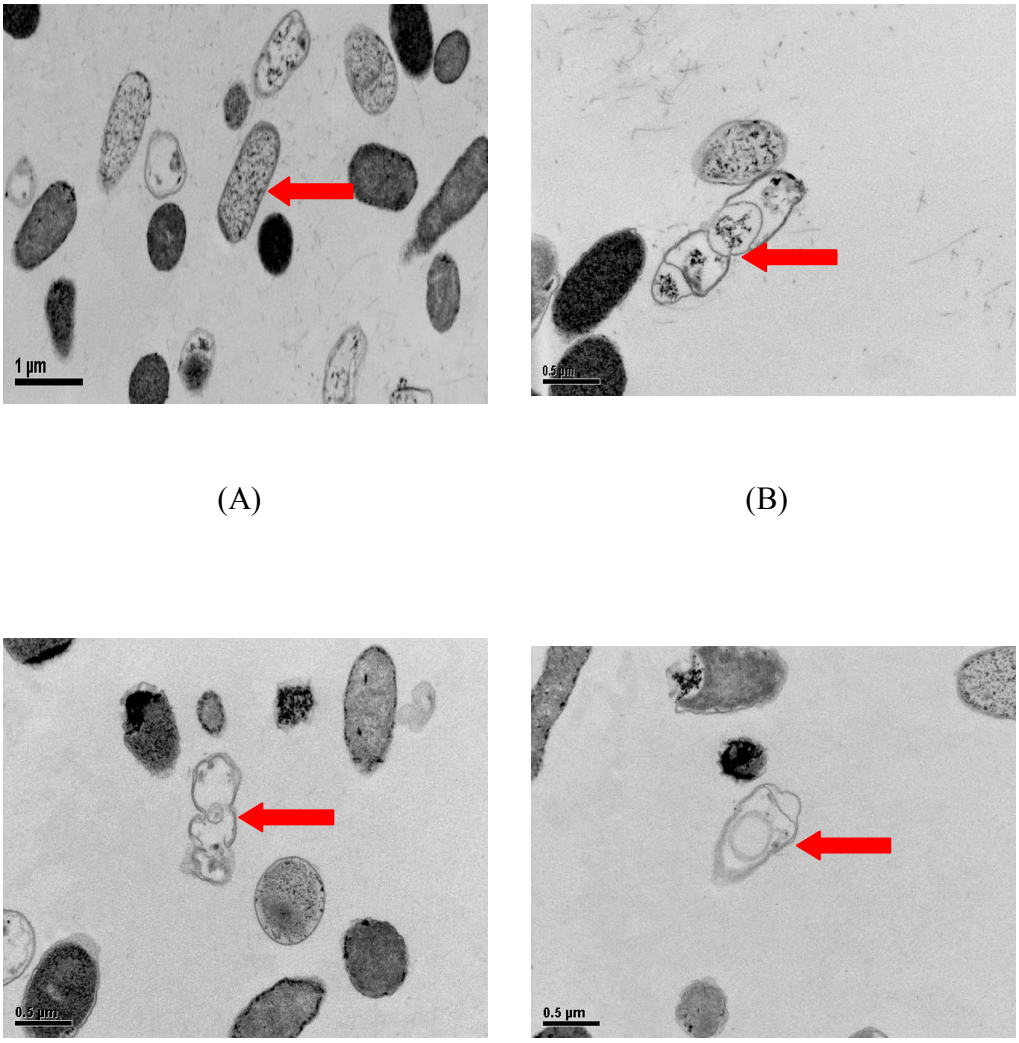


Figure 4.13 The effect of cardanol on the morphology of *E. coli* by SEM. Untreated cells were photographed by using 15,000x magnification (A) and 10,000x magnification (B), respectively. Treated cells were photographed by using 15,000x magnification (C) and 10,000x magnification (D), respectively.

4.8.2 By transmission electron microscope (TEM)

Besides SEM, the morphology of untreated and treated cells were additionally observed by using TEM (JEOL, model JSM-1230 80KV-120KV). Untreated cells of *E.coli* were completely rod-shaped with normal growth (Figures 4.15A and 4.15B).

However, for treated cells, unusual shape, especially dividing cells, could be distinguished. Some cells looked damaged and dead (Figures 4.15C and 4.15D).



(A) (B)

(C) (D)

Figure 4.14 The effect of cardanol on the morphology of *E. coli* by TEM. Untreated cells were photographed by using 15,000x (A) and 25,000x magnification (B), respectively. Treated cells were photographed by using 25,000x (C) and 25,000x magnification (D), respectively but in the different fields.

CHAPTER V

DISCUSSION

Apis mellifera is native to the continents of Europe and Africa (Oldroyd and Wongsiri, 2006). However, it was imported to Thailand for bee farming a long time ago due to its excellent foraging behavior. Honey which is its main product is economic to the country. Other than honey, bee products are royal jelly, bee pollen, bee venom, wax, and propolis. In this research, propolis from *A. mellifera* was focused. It was used to test for the antibacterial activity against *Staphylococcus aureus* (ATCC 20651), *Escherichia coli* O157: H7, and *Paenibacillus larvae larvae* (PL 44). Many previous studies reported that propolis had many bioactivities and various chemical compounds which depended mainly on plant sources, bee species, season, harvesting periods, geographical areas, and other external factors (Bankova *et al*., 2000; Abd El Hady and Hegazi, 2002; Trusheva *et al*., 2006; Moreira *et al*., 2008).

In 2011, Bonvehí and Gutiérrez reported that the anti-*S. aureus* activity of propolis collected from different regions in Basque, the northeastern part of Spain was varied from their origin. The inhibition zone from those samples was in the range of 1.0 - 1.6 cm. Thus, propolis of *A. mellifera* used in this research was collected from Nan province locating in the northern part of Thailand. Considering

the geography of Nan province, it is forestry and mountainous although some areas were already modified for Agriculture. The weather is much different in the summer and the winter. Furthermore, there are various plant types which may provide interesting active compounds on the antibacterial activity.

Nowadays, searching for a new antibacterial agent is still important because the mutation rate of bacteria is high. Antibiotic-resistant bacteria are widely spread and can cause the problem for treatment.

Considering Table 4.4, Gram positive bacteria were more sensitive to CME than Gram negative bateria. The result was coincided to crude ethanol extract of proplis (CEE) from Mongolia, Albania, Egypt, and Brazil. They were more effective to *S. aurues* than *E. coli* (Kujumgiev *et al*., 1999). Also, CEE from Brazil showed the better anti-*S. aureus* activity than anti-*E. coli* activity (Gonsales *et al*., 2006). Furthermore, alcoholic extract of propolis from Argentina presented the better antimicrobial activity to Gram positive bacteria (Nieva Moreno *et al*., 1999).

From the above, it seemed to be that alcohol, both methanol and ethanol, were the suitable solvent to extract propolis. This could be explained that alcohol could remove wax which was one of main components in propolis. Thus, the more purity of crude extract was obtained. In this research, only CME was effective but not CDE and CHE. It could be implied that our active compound (s) should be polar. As known, methonol is high polar while CH_2Cl_2 and hexane are medium and low polar, respectively.

However, Nowacka-Krukowska and Ludwicki (1999) was successful to use hexane to extract propolis and beeswax to get bromfenvinphos which provided many interesting bioactivities. In addition, Pereira *et al*. (1999) used hexane to extract propolis from Brazil to get pentacyclic triterpenoid alkanoates which were effective in many bioactivites. Besides hexane, Tazawa *et al*. (1999) could identify seven new *p*-coumaric acid derivatives along with seventeen known compounds, including four flavonoids, one prenylated phenolic acid, four diterpenoic acids, one lignan, two p-coumaric acid esters and five cinnamic acid derivatives from the ethyl acetate soluble fraction of 75% ethanol extract of Brazilian propolis.

Considering Tables 4.6 and 4.7, after propolis was purified, the better anti-*E. coli* could be noticed. Thus, it was possible that inert compounds or inactive compounds for *E. coli* inhibiting obstacles were more removed. Alternatively, it could be concluded that synergistic effect among compounds could be out of concern at this step. In some cases, crude extract of natural products provided the better result than purified form. For example, crude honey of *Tetragonula laeviceps* provided the better antibacterial activity and antiproliferation than the purified forms (Chartthai, 2010; Jirakannwisal, 2010; Tasaniyananda, 2010).

From Table 4.7, three active fractions (Q3-Q5) were effective to inhibit both *S. aureus* and *E. coli* with MIC values of 31.25 and 6.25 µg/ml, respectively. This result was supported by Koru *et al*. (2007) which the purified form of propolis was active againt Gram positive and Gram negative bacteria. Furthermore, it was

reported that German propolis was very active against *S. aureus* and *E. coli* (Hegazi *et al*., 2000). Unlike our work, some purified forms of propolis had no effect on *E. coli* at all (Kujumgiev *et al*., 1999; Stepanovi *et al*., 2003; Gonsales *et al*., 2006).

After adsorption chromatography, all obtained fractions could inhibit the growth of both bacteria but the MIC values could not be estimated. Thus, the IC_{50} values were calculated instead (Table 4.8). At this step, synergistic effect of compounds need to be considered. In 1999, Scheller *et al*. reported synergism of active compounds in CEE of propolis against the growth of mycobacteria causing tuberculosis. They investigated whether the same synergism and correlation existed between CEE and some anti-tuberculosis drugs on tuberculosis mycobacteria with different degrees of virulence. Furthermore, CEE was found to have a synergistic effect with antibiotics on growth of *S. aureus*. In this case, antagonism was recorded only in one case when *S. aureus* was treated with a mixture of CEE and ethambutol. Thus, it was suggested that a chemical bond could have been formed between this anti-tuberculosis antibiotic and one of the active components of the CEE.

After the MIC values of tested samples were obtained, all treated cultures were spread on either LB or BHI agar plates. The results showed that bacterial colonies could still be appeared (>10 colonies) on any chosen agar plates. Hence, it indicated that our propolis extracts could not kill bacterial colonies totally or no MBC values could be estimated.

After being analyzed by NMR, it was shown that the active compound in the purified fraction A1A was a chemical compound in cardanol group. It was previously reported that cardanol group from Brazilian propolis could inhibit the growth of bacteria (Silva *et al*., 2008). In addition, it was previously reported that cardanol could have a broader antimicrobial effect against Gram positive bacteria (Celis *et al*., 2011). Cardanol belonged to phenolic compounds. Thus, the more content of phenolic compounds should present the better antimicrobial activity. This was supported by the Basque propolis. It had the highest total phenolic content and showed the best antimicrobial activity, comparing to propolis from other parts of Spain (Bonvehí and Gutiérrez, 2011).

Phenolic compounds possesses many bioactivities such as analgesic (pain-killing), anti-inflammatory, anti-asthmatic, antibacterial, anti-carcinogenic, and other effects (Jang *et al*., 2010; Huang *et al*., 2009). These compounds were worldwide found in tropical plants of family Anacardiaceae, both in the native and cultivated culture (Trevisan *et al*., 2006). Economic plants in this family were cashew nut, mango, and ginkgo (Gellerman *et al*., 1976). Since many components in propolis were from plants, it was reasonable that phenolic compounds were from plants.

Considering time-kill by cardanol on *E. coli*, during 1-4 h, it seemed that the targeted sample could inhibit the growth of *E. coli* with a range of 5.523 $\log_{10}$ cfu/ml to 7.039 $\log_{10}$ cfu/ml (Table 4.9 and Figure 4.13). Thus, it could be said that the anti-*E. coli* activity of cardanol was dose- and time- dependent. The activity like this

could always been observed in many natural products (Nostro *et al*., 2001; Umthong *et al*., 2009).

In this research, a mechanism on how cardanol affects the bacteria was still unknown but the change in morphology was already reported. Previously, it was reported that treated bacterial cell membrane could be destroyed because of the instability of enzymes involving in the DNA repair pathway. Also, dead cells could occur due to the misfunction of poly ADP ribose polymerase (PARP) (Satoh and Lindahl, 1992).

The very interesting result of this work was in Table 4.7, our cardanol gave us the better MIC value (6.25 μg/ml) than streptomycin (12.5 μg/ml). Thus, it is a promising anti-*E. coli* agent. In the future, more experiments need to be performed which later may lead to synthesize it as a pure compound and develop it as a pharmaceutical product.

CHAPTER VI

CONCLUSIONS

1. *S. aureus* (a representative of Gram positive bacteria), *P. larvae larvae* (Gram positive bacteria), and *E. coli* (a representative of Gram negative bacteria) were confirmed by Gram staining (except *P. larvae larvae*) and partial *16S rDNA* gene analysis. Purple cocci shape and red rod were observed for *S. aureus* and *E. coli*. By PCR amplification, DNA sequencing, and blastN, the maximal identity with 97%, 86%, and 99% to *S. aureus*, *P. larvae larvae,* and *E. coli,* respectively.

2. Propolis of *Apis mellifera* collected from Nan province was assayed for the antibacterial activity of those bacteria by agar well diffusion and microbroth dilution assay. Among CME, CDE, and CHE, CME provided the best antibacterial activity with the MIC value of 5 mg/ml and was effective to inhibit both *S. aureus* and *E. coli*. In addition, CME was effective to inhibit *P. larvae* with the MIC value of 6.25 mg/ml.

3. CME was further purified by quick column chromatography. Fractions Q3-Q5 were able to inhibit the growth of *E. coli* with the MIC value of 6.25 µg/ml while the MIC value of streptomycin was 12.5 µg/ml.

4. After NMR analysis, the active compound in the purified fraction A1A was in a cardanol group.

5. The morphology of *E. coli* treated with cardanol at the 10x concentration of IC_{50} value was changed by observing under SEM and TEM. Unusual, smaller shape and damage outer membrane of treated cells could be revealed.

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APPENDICES

APPENDIX A

Raw data of the inhibition zone diameter in cm from agar well diffusion assay.

Table A: The Inhibition zone diameter in cm from agar well diffusion assay by crude propolis of *Apis mellifera*.

Microorganism / Sample	*E. coli*			*S. aureus*			*P. larvae*		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Streptomycin (200 µg)	2.1	2	2.1	2.1	2.2	2.1	2.2	2.0	2.0
CME (100 µg)	1.4	1.3	1.4	1.8	1.7	1.7	1.5	1.6	1.5
CDE (100 µg)	-	-	-	-	-	-	-	-	-
CHE (100 µg)	-	-	-	-	-	-	-	-	-

Remark: The symbol of "-" means no inhibition zone.

Table B: Inhibition zone diameter in cm from agar well diffusion assay on antibacterial activity of all fractions by column chromatography.

Test microorganisms	*E. coli*			*S. aureus*			*P. larvae*		
	1	**2**	**3**	**1**	**2**	**3**	**1**	**2**	**3**
Fraction Q1 (200 µg)	-	-	-	-	-	-	ND	ND	ND
Fraction Q2 (200 µg)	-	-	-	-	-	-	ND	ND	ND
Fraction Q3 (200 µg)	1.4	1.5	1.5	-	-	-	ND	ND	ND
Fraction Q4 (200 µg)	1.2	1.2	1.1	1.5	1.5	1.5	ND	ND	ND
Fraction Q5 (200 µg)	1.5	1.4	1.4	1.5	1.6	1.6	ND	ND	ND
Fraction Q6 (200 µg)	-	-	-	-	-	-	ND	ND	ND
Fraction Q7 (200 µg)	-	-	-	-	-	-	ND	ND	ND
Purified fraction A1A (200 µg)	1.4	1.2	1.3	-	-	-	1.37	1.51	1.42

Remark: The symbol of "-" means no inhibition zone and symbol of "ND" means no test on antimicrobial activity by agar well diffusion assay.

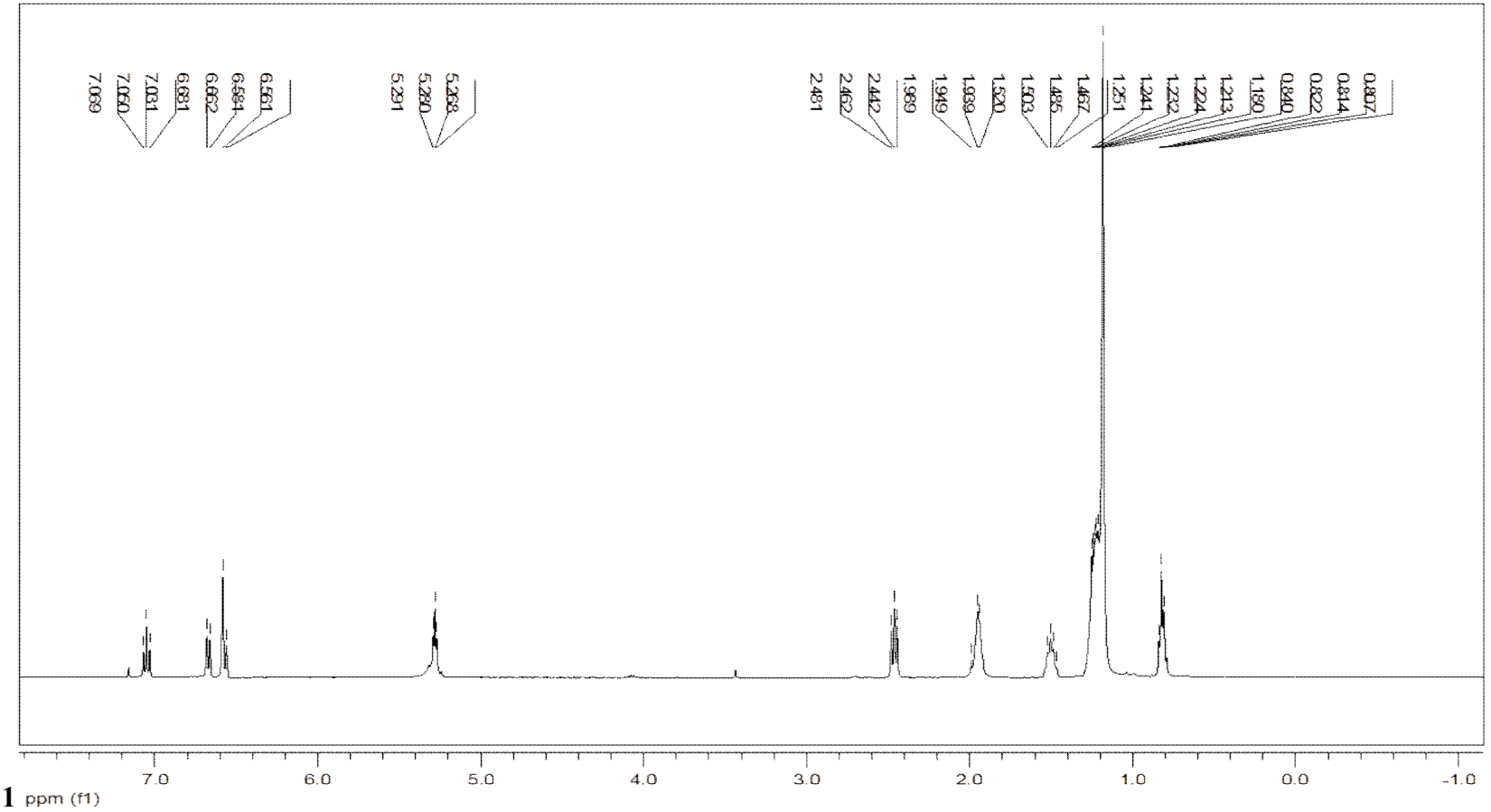
APPENDIX B

Raw data of the absorbance values at 600 nm of *E. coli* and *P. larvae* after treating by purified fraction A1A.

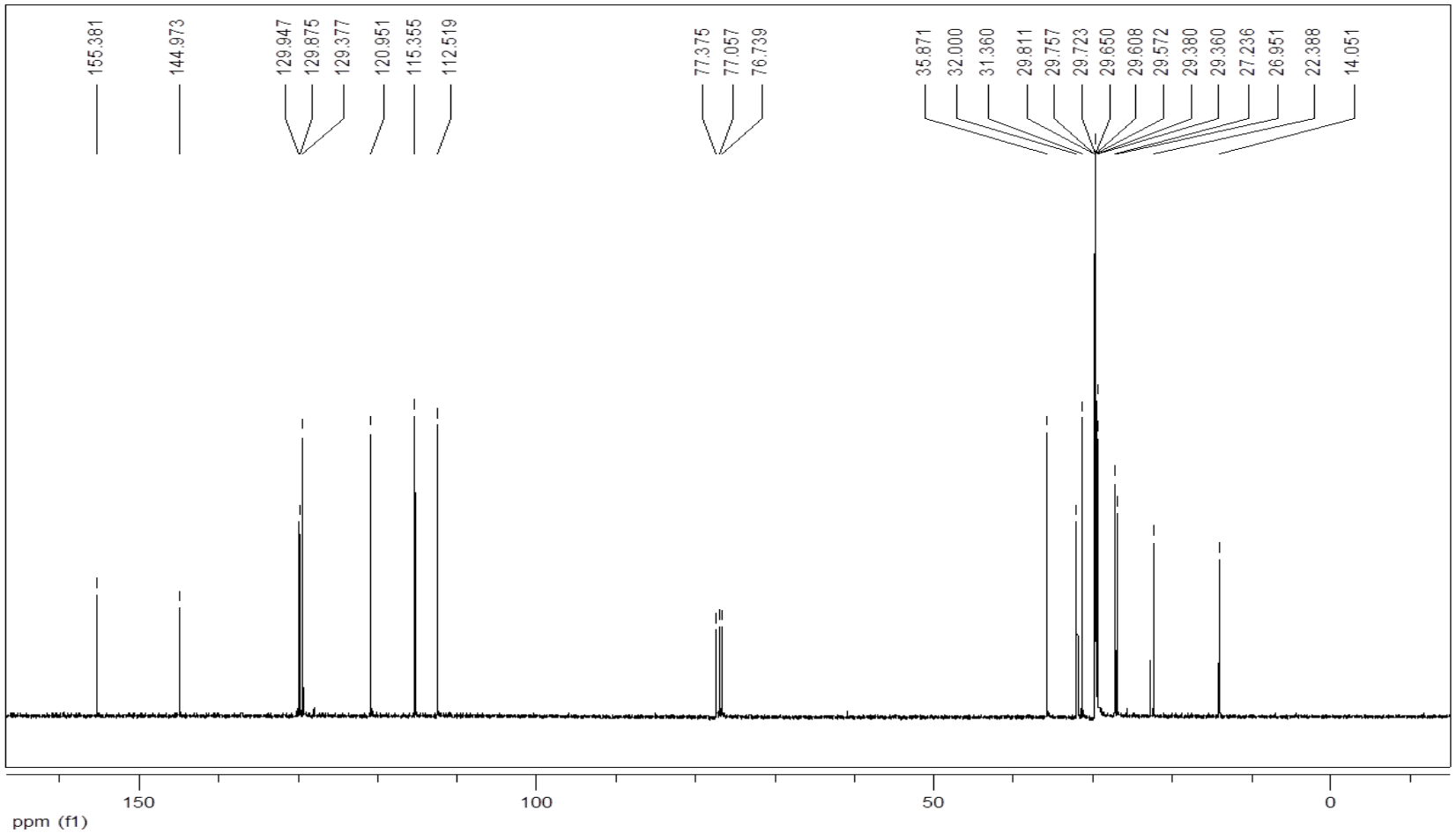
Bacteria	purified fraction A1A (µg/ml)														
	Control	**0.0061**	**0.01**	**0.024**	**0.048**	**0.097**	**0.195**	**0.39**	**0.78**	**1.56**	**3.125**	**6.25**	**12.5**	**25**	**50**
E. coli	0.809	0.483	0.537	0.518	0.523	0.526	0.390	0.353	0.374	0.428	0.422	0.491	0.476	0.479	0.498
	0.778	0.468	0.514	0.503	0.516	0.52	0.382	0.341	0.37	0.395	0.378	0.478	0.474	0.472	0.495
	0.852	0.507	0.551	0.548	0.545	0.547	0.407	0.371	0.384	0.467	0.445	0.492	0.478	0.483	0.501
Average	0.813	0.486	0.534	0.523	0.528	0.531	0.393	0.355	0.376	0.43	0.415	0.487	0.476	0.478	0.498
Percentage of survival	100.00	59.78	65.68	64.33	64.94	65.31	48.34	43.91	46.25	52.89	51.05	59.9	58.55	58.79	61.01
P. larvae	0.923	0.419	0.385	0.366	0.349	0.410	0.347	0.411	0.522	0.424	0.433	0.524	0.485	0.540	0.598
	0.926	0.412	0.385	0.364	0.347	0.407	0.341	0.413	0.487	0.392	0.415	0.513	0.476	0.540	0.622
	0.923	0.414	0.376	0.356	0.342	0.398	0.344	0.409	0.458	0.384	0.385	0.493	0.473	0.522	0.559
Average	0.925	0.415	0.382	0.362	0.346	0.405	0.344	0.411	0.489	0.400	0.411	0.510	0.478	0.534	0.593
Percentage of survival	100.00	44.86	41.30	39.14	37.41	43.78	37.19	44.43	52.86	43.24	44.43	55.14	51.68	57.73	59.68

APPENDIX C

The proton NMR spectrum data of fraction A1A



The carbon NMR spectrum of fraction A1A



APPENDIX D

Raw data of the absorbance values at 600 nm to test the effect of cardanol on the viability of *E. coli*.

The viability of cell	**Time (h)**								
	0	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Untreated	-0.009	0.031	0.075	0.400	0.688	0.861	0.965	1.006	1.104
	-0.009	0.031	0.076	0.420	0.702	0.871	0.964	1.008	1.100
	-0.009	0.028	0.074	0.380	0.686	0.854	0.972	1.004	1.090
Average	-0.009	0.030	0.075	0.400	0.692	0.862	0.967	1.006	1.098
Treated	-0.009	-0.008	0.010	0.088	0.328	0.667	0.830	0.889	0.994
	-0.009	-0.009	0.009	0.087	0.323	0.664	0.827	0.888	0.990
	-0.009	-0.007	0.011	0.092	0.333	0.667	0.833	0.893	0.995
Average	-0.009	-0.008	0.010	0.089	0.328	0.666	0.830	0.890	0.993

APPENDIX E

Preparation of 0.5 Mc Farland

- 1% (v/v) Sulfuric acid (H_2SO_4)

- 1.175% (w/v) Barium chloride ($BaCl_2$)

Preparation of agarose gel electrophoresis

- 1.2% (w/v) agarose gel

agarose	0.3 g
1x TBE buffer	25 ml

- 1x Tris Boric EDTA buffer (TBE buffer), pH 8.0

Tris aminomethane (50 mM)	108 g
EDTA (0.65 mM)	7.44 g
Boric acid (50 mM)	50.4 g

Adjust pH to be 8.0 and quantitate the volume to be 1,000 ml.

BIOGRAPHY

Miss Pattaraporn Boonsai was born on March 14th, 1988 in Sakon Nakhon province, Thailand. She graduated with a Bachelor's Degree in Biology from Department of Biology, Faculty of Science, Chulalongkorn University in 2009. At present, she is a graduate candidate in Master's Degree in Zoology, Department of Biology, Faculty of Science, Chulalongkorn University.

Presentation:

1. **Boonsai, P**., Phuwapraisirisan, P., and Chanchao, C. 2012. Antibacterial activity and chemical compositions of *Apis mellifera* propolis extracts from Nan province, Thailand. Proceedings. The 1st Asean Plus Three Graduate Research Congress, Chiang Mai, Thailand. Number ST62, p. 335-340.
2. **Boonsai, P**., Phuwapraisirisan, P., and Chanchao, C. 2012. Antibacterial activity and chemical compositions of *Apis mellifera* propolis extracts from Nan province, Thailand. Abstract. The 17th Biological Sciences Graduate Congress, Bangkok, Thailand. p. 60.