การศึกษาฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ลดระดับน้ำตาลในเลือดและฤทธิ์ต้านแบคทีเรียของสารสกัดน้ำจากใบกฤษณา *AQUILARIA CRASSNA*

นางสาวศิริลักษณ์ กมลวรรณสิทธิ์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

สาขาวิชาชีวเวชศาสตร์

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ปีการศึกษา 2556

STUDY ON ANTIOXIDANT, ANTIHYPERGLYCEMIC AND ANTIBACTERIAL ACTIVITIES OF THE AQUEOUS EXTRACT OF *AQUILARIA CRASSNA* LEAVES

Sirilak Kamonwannasit

A Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Doctor of Philosophy in Biomedical Sciences

Suranaree University of Technology

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STUDY ON ANTIOXIDANT, ANTIHYPERGLYCEMIC AND ANTIBACTERIAL ACTIVITIES OF THE AQUEOUS EXTRACT OF *AQUILARIA CRASSNA* LEAVES

Suranaree University of Technology has approved this thesis submitted in partial fulfillment of the requirements for the Degree of Doctor of Philosophy.

Thesis Examining Committee

_____

(Asst. Prof. Dr. Rungrudee Srisawat)

Chairperson

_____

(Assoc. Prof. Dr. Nuannoi Chudapongse)

Member (Thesis Advisor)

_____

(Assoc. Prof. Dr. Sajeera Kupittayanant)

Member

_____

(Asst. Prof. Dr. Duangkamol Maensiri)

Member

_____

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Member

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ศิริลักษณ์ กมลวรรณสิทธิ์ : การศึกษาฤทธิ์ต้านอนุมูลอิสระ ฤทธิ์ลดระดับน้ำตาลในเลือด และฤทธิ์ต้านแบคทีเรียของสารสกัดน้ำจากใบกฤษณา *AQUILARIA CRASSNA* (STUDY ON ANTIOXIDANT, ANTIHYPERGLYCEMIC AND ANTIBACTERIAL ACTIVITIES OF THE AQUEOUS EXTRACT OF *AQUILARIA CRASSNA* LEAVES) อาจารย์ที่ปรึกษา : รองศาสตราจารย์ ดร.นวลน้อย จูฑะพงษ์, 98 หน้า.

Agarwood หรือ กฤษณา (ไม้หอม) เป็นพืชจัดอยู่ในจีนัส *Aquilaria* (วงศ์ Thymelaeaceae) ในเอเชียพืชชนิดนี้ถูกนำไปผลิตเครื่องหอมและน้ำหอม นอกจากนั้นยังถูกนำมาใช้เป็นยาแผนโบราณเพื่อสงบประสาท แก้ปวดและยาช่วยในการย่อยอาหารในประเทศแถบเอเชียตะวันออกและประเทศจีน มีการนำใบอ่อนของพืชกฤษณา สปีชีส์ *Aquilaria crassna* ที่ปลูกโดยเกษตรกรมาผลิตเป็นชาเพื่อสุขภาพกันอย่างแพร่หลายในประเทศเวียดนาม กัมพูชาและไทย ผู้ผลิตได้ทำการประชาสัมพันธ์เพื่อส่งเสริมการขายว่ามีฤทธิ์ทางเภสัชวิทยาหลายประการโดยปราศจากข้อมูลสนับสนุนทางวิทยาศาสตร์ การศึกษาวิจัยนี้มีวัตถุประสงค์เพื่อศึกษาฤทธิ์ทางเภสัชวิทยาของสารสกัดจากใบกฤษณา ได้แก่ ฤทธิ์และกลไกการออกฤทธิ์ในการต้านเชื้อแบคทีเรีย รวมทั้งฤทธิ์ในการลดระดับน้ำตาลในเลือด นอกจากนั้นยังได้ทำการตรวจสอบพฤกษเคมีเบื้องต้นและฤทธิ์ต้านอนุมูลอิสระตลอดจนทดสอบความเป็นพิษอย่างเฉียบพลันของสารสกัดด้วย

การตรวจสอบพฤกษเคมีเบื้องต้นพบว่าสารสกัดน้ำจากใบกฤษณา *A. crassna* ประกอบด้วย แทนนิน ฟลาโวนอยด์ ซาโปนิน คาร์ดิแอกไกลโคไซด์ และสารประกอบฟีนอลิก การตรวจสอบฤทธิ์การต้านอนุมูลอิสระซึ่งทำโดยวิธี DPPH ABTS และ FRAP scavenging methods แสดงให้เห็นว่าสารสกัดมีฤทธิ์ในการต้านอนุมูลอิสระค่อนข้างแรงเมื่อเปรียบเทียบกับสารมาตรฐาน ascorbic acid และ butylated hydroxytoluene การศึกษาฤทธิ์ต้านแบคทีเรียโดยวิธี macro-dilution พบว่าสารสกัดสามารถยับยั้งการเจริญของเชื้อ *Staphylococcus epidermidis* โดยมีค่า MIC เท่ากับ 6 มิลลิกรัมต่อมิลลิลิตร ขณะที่ค่า MBC เท่ากับ 12 มิลลิกรัมต่อมิลลิลิตร ผู้วิจัยทำการสังเกตการเปลี่ยนแปลงทางด้านสัณฐานวิทยาด้วยจุลทรรศน์อิเล็กตรอนแบบส่องกราดและศึกษาฤทธิ์ต่อการสร้างไบโอฟิล์มด้วยการใช้กล้องจุลทรรศน์แบบเฟสคอนทราสท์ พบว่าสารสกัดไม่เพียงแต่มีผลทำให้เซลล์บวมและบิดเบี้ยวเท่านั้นแต่สามารถยับยั้งการสร้างไบโอฟิล์มได้อีกด้วย และจากการใช้กล้องจุลทรรศน์อิเล็กตรอนแบบส่องผ่านพบว่าหลังจากบ่มสารสกัดนาน 24 ชั่วโมง ผนังเซลล์แบคทีเรียเกิดการแตกเสียหายขึ้น สำหรับการศึกษาฤทธิ์ลดระดับน้ำตาลในเลือดได้ทำการศึกษาโดยใช้หนูขาวที่ถูกชักนำให้เกิดภาวะเบาหวานด้วยสเตรปโตโซโตซิน จากการทดลองพบว่าระดับน้ำตาลในเลือดไม่ลดลงหลังจากหนูได้รับสารสกัดเป็นเวลา 15 วัน ต่างจากกลุ่มของหนูที่ได้รับยาต้านเบาหวานไกลเบนคลาไมด์ ผลการทดลองในครั้งนี้ชี้ให้เห็นว่าสารสกัดน้ำจากใบกฤษณา

A. crassna ไม่มีฤทธิ์ลดระดับน้ำตาลในเลือด การทดสอบพิษเฉียบพลันในหนูถีบจักร ไม่พบว่าหนูทดลองเกิดความเป็นพิษและไม่มีหนูทดลองตายในระหว่างการทดสอบ หลังจากได้รับสารสกัดในขนาด 2,000 และ 15,000 มิลลิกรัมต่อกิโลกรัมของน้ำหนักตัว

จากการศึกษาในครั้งนี้สรุปได้ว่า สารสกัดน้ำจากใบกฤษณา *A. crassna* มีฤทธิ์ต้านเชื้อแบคทีเรีย *S. epidermidis* โดยไม่พบว่าเกิดพิษเฉียบพลันต่อหนูทดลอง สารสกัดมีกลไกการออกฤทธิ์ไปรบกวนการสร้างผนังเซลล์ของแบคทีเรียและยับยั้งการสร้างไบโอฟิล์ม อย่างไรก็ตามข้อมูลที่ได้จากการศึกษานี้บ่งชี้ให้เห็นว่าสารสกัดไม่มีฤทธิ์ต้านเบาหวานที่ถูกชักนำด้วยสเตรปโตโซโตซิน

สาขาวิชาเภสัชวิทยา ลายมือชื่อนักศึกษา_____

ปีการศึกษา 2556 ลายมือชื่ออาจารย์ที่ปรึกษา_____

ลายมือชื่ออาจารย์ที่ปรึกษาร่วม _____

SIRILAK KAMONWANNASIT : STUDY ON ANTIOXIDANT, ANTIHYPERGLYCEMIC AND ANTIBACTERIAL ACTIVITIES OF THE AQUEOUS EXTRACT OF *AQUILARIA CRASSNA* LEAVES. THESIS ADVISOR : ASSOC. PROF. NUANNOI CHUDAPONGSE, Ph.D. 98 PP.

AQUILARIA CRASSNA/*STAPHYLOCOCCUS EPIDERMIDIS*/ANTIOXIDANT/ ANTIBACTERIAL/CELL WALL/ACUTE TOXICITY/ANTIDIABETIC

Agarwood or Krisana (Mai-Hom) is mainly produced by trees in the species of *Aquilaria* (family Thymelaeaceae). These plants are used for the production of incense and perfumes in Asia. It is also used as a traditional sedative, analgesic and digestive medicine throughout East Asia and China. The young leaves of cultivated *Aquilaria crassna* are widely used for production of Krisana health tea in Viet Nam, Cambodia and Thailand. It has been promoted by local manufacturers that agarwood tea possesses many pharmacological activities without any scientific evidences supporting the promotion. The main objectives of this study were to examine the antibacterial activity of the *A. crassna* leaf extract and its underlying mechanism. Anti-hyperglycemic action of the extract, as it is claimed, was also investigated. Moreover, phytochemical screening, antioxidant activity and acute toxicity were studied as well.

Phytochemical evaluation of *A. crassna* showed the presence of tannins, flavonoids, saponins, cardiacglycoside and phenolic compounds in the aqueous extract of *A. crassna* leaves. Antioxidant activities were examined by DPPH, ABTS, and FRAP scavenging methods. The results indicated that the extract exhibited quite considerable antioxidant activity compared to the standards, ascorbic acid and

butylated hydroxytoluene. By macro-dilution method, it was found that *Staphylococcus epidermidis* was susceptible to the extract with the MIC and MBC of 6 and 12 mg/ml, respectively. Morphological changes of the microbe were observed by scanning electron microscopy, while the inhibitory effect on biofilm formation was evaluated by phase contrast microscopic analysis. The extract not only caused swelling and distortion of bacterial cells but also inhibited bacterial biofilm formation. Rupture of bacterial cell wall occurred after treated with the extract for 24 h, observed by transmission electron microscopy. For anti-hyperglycemic examination, streptozotocin-induced diabetic rats were used. The results showed that after oral administration for 15 days, blood glucose level of the rats that received *A. crassna* leaves extract did not decrease compared to those of the groups that received glybenclamide, an antidiabetic drug. This result suggested that the aqueous extract of *A. crassna* leaves did not possess the hypoglycemic activity. Acute toxicity in mice was conducted in accordance with the OECD for Testing of Chemicals (2001) guidelines. It showed no sign of toxicity or death of the rats at the doses of 2,000 and 15,000 mg/kg body weight.

In conclusion, the aqueous extract of *A. crassna* leaves possesses an *in vitro* antibacterial activity against *S. epidermidis*, with no sign of acute oral toxicity in mice, probably by interfering with bacterial cell wall synthesis and inhibiting biofilm formation. However, the data from this study suggests that the extract does not have anti-diabetic activity induced by streptozotocin.

School of Pharmacology
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Student's Signature_____
Advisor's Signature_____
Co-advisor's Signature_____

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LIST OF ABBREVIATIONS

ABTS	=	2, 2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging
BHA	=	Butylated hydroxyanisole
DPPH	=	2, 2-diphenyl-1-picrylhydrazyl radical scavenging capacity
DPP-IV	=	Dipeptidyl peptidase-IV
$FeCl_3$	=	Ferric chloride solution
FRAP	=	Ferric reducing antioxidant power
GAE	=	Gallic acid equivalents
GLP-1	=	Glucagon like polypeptide-1
IDDM	=	Insulin dependent diabetes mellitus
MBC	=	Minimum bactericidal concentration
MHB	=	Mueller Hinton broth
MIC	=	Minimum inhibitory concentration
NIDDM	=	Non- insulin dependent diabetes mellitus
PBS	=	Phosphate buffer solution
STZ	=	Streptozotocin
TBHQ	=	Tertiary butylhydroquinone
TEM	=	Transmission electron microscopy
TISTR	=	Thailand institute of scientific and technological research

LIST OF ABBREVIATIONS (Continued)

TNF-α	=	Tumour necrosis factor-alpha
TSB	=	Tryptic soy broth
WHO	=	World health organization

CHAPTER I

INTRODUCTION

Plant and plant products have been used as sources of medicine since long. Traditional medicines and food from plants are more likely to yield pharmacologically active compounds. Bioactive compounds found in several medicinal plants have been shown to have health benefits, for example, antioxidant, antimutagenic, anticarcinogenic, antihypertensive, antidiabetic and antimicrobial activities (Cao and Cao, 1999; Geleijnse, Launer, Hofman, Pols and Witteman, 1999; Kahkonen *et al*., 1999; Yen, Duh and Tsai, 2002). Many medicinal properties of plants have been scientifically investigated and proved for low toxicity (Valiathan, 1998). In addition to being relatively safe, accessibility and affordability are more advantages of herbal medicines.

Agarwood or Krisana (Mai-Hom) is mainly produced by trees in the species of *Aquilaria* (family Thymelaeaceae). These plants provide an economically important natural product which is used for the production of incense, perfumes and traditional medicines in Asia (Feng, Yang and Wang, 2011). In Thailand, at least four species of agarwood trees are found in tropical rainforest areas of Thailand, namely *Aquilaria crassna* Pierre ex Lecomte, *A. subintegra*, *A. malaccensis* and *A. rugosa* (Eiadthong, 2007). The plants are not only one of the most valuable plants traded internationally but also have been used in Thai pharmaceutical products,

such as carminative and antispasmodic agents. It has been reported that agarwood is used as a traditional sedative, analgesic and digestive medicine throughout East Asia and China (Hu, Gao, Ling and Liu, 2009; Feng, Yang and Wang, 2011). *Aquilaria* species has been used as one of the active ingredients in the traditional Thai pharmaceutical preparation Krisanaglun as an antispasmodic, antidiarrheal and antiflatulence agent for more than fifty years. It has also been used as the ingredient of Ya-hom as a cardiovascular function enhancer in fainted patient (Miniyar, Chitre, Karve, Deuskar and Jain, 2008). Based on Thai folklore information, several parts of agarwood have been used for a long time in the treatment of infectious diseases such as diarrhoea, dysentery and skin diseases. However, scientific study with respect to its usefulness in the treatments of infectious diseases is very limited. Infectious diseases represent an important cause of morbidity and mortality among population, particularly in developing countries. Generally, species of the genus *Staphylococcus* are normal and abundant colonizer of the human skin and mucous membranes. *Staphylococcus epidermidis* is nowadays seen as an important opportunistic pathogen. *S. epidermidis* represents the most common source of infections on indwelling medical devices or contact lenses. It is now the most frequent cause of hospital-acquired infections (Raad, Alrahwan and Rolston, 1998). Treatment of the infection caused by this microorganism is complicated due to its specific antibiotic resistance genes and the formation of biofilms and multicellular agglomerations that have intrinsic resistance to antibiotics and mechanisms of host defense (Costerton, Stewart and Greenberg, 1999). The increasing failure of chemotherapeutic agents and antibiotic resistance exhibited by pathogenic microbial infectious agents has led to the

screening of several medicinal plants for their potential antimicrobial activity (Sharma, Patel, Rawat, Ramteke and Verma, 2010).

Owing to over-exploitation, many species of agarwood in the genus Aquilaria are nowadays considered as endangered species of Southeast Asia. Therefore, cultivation of agarwood is encouraged to reduce the harvest from wild populations. Since it takes about 10 years before the wood can produce valuable essential oil or resin, leaves from young agarwood are collected for production of healthy tea in Viet Nam, Cambodia and Thailand. However, it has been promoted by local manufacturers that agarwood tea possess many pharmacological activities, such as lipid lowering effect, anti-gout and antidiabetic effects without scientific evidence to support.

Diabetes is a chronic metabolic disease characterized by hyperglycemic caused by dysfunction in carbohydrate, protein and fat metabolism due to the insulin produced by the body is insufficient, or cells do not respond properly to the insulin that is produced (Ramachandran, Naveen, Rajinikanth, Akbar and Rajasekaran, 2012). It is one of the alarming worldwide health problems at present leading to urinary tract infections, papillary necrosis and glomerular lesions (Li *et al*., 2012). World Health Organization (WHO) estimates that more than 220 million people worldwide have diabetes and this number is likely to double by 2030 (Aragãoa *et al*., 2010). The high prevalence of diabetes as well as its long term complications has led to an ongoing search for hypoglycemic agents from natural sources (Nicasio, Santamaría, Aranda, Ortiz and González, 2005). It is significant to find evidence whether the aqueous extract from *A. crassana* leaves which are used in production of healthy tea can lower blood glucose *in vivo*.

Free radicals have been claimed to play an important role in affecting human health by causing several diseases including cancer, hypertension, heart attack and diabetes. These free radicals are generated during body metabolism. Exogenous intake of antioxidants can help the body scavenge free radicals effectively. On the other hand, concern about the safety of the commonly used synthetic antioxidants such as butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) have led to increased interest on natural antioxidants which occur in plants as secondary metabolites (Raghuveer and Tandon, 2009). It has never been reported regarding to antioxidant activities of agarwood leaf extract.

Herbal remedies are widely used for the treatment and prevention of various diseases and often contain highly active pharmacological compounds. There is increasing concern about the safety of medicinal plants and their ability to produce toxicity and adverse effects. Accidental herbal toxicity occurs not only as a result of a lack of pharmaceutical quality control in harvesting and preparation, but also because herbal remedies are believed to be harmless. Therefore, contrary to popular belief, the use of herbal remedies can pose serious health risks (Alastair and Wood, 2002). Thus, it is necessary that toxicity study should be done along with pharmacological activity study to confirm the capability in clinical use.

The aim of this study is to investigate whether the aqueous extract of *A. crassna* leaves produces antihyperglycemic effect in rats. The data obtained from this experiment will provide scientific evidence showing whether the herbal tea of *A. crassna* possesses an antidiabetic activity as claimed in commercial advertisements. In addition, antibacterial activity of the extract was determined since bacterial infection is a common cause of gastrointestinal disorders and *Aquilaria* extract is a

component in a traditional Thai pharmaceutical preparation for GI illness. In the present study, antibacterial activity of the extract against *S. epidermidis* was found and the mechanisms underlying was proposed. In the present study, phytoconstiuents of this plant, such as alkaloids, cardiac glycosides, flavonoids, tannins and saponin were determined. Finally, phenolic content, antioxidant properties and acute toxicity of the extract were studied as well.

CHAPTER II

LITERATURE REVIEWS

2.1 *Aquilaria* species

Agarwood (*Aquilaria* spp.) is well known for its aromatic resin. Synonyms were Gaharu wood and Krisana, or Mai-hom in Thai. There are about 15 species of *Aquilaria*, which belong to Thymelaeaceae family, distributed throughout tropical Asia. Natural distribution occurs in South and Southeast Asia, such as China (Hongkong, Hainan), Pakistan, India, Nepal, Bhutan, Bangladesh, Sri-Lanka, Cambodia, Vietnam, Laos and Thailand. The 15 species of *Aquilaria* are *A. apiculata*, *A. baillonii*, *A. banaense*, *A. beccariana*, *A. brachyantha*, *A. cumingiana*, *A. filaria*, *A. hirta*, *A. khasiana*, *A.malaccensis*, *A. microcarpa*, *A. rostrata*, *A. sinensis*, *A. subintegra*, and *A. crassna*. In Thailand, there are four species of *Aquilaria*, including *A.crassna*, *A. malaccensis*, *A. subintegra* and *A. hirta*. They are naturally distributed in central, northeast and eastern of Thailand, such as Nakhon Ratchasima, Nakhon Nayok, Prachin Buri, Buri Ram and Chantaburi provinces (Matsui, 2005).

Krisana is used for the production of high valued incense, cosmetics and pharmaceutical products in Asia (Eurlings, Beek and Gravendeel, 2010) and their leaves are also cultivated as a healthy tea in South East Asia countries such as Viet Nam, Laos and Thailand. Having been heavily poached, nowadays Krisana has become endangered species. The Thai government encourages cultivating of Krisana

for preventing species extinction. However, it takes at least 5 years before they can provide precious resin. During this period, leaves of young *A. crassna* are used to produce commercial healthy herbal tea as it is believed that tea from *A. crassna* leaves has antidiabetic, antigout and antihyperlipidemia activities. Characteristics of four agarwood species found in Thailand are the followings.

Aquilaria malaccensis

A. malaccensis reaches up to 20-40 m tall and 60 cm in diameter. Young bark is light brown with fine hairs, older bark is smooth and whitish in color. Leaves are characterized by alternate, elliptic or lanceolate, 3-3.5 cm wide and 6-8 cm long with 12-16 pairs of veins. Inflorescens are a terminal or a axillary umbel.

Figure 2.1 *A. malaccensis*.(1) twig (2) flower (3) longitudinal section of flower (4) fruit (5) longitudinal section of fruit (Adelina, 2004).

Flowers are hermaphroditic, up to 5 mm long, fragrant and yellowish green or white. Fruit is green in color, egg-shaped capsule and leathery exocarp with fine hairs, 4 cm long and 2.5 cm wide. There are two seeds per fruit. Seed is blackish brown in

color, ovoid and densely covered with red-brown hair (Sitepu, Santoso and Turjaman, 2011).

Aquilaria subintegra

A. subintegra is a small tree up 2 m tall. Leaves are acuminate, base cuneate to obtuse petiole pubescent or glabrous, 5-10 mm long. The flowers are white. Its pedicels are puberulous 6-13 mm long. The calyx-tube is 5-12 mm long. The fruit is elliptic. The seed is narrowly elliptic, sparsely puberulous (Santisuk, 1997).

Figure 2.2 *A. subintegra* (A) flowers (B) leaves (Source: Forest Herbarium, 2003).

Aquilaria hirta

A. hirta is tree up to 14 m tall. Leaves are acuminate, base cuneate to obtuse or rounded, 6.5-14 by 2.5-5.5 cm. The flowers are white or light yellow. The pedicel are up to 20 mm long. The calyx-tube is 6-8 mm long. The seed is ovoid, puberulous, glabrescent and shortly beaked at the apex (Santisuk, 1997).

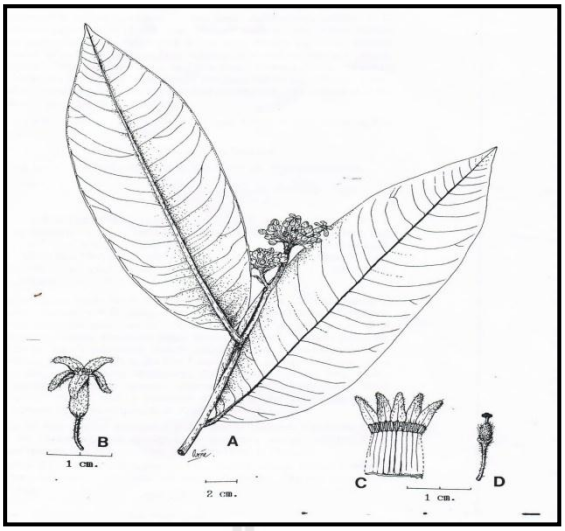

Figure 2.3 *A. hirta* (A) twig and iflorescence (B) flower (C) flower cutting open to show stamens (D) ovary (Santisuk, 1997).

Aquilaria crassna

A. crassna is an evergreen tree up to 30 meters with narrow crown and slender tree (Matsui, 2005). Its bark is brownish grey, shallowly fissured and flaking in thin strip. Inner bark is wet with much water. Leaves are alternate, coriaceous, elliptical, petiole 4-5 mm, blade 7-11.5 cm long 2.5-5 cm wide. The flowers are small, pale blue-yellow, fragrant and fine-haired. Its pedicels are up to 0.6-0.8 cm long and bisexual. The fruit is 4 cm long, 2.2 cm wide, bright green and pubescent. The seed is glossy black, long and tail-like pubescent appendage.

The main differences of botanical characteristics between *A. crassna* and *A. malaccensis* are the length of pedicels and the size of calyx-lobes. The length of pedicels of *A. crassna* is 5-10 mm larger than 2-5 mm in *A. malaccensis*.

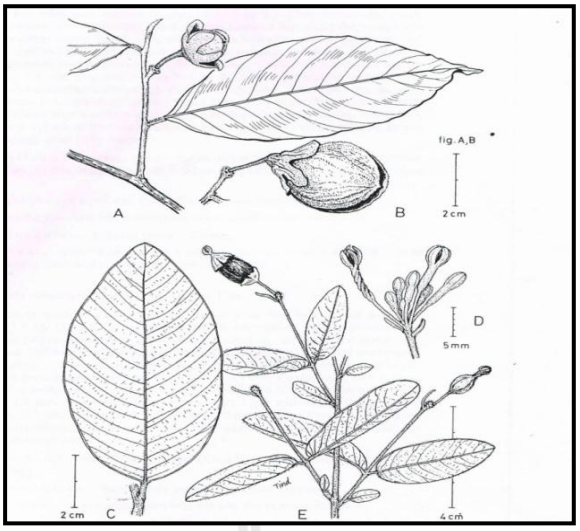

Figure 2.4 *A. crassna* (A) fruiting branch (B) fruit (C) leaf seen from beneath (D) inflorescence (E) fruiting branch (Santisuk, 1997).

The size of calyx-lobes of *A. crassna* is 3-4 x 2-3.5 mm large as compared to 2-3 x 1.5 -2 mm in *A. malaccensis*. The calyx-lobes of *A. crassna* during fructification are much enlarged up to 15 mm. In addition, the height of *A. crassna* tree is 30-40 m as compared to 40 m in *A. malaccensis*. The leaf size of *A. crassna* is 8-16 x 2-5.5 cm as compared to 7-12 x 2.5-5.5 cm in *A. malaccensis*. Moreover, they can be distinguished by measuring the size of leaflet. The size of leaflet of *A. subintegra* is 7-10.5 x 19-27.5 cm large as compared to 2-5.2 x 5-15 cm in *A. crassna* and 2.5-5 x 7-11.5 cm in *A. malaccensis* and 2.5-5.5 x 6.5-14 cm in *A. hirta* (Matsui, 2005).

2.2 Phytochemicals of *Aquilaria* species

Plants have many phytochemicals which are potential sources of natural antioxidants, e.g. phenolic diterpenes, flavonoids, tannins and phenolic acids (Dawidowicz, Wianowska and Baraniak, 2006). Medicinal plants are a source of bioactive activity and are of great utility for developing some novel therapeutic

agents. The medicinal value of these plants lies in some active chemical substances called phytochemicals that produce a definite physiological action on the human body. The most important of these chemically active (bioactive) constituents of plants are alkaloids, tannins, flavonoids and phenolic compounds (Edeoga, Okwu and Mbaebie, 2005). Phytochemical studies of some species of *Aquilaria* have been reported. The presence of alkaloids, flavonoids, triterpenoids, steroids and saponins were revealed in *A. malaccensis* leaves extract (Huda, Munira, Fitrya and Salmah, 2009). The phytoconsituents isolated from this leaves are kusunol, jinkoheremol, jinkohol II, α-agarofuran, (-)-10-epi-γ-eudesmol, oxo-agarospirol, 10-epi-γ-eudesmol and six new 2-(2-Phenylethyl) chromone compounds (Konishi, Konoshima, Shimada and Kiyosawa, 2002). *A. malaccensis* leaves contained sesquiterpene alcohols, which was 10-epi-γ-eudesmol. Four new compounds were isolated from the n-butanol fraction of the 70% aqueous ethanolic of *A. sinensis*, including aquilarisinin, aquilarisin, hypolaetin 5-*O*-β-D-glucuronopyranoside and aquilarixanthone. In addition, four known compounds, including mangiferin, iriflophenone 2-*O*-α-L-rhamnopyranoside, iriflophenone 3-*C*-β-D-glucooside and iriflophenone 3,5-*C*-β-D-diglucopyranoside, extracted from the ethyl acetate fraction showed an α-glucosidase inhibitor activity (Feng, Yang and Wang, 2011). A new spirovetivane - type sesquiterpene along with a known 2-(2-phenylethyl) chromone was found in the 70% ethanol extract of Vietnamese agarwood (Ueda *et al*., 2006). Three novel diepoxy tetrahydrochromones, oxidoagarochromone A, B, and C, were isolated from agarwood artificially produced by intentional wounding of *A. crassna* (Yagura, Shibayama, Ito, Kiuchi and Honda, 2005). However, phytochemical report regarding to the constituents of *A. crassna* leaves is very limited.

2.3 Pharmacological properties of *Aquilaria* species

Aquilaria species have been used as a Thai traditional medicine for a long time. It is one of several ingredients in the commercial preparation Krisanaglun for treatment of gastrointestinal illness, such as stomach ache, diarrhea and flatulence. *A. crassna* extract has been using as an ingredient of the Thai traditional product so called Ya-hom for treatment of fainting by targeting the cardiovascular system. It has also been reported that Ya-hom inhibited both histamine and carbachol induced gastric acid, pepsin and soluble mucus secretions in a dose-dependent manner. Ya-hom had a maximum inhibition on the acid-stimulating effects of the histamine than that of carbachol. (Suvitayavat, Kodchawongs, Thirawarapan and Bunyapraphatsara, 2004). However, there are many more active ingredients in Ya-hom that could be contribute to this action.

Some pharmacological activities of *Aquilaria* species have been studied. For example, it has been described that the ethyl acetate fraction of the 70% aqueous ethanol extract from the leaves of *A. sinensis* has inhibitory effects against α-glucosidase activity (Feng, Yang and Wang, 2011). Kim (1997) also reported that the aqueous extract of *A. agallocha* stems inhibits the immediate hypersensitivity reaction by inhibition of histamine release from mast cells. The leaves of *A. sinensis* which have been used traditionally in China for treatments of inflammation and anaphylaxis were found to exhibit notable analgesic, antiinflammatory (Zhou *et al*., 2008).

It was claimed that agarwood (Jinkoh in Japanese) is used traditionally as a sedative in Asia (Okugawa, Ueda, Matsumoto, Kawanishi and Kato, 1993). Neuropharmacological studies have been conducted with the petroleum ether, benzene, chloroform, and water extracts from *A. malaccensis* in mice. The benzene

extract showed a reducing effect in spontaneous motility, a prolonging effect on hexobarbiturate-induced sleeping time, a hypothermic effect in terms of rectal temperature and a suppressive effect on acetic acid-writhing by oral administration. Fraction 1 of the three fractions which were obtained from the benzene extract by column chromatography was found to produce more positive effects on these neuropharmacological tests than the original benzene extract. These result suggested that the benzene extractable compounds of agarwood possess potent central nervous system depressant activities (Okugawa, Ueda, Matsumoto, Kawanishi and Kato, 1993). The jinkoh-eremol and agarospirol were obtained from the benzene extract. They also gave positive effects on the central nervous system by peritoneal and intracerebroventricular administration. They decreased both methamphetamine and apomorphine-induced spontaneous motility. Therefore, jinkoh-eremol and agarospirol can be considered to be neuroleptic (Okugawa, Ueda, Matsumoto, Kawanishi and Kato, 1996).

Recently, the underline mechanism of anti-inflammatory effect of the ethyl acetate extract of *A. crassna* has been studied using isolated human peripheral blood mononuclear cell. It was proposed that the anti-inflammatory effect was produced by inhibition of tumour necrosis factor-alpha (TNF-α) gene expression and secretion (Kumphune, Prompun, Phaebuaw, Sriudwong, Pankla, and Thongyoo, 2011). In addition, the ethyl acetate extract of *A. crassna* could reduce ischemia induced injury and cell death in cardiac myoblast cell line (Jermsri and Kumphune, 2012). It has been reported that the water and ethanol extract of *A. crassna* process antimicrobial activity against some bacteria, such as *Staphylococcus aureus*, *Clostridium difficile*, and *Bacteroides* spp. but not *Escherichia coli* and lactic acid bacteria including

Enterococcus faecalis, Bifidobacterium longum and *Bifidobacterium adolescentis*) (Kakino *et al*., 2012). However, the antimicrobial activity of the extract from *A. crassna* leaves against *Psuedomonas auruginosa* has never been reported.

2.4 Antioxidant activity of medicinal plants

Antioxidant compounds in food play an important role as a health protecting factor. Scientific evidence suggests that antioxidants reduce the risk for chronic diseases including cancer, diabetes and heart disease. Primary sources of naturally occurring antioxidants are whole grains, teas, herbs, fruits and vegetables (Aiyegoro and Okoh, 2010). Amongst the antioxidants, several groups of polyphenols (anthocyanins, tannins, flavonones, isoflavones, resveratrol and ellagic acid) are currently used in the industry as nutraceuticals and/or functional foods (Espin, Conesa, Teresa and Barberan, 2007). Some antioxidant compounds are extracted from easy sources, such as agricultural and horticultural crops or medicinal plants. These antioxidant properties are due to some vitamins, terpenoids (carotenoids and essential oils) and phenolic compounds which are important in plants themselves for normal growth development and defense against infection and injury. The presence of phenolics in injured plants may have an important effect on oxidative stability and microbial safety (Tadhani, Patel and Subhhash, 2007). Additionally to their role as antioxidant, these compounds exhibit a wide spectrum of medicinal properties, such as antiallergic, antiinflammatory, antithrombotic, cardio-protective and vasodilatory effects (Balasundram, Sundram and Samman, 2006). Several studies have described the antioxidant properties of medicinal plants, food and beverages which are rich in phenolic compounds because of their highly redox potentials. Such compounds act as

reducing agents, hydrogen donors, singlet oxygen quenchers, free radical scavengers and as chelating agents of pro-oxidants metals (Chanwitheesuk, Teerawutgulrag and Rakariyatham, 2005; Tsao and Deng, 2004). It has been shown that phenolic antioxidants in herbs are mainly composed of phenolic acids, flavonoids and catechins which have capacities of quenching lipid peroxidantion, preventing DNA oxidative damage and scavenging reactive oxygen species.

Testing the activity by more than one assay is desirable because different methods measure different characteristics of the antioxidant. Several approaches are used to test the antioxidants in foods and biological systems. In the present study, three different methods were used to determine antioxidant activity of the extract of *A. crassna* leaves as follow:

2.4.1 2, 2-diphenyl-1-picrylhydrazyl radical scavenging capacity (DPPH) assay

2, 2-diphenyl-1-picrylhydrazyl radical scavenging capacity assay is hydrogen atom transfer process widely used evaluate the antioxidant activity of reductants (plant extracts, phytochemical or pharmaceutical drugs) (Kaviarasan, Naik, Gangabhagirathi, Anuradha and Priyadarsini, 2007). The DPPH free radical is reduced to the corresponding hydrazine when it reacts with hydrogen donors (antioxidant) using decolouration mechanism (purple to yellow), which are monitored by the decrease in absorbance at 515-528 nm (Rekha *et al*., 2012).

2.4.2 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging (ABTS) assay

2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) cation radical scavenging assay is based on the reduction of blue/green $ABTS^{\bullet+}$ chromophore

generated from the reaction between ABTS and potassium persulphate ($K_2S_2O_8$) by an electron-donating antioxidant. The decolourization of the $ABTS^{\bullet+}$ chromophore is measured spectrophotometrically at 734 nm. Thus the extent of decolorization as percentage inhibition of the $ABTS^{\bullet+}$ radical cation is determined as a function of concentration and time and calculated relative to the reactivity of a standard (Re *et al*., 1999).

2.4.3 Ferric reducing antioxidant power (FRAP) assay

Ferric reducing antioxidant power assay is based on the reduction at low pH, of a colorless ferric complex to an intense blue colored ferrous complex by an electron donating antioxidant. The reduction of ferric complex is monitored by measuring the increase in absorbance at 593 nm. The FRAP assay is a robust and potentially useful test using inexpensive reagents and speedy reaction applicable over a wide concentration range (Benzie and Szeto, 1999).

2.5 Diabetes mellitus

Diabetes mellitus is a major endocrine disorder affecting nearly 10% of the population all over the world. Diabetes is one of the leading causes of death in humans if not treated. It is responsible for many complications affecting various organs in the body. The chronic hyperglycemic of diabetes is associated with long term damage, dysfunction and failure of various organs (Grover, Yadav and Vats, 2002; Burke *et al*., 2003).

2.5.1 Classification of diabetes

Diabetes mellitus is among the most common metabolic diseases. It is a chronic disorder of carbohydrate, lipid and protein metabolism characterized by

high levels of blood glucose by defective insulin production or complete cessation of insulin synthesis (Leahy, 2005).

Insulin dependent diabetes mellitus (IDDM) or Type 1 diabetes accounts for about 5-15% of all cases of diabetes and occurs most often in young people. This is autoimmune condition, resulting in destruction of pancreatic β-cells.

Non-insulin dependent diabetes mellitus (NIDDM) or Type 2 diabetes. Most diabetics are type 2. The disease is influenced by aging, obesity and peripheral insulin resistance. Type 2 diabetes are often obese. Type 2 diabetes is frequently accompanied by the lack of sensitivity of target organs to either endogenous or exogenous insulin. This resistance to insulin is considered to be a major cause of this type of diabetes (Finkel, Clark and Cubeddu, 2009). Summary of the principle characteristics of each type are shown in Table 2.1.

2.5.2 Insulin

Insulin is synthesized in significant quantities only in β-cells in the pancreas. Insulin stimulates glucose transport and metabolism, enhances glycogen synthesis and stimulates lipogenesis to promote storage of fuel for energy. It also has important growth-promoting actions in a variety of tissues. Insulin is a polypeptide composed of 51 amino acid and formed by an A chain and a B chain linked by disulfide bonds. The insulin mRNA is translated as a single chain precursor called preproinsulin, and removal of its signal peptide during insertion into the endoplasmic reticulum generates proinsulin. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide in the middle known as the C peptide. Structure of proinsulin is shown in Figure 2.5.

2.5.3 Insulin secretion

Insulin is released from β-cells by exocytosis which is initiated by the increase in intracellular calcium through voltage-dependent calcium channel. When glucose enters β-cells of pancreas, it is metabolized to ATP. The increasing of ATP causes a closure of ATP-sensitive potassium channels, which leads to a decreased potassium influx and subsequent depolarization of the β-cells membrane. Voltage-dependent calcium channels open and result in an influx of calcium, causing translocation and exocytosis of secretory granules of insulin to the cell surface.

Table 2.1 Summary of the main features of Type 1 diabetes and Type 2 diabetes.

	Type 1 diabetes	**Type 2 diabetes**
Aetiology	Autoimmune (β-cell destruction)	Insulin resistance and β-cell destruction
Peak age	12 years (can occur at any age)	60 years (increasingly seen at a young age due to obesity)
Prevalence	0.3%	Around 6%
Presentation	Osmotic symptoms (days to weeks), weight loss, DKA Patient usually slim	Osmotic symptoms (months to years), diabetic complications Patient usually obese
Treatment	Diet and insulin	Diet, exercise (weight loss), oral hypoglycaemic agents, insulin later

DKA, diabetic ketoacidosis (Lechago and Gould, 1997; Ajjan, 2009).

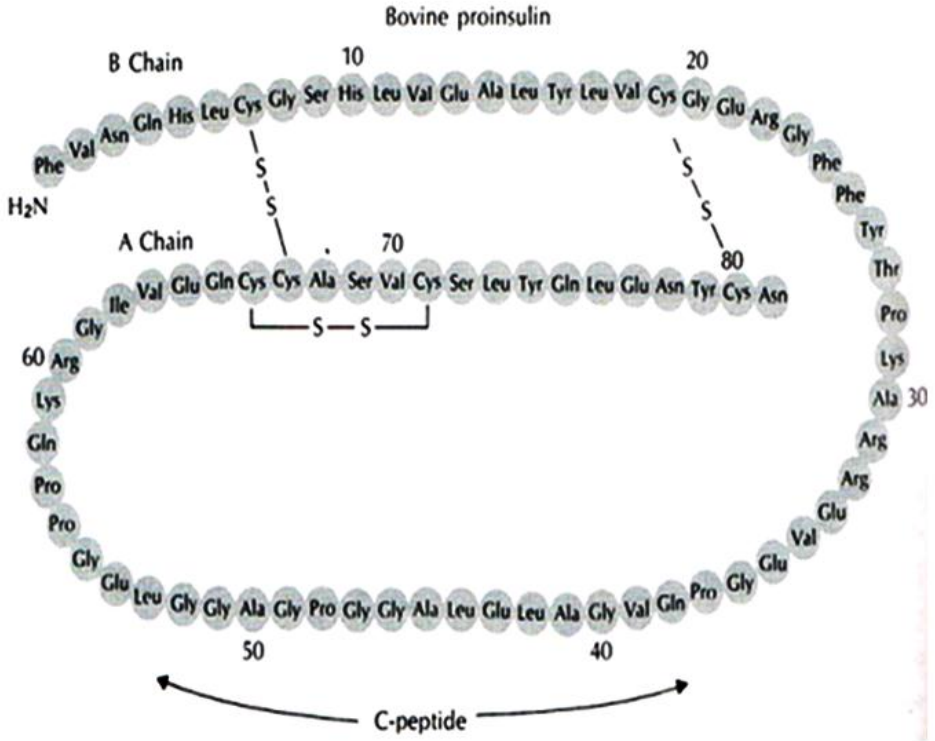

Figure 2.5 Structure of proinsulin (Clark, Brater and Johnson, 1992).

2.5.4 Oral antidiabetic agents

Oral antidiabetic agents available in markets include sulfonylureas, biguanide, α-glucosidase inhibitors and dipeptidyl peptidase-IV inhibitor. Each of them produce hypoglycemic effect by different mechanisms as the followings.

2.5.4.1 Insulin secretagogues: Sulphonylurea

The sulphonylurea group of drugs has been available for the last 50 years. All of them (glibenclamide, glipizide and gliclazide) have a similar mechanism of action that is mediated by inducing closure of the ATP-sensitive potassium channels. The intracellular retention of ATP-sensitive potassium changes the membrane potential resulting in depolarization of the β-cell and opening of the voltage-dependent calcium channels (Ca^{2+}). This facilitates movement of Ca^{2+} into the cell, stimulating exocytosis of insulin into the circulation (Joshi and Joshi, 2009).

2.5.4.2 Insulin sensitisers: Biguanides

Hepatic sensitivity to insulin is increased, thereby reducing gluconeogenesis as well as glycogenolysis, which contribute to the post-prandial plasma glucose lowering effects. Skeletal muscle and adipocytes undergo up-regulation of the insulin-sensitive GLUT-4 and GLUT-1 transporters to the cell membranes, thereby increasing glucose uptake. Further metabolic effects include suppression of fatty acid oxidation as well as triglyceride lowering (Bösenberg and Zyl, 2008).

2.5.4.3 α-glucosidase inhibitors: Acarbose

The α-glucosidase inhibitors inhibit the activity of the glucosidase enzymes which are present in the brush border of enterocytes in the intestinal villi. These drugs are taken at the beginning of meals. They act by delaying the digestion of carbohydrates, thereby resulting in lower postprandial glucose levels (Finkel, Clark and Cubeddu, 2009).

2.5.4.4 Dipeptidyl peptidase-IV inhibitor: Sitaglitin

Incretin is a peptide that is produced in the gastrointestinal tract. One of these incretins is glucagon like polypeptide-1 (GLP-1). GLP-1 increases insulin secretion and decreases glucagon release. GLP-1 is metabolized by dipeptidyl peptidase-IV (DPP-IV) and has a very short half-life. Thus, DPP-IV inhibitors help increase insulin release in response to meals (Clayton, Stock and Cooper, 2009; Finkel, Clark and Cubeddu, 2009; Nolte, 2009).

2.5.5 Chemical induction of diabetes

Streptozotocin (2-deoxy-2-(3-methyl-3-nitrosoureido)-glucopyranose) (STZ) is synthesized by *Streptomycetes achromogenes* and is used to induce both IDDM and NIDDM (Szkudelski, 2001). STZ is widely used to induce experimental diabetes in animals. The mechanism of their action in β-cells of the pancreas has been intensively investigated and now is quite well understood. STZ enters insulin secreting pancreatic β-cells through GLUT-2 and causes DNA damage and finally triggers pancreatic.

Table 2.2 Summarized mechanism of action of antidiabetic drugs (Clayton, Stock and Cooper, 2009; Laurence, Bennett and Brown, 1997).

Antidiabetic drugs	Mode of action
1. Sulphonylureas - Tolbutamide - Chlorpropamide - Glibenclamide	- Stimulate insulin release from β-cell
2. Biguanide - Metformin	- Reduce hepatic gluconeogenesis - Slow glucose absorption from gastrointestinal - Increase insulin action in peripheral tissue
3. α-glucosidase inhibitors - Acarbose	- Inhibit α-glucosidase
4. Dipeptidyl peptidase-IV Inhibitor - Sitagliptin	- Increase incretin levels; GLP-1 and glucose-dependent insulinotropic polypeptide which inhibit glucagon release

β-cells necrosis (Botolin and Mccabe, 2007). The induction of experimental diabetes in the rat using chemical which selectively destroys pancreatic β-cells is very convenient and simple to use. The range of the STZ dose is between 40 and 60 mg/kg, but higher doses are also used. STZ is also efficacious after intraperitoneal administration of a similar or higher dose, but single dose below 40 mg/kg may be ineffective. STZ action in β-cells is accompanied by characteristic alterations in blood insulin and glucose concentrations. Two hours after injection, the hyperglycemic is observed with a concomitant drop in blood insulin. About six hours later, hypoglycemic occurs with high levels of blood insulin. Finally, hyperglycemic develops and blood insulin levels decrease (Szkudelski, 2001).

Although plants and herbal preparations are being used in traditional medicine for various ailments since ancient time, the efficacious attributes of these have been experimentally validated only in recent decades. Several herbs and trees have found potential use as antidiabetic in the folk medicine (Roy, Harris, Dennison, Phoenix and Singh, 2005).

2.6 *Staphylococcus epidermidis*

In principle, bacteria are divided into two main groups, Gram-negative and Gram-positive bacteria, based on their cell wall structures resulting in different Gram-stain retention.

Cell wall structure of Gram-negative and Gram-positive bacteria are shown in Figure 2.6. Normally, the cell wall Gram-positive bacteria has a peptidoglycan layer that is relatively thick (20-80 nm) and comprises approximately 90% of the cell wall. Peptidoglycan forms the outer layer of the Gram-positive bacteria cell wall and does

not contain lipoproteins. The cell wall of Gram-positive bacteria has teichoic acids, which are acidic anionic polysaccharides. Teichoic acids which contain a carbohydrate such as glucose, phosphate and an alcohol are bonded to the peptidoglycan, making them an integral part of the Gram-positive cell wall structure. The peptidoglycan layer of the Gram-negative cell wall is very thin (5-10 nm) and overlaid by an outer membrane, anchored to lipoprotein molecules in the peptidoglycan layer. Teichoic acids do not occur in Gram-negative bacterial cell walls. Rather, lipoproteins (lipids linked to protein molecules) are bonded to the peptidoglycan, forming an integral part of the Gram-negative bacterial cell wall. Additionally, there are layers of lipopolysaccharide (lipids linked to carbohydrate molecules), phospholipids and proteins outside the peptidoglycan layer (Atlas, 1994).

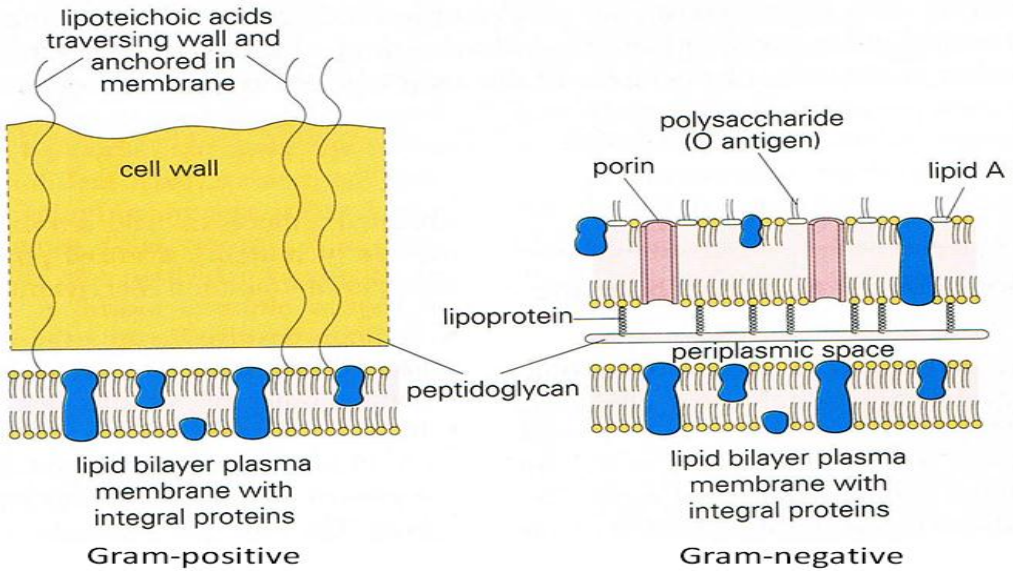

Figure 2.6 Construction of the cell wall of Gram-positive and Gram-negative bacteria (Mims *et al*., 2004).

Table 2.3 Natural medicines used for diabetes therapy.

Plant	Part used	Dose	Model used	References
Averrhoa bilimbi Linn	leaves	125 mg/kg	60 mg/kg STZ rats	Pushparaj, Tan and Tan, 2000
Cichorium intybus	Whole plant	125 mg/kg	50 mg/kg STZ rats	Pushparaj, Low, Manikandan and Tan, 2007
Coriandrum sativum L.	seeds	200,250 mg/kg	70 mg/kg STZ rats	Eidi *et al*., 2009
Chamaemelum nobile	aerial plart	20 mg/kg	20 mg/kg STZ rats	Eddouksa, Lemhadria, Zeggwagha and Michel, 2005
Fraxinus excelsior L.		10 mg/kg	65 mg/kg STZ rats	Eddouks, Lemhadri and Michel, 2004
Clausena anisata	root	800 mg/kg	90 mg/kg STZ rats	Ojewole, 2002
Eclipta alba	leaf	2 g/kg	150 mg/kg alloxan rats	Ananthi, Prakasam and Pugalendi, 2003
Caesalpinia bonducella	seed	300 mg/kg	150 mg/kg alloxan rats	Kannur, Hukkeri and Akki, 2006
Bauhinia forficate	leaves	200, 400 mg/kg	60 mg/kg alloxan rats	Lino *et al*., 2004
Andrographis paniculata	leaf	400 mg/kg	45 mg/kg STZ rats	Dandu and Inamdar, 2009
Adansonnia digitata	Stem bark	100 mg/kg	60 mg/kg STZ rats	Tanko *et al*., 2008

S. epidermidis, which belongs to Staphylococcaceae family, is an aerobic Gram-positive cocci, arranged in grape-like clusters. It forms white, raised, cohesive colonies. The regular size/diameter of *S. epidermidis* is approximately 0.5 to 1.5 µm. *S. epidermidis* is primarily a normal inhabitant of the healthy human skin and mucosal microflora. It has emerged as a common cause of numerous nosocomial infections. *S. epidermidis* as one of the most often isolated bacterial pathogens in hospitals and as the most important pathogen involved in nosocomial blood stream infections, cardiovascular infections, and infections of the eye, ear, nose, and throat. *S. epidermidis* very often becomes the major infective agent in compromised patients, such as drug abusers and immuno-compromised patients (patients under immunosuppressive therapy and premature newborns). The port of entry into the human body in all of these infections is usually an intravascular catheter or implanted device or contact lenses (Lim and Web, 2005; Ziebuhr *et al*., 2006; Lindsay, 2008). *S. epidermidis* infections are generally treated with vancomycin. When *S. epidermidis* is growing on an implanted device in a debilitated patient (Walker, 1999).

2.6.1 Virulence of *S. epidermidis*

The ability to form biofilm, a slimy layer with embedded microcolonies, is one of the most important and one of the most widespread virulence factors occurring in microbes. This ability can be found in bacteria living in the outer environment as well as in pathogens and potential pathogens of humans. The biofilm-forming ability helps bacteria to resist the conditions of the surrounding environment. Biofilms grow easily on surfaces of artificial materials used for catheters and prosthetic devices (Stewart and Costerton, 2001) and it is estimated that biofilms are associated with about 65% of nosocomial infections. These infections are usually

chronic and difficult to treat. The higher incidence of biofilm-associated infections is associated with the frequent use of artificial implants and medical devices nowadays and bacteria most commonly isolated from these infections are those of the *Staphylococcus* genus (Deightoni and Balkau, 1990; Gelosia, Baldassarri, Deighton and Nguyen, 2001). The most important group of infection caused by *S. epidermidis* is infections on foreign bodies. Biofilms are often the cause for the difficulty to eradicate *S. epidermidis* infections on an indwelling device, due to an impaired penetration of antibiotics to the target and a decreased immune response. Numerous studies in recent years have clearly indicated that the ability to form thick, multilayered biofilms on insert surfaces, such as polymers or metals, represents a typical feature of many nosocomial isolates (Götz, 2002). Biofilms, in the narrower sense, are communities of microorganisms that stick to each other and/or to surfaces by the production of an extracellular matrix that mostly consists of polysaccharides and proteins and illustrate some of the factors involved in the biofilm formation of staphylococci, which proceeds in two steps. First, bacteria adhere to a surface by unspecific factors, such as hydrophobicity and surface charge (Vacheethasanee *et al*., 1998), but also by means of cell wall teichoic acids and proteins, such as autolysins or cell wall-associated proteins that interfere with collagen, fibronectin or other matrix proteins (Hussain, Heilmann, Peters and Herrmann, 2001). Second, the initial adherence stage is followed by the actual accumulation of the biofilm, which is characterized by the production of factors that mediate bacterial cell-to-cell contacts. A major component of *S. epidermidis* biofilms is polysaccharide intercellular adhesion, which represents a β-1,6-linked glucose aminoglycan substituted with different side groups. Moreover, it has been shown recently that *S. epidermidis*

biofilms contain, depending on the growth conditions, considerable amounts of extracellular teichoic acids (Sadovskaya, Vinogradov, Flahaut, Kogan and Jabbouri, 2005).

2.7 Antibiotics

Pharmacological industries have produced a number of new antibiotics in the last three decades, in spite of that resistance to these drugs by microorganisms has also been increased. In general, bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents (Chitravadivu, Manian and Kalaichelvi, 2009). Antibiotics have long been known to have multiple effects on target cells. They have been interfered with essential functions of bacteria and least effect to host cell. Antibiotics may have a killing effect or an inhibitory effect on a range of microbes. Most antimicrobial agents used for the treatment of bacterial infections may be categorized according to their principal mechanism of action. There are 4 major modes of action (Figure 2.7): (1) inhibition of cell wall synthesis (2) inhibition of protein synthesis (3) inhibition of nucleic acid synthesis and (4) inhibition of a metabolic pathway (Page, Curtis, Sutter, Walker and Hoffman, 2002).

2.7.1 Drugs that inhibit bacterial cell wall synthesis

The major structural component of bacterial cell wall is the peptidoglycan layer. The basic structure is a chain of 10 to 65 disaccharide residues consisting of alternating molecules of N-acetylglucosamine and N-acetylmuramic acid. These chains are then cross-linked with peptide bridges that create a rigid mesh coating for the bacteria. The building of the chains and cross-links is catalyzed by

specific enzymes (e.g., transpeptidases, transglycosylases, carboxypeptidases) that are members of a large family of serine proteases. These regulatory enzymes are also called penicillin-binding proteins (PBPs) because they can be bound by β-lactam antibiotics. When growing bacteria are exposed to these antibiotics, the antibiotic binds to specific PBPs in the bacterial cell wall and inhibits formation of cross-links between peptidoglycan chains.

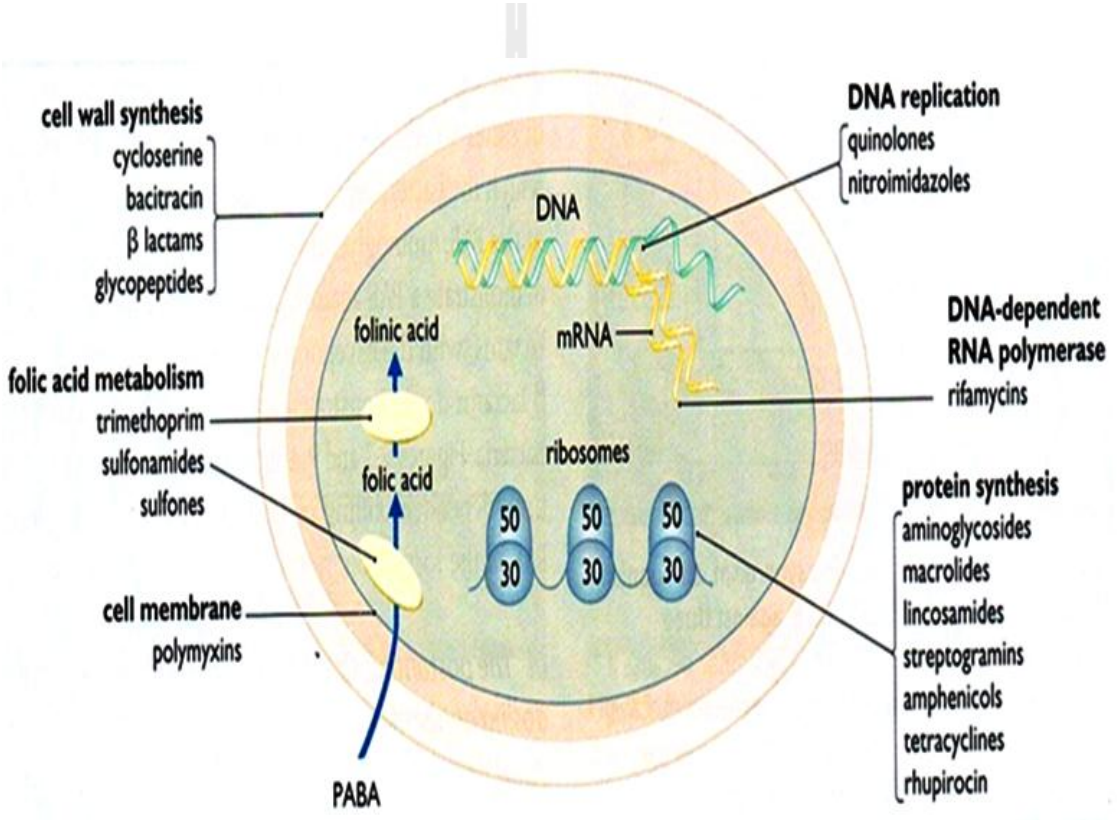

Figure 2.7 The Mechanisms of antibiotic drugs (Page, Curtis, Sutter, Walker and Hoffman, 2002).

This in turn activates autolysins that degrade the cell wall, resulting in bacterial cell death. Thus the β-lactam antibiotics generally act as bactericidal agents.

Vancomycin is a relatively large glycopeptide antibiotic. It is active against most Gram-positive bacteria, including streptococci, staphylococci and

enterococci. Vancomycin is a prominently bactericidal antibiotic that inhibits bacterial cell wall synthesis by blocking the important substrates for cell wall-synthesizing machinery such as the D-alanyl-D-alanine residue at the free carboxyl end of the pentapeptide (Figure 2.8). Therefore, it inhibits utilization of the substrates by glycosyltransferase to produce the nascent peptidoglycan chain. In this study, vancomycin was used as a positive control antibacterial agent against *S. epidermidis*.

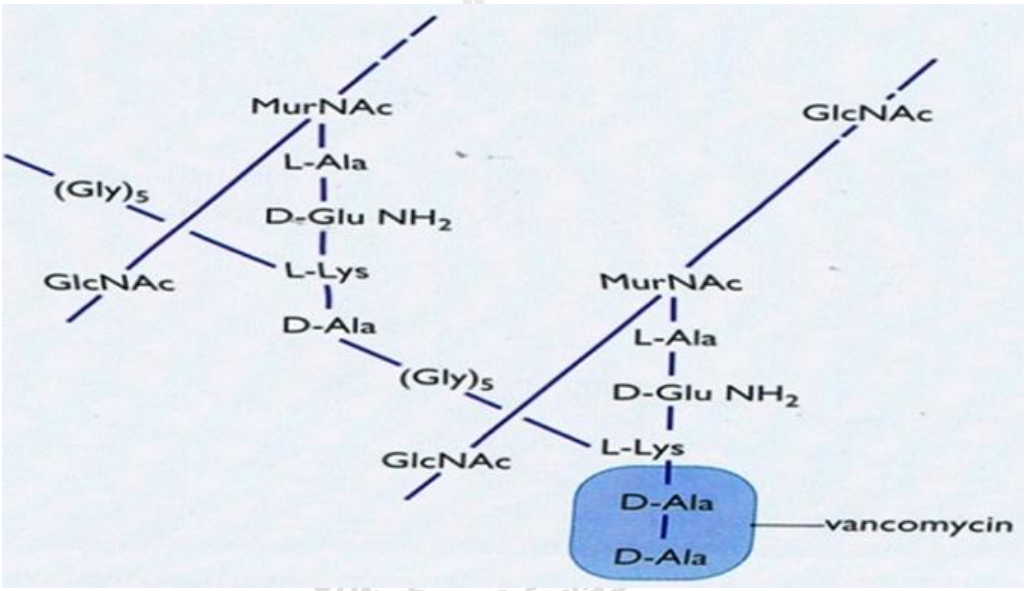

Figure 2.8 Vancomycin inhibits peptidoglycan synthesis (Page, Curtis, Sutter, Walker and Hoffman, 2002).

2.7.2 Drugs that inhibit bacterial protein synthesis

The aminoglycosides antibiotics consist of amino sugars linked through glycosidic bonds to an aminocyclitol ring. Streptomycin, neomycin, kanamycin, and tobramycin were originally isolated from *Streptomyces* species and gentamicin and sisomicin were isolated from *Micromonospora* species. Amikacin and

netilmicin are synthetic derivatives of kanamycin and sisomicin, respectively. These antibiotics exert their effort by passing through the bacterial outer membrane (in Gram-negative bacteria), cell wall and cytoplasmic membrane to the cytoplasm, where they inhibit bacterial protein synthesis by irreversibly binding to the 30S ribosomal proteins. This attachment to the ribosomes has two effects: production of aberrant proteins as the result of misreading of the messenger RNA (mRNA) and interruption of protein synthesis by causing the premature release of the ribosome from mRNA. The aminoglycosides are bactericidal because of their ability to bind irreversibly to ribosomes and are commonly used to treat serious infections caused by many Gram-negative rods and some Gram-positive organisms.

Tetracyclines

The tetracyclines are broad-spectrum, bacteriostatic antibiotics that inhibit protein synthesis in bacteria by binding reversibly to the 30S ribosomal subunits, thus blocking the binding of aminoacyl-transfer RNA (tRNA) to the 30S ribosome-mRNA complex. Tetracyclines (i.e., tetracycline, doxycycline, minocycline) are effective in the treatment of infections caused by *Chlamydia*, *Mycoplasma* and *Rickettsia* species and other selected Gram-positive and Gram-negative bacteria.

Oxazolidinones

Linezolid blocks initiation of protein synthesis by interfering with the formation of the initiation complex consisting of tRNA, mRNA and the ribosome. The drug binds to the 50S ribosomal subunit, which distorts the binding site for tRNA, thus inhibiting formation of the 70S ribosomal subunit initiation complex. Because of this unique mechanism, cross-resistance with other protein inhibitors does not occur.

Chloramphenicol

Chloramphenicol exerts its bacteriostatic effect by binding reversibly to the peptidyl transferase component of the 50S ribosomal subunit, thus blocking peptide elongations.

Clindamycin

Clindamycin blocks protein elongation by binding to the 50S ribosome. It inhibits peptidyl transferase by interfering with the binding of the amino acid-acyl-tRNA complex. Clindamycin is active against staphylococci and anaerobic Gram-negative rods but is generally inactive against aerobic Gram-negative bacteria.

2.7.3 Drugs that inhibit nucleic Acid Synthesis

Quinolones

The quinolones are one of the most widely used classes of antibiotics. These are synthetic chemotherapeutic agent that inhibits bacterial DNA topoisomerase type II (gyrase) or topoisomerase type IV, which are required for DNA replication, recombination and repair. The DNA gyrase-A subunit is the primary quinolone target in Gram-negative bacteria, whereas topoisomerase type IV is the primary target in Gram-positive bacteria.

Rifampin and Rifabutin

Rifampin, a semisynthetic derivative of rifamycin B produced by *Streptomyces mediterranei,* binds to DNA-dependent RNA polymerase and inhibits the initiation of RNA synthesis. Rifampin is very active against aerobic Gram-positive cocci, including staphylococci and streptococci.

2.7.4 Drugs that inhibit bacterial metabolites

The sulfonamides are antimetabolites that compete with p-aminobenzoic acid, thereby preventing the synthesis of the folic acid required by certain microorganism. Trimethoprim is another antimetabolite that interferes with folic acid metabolism by inhibition dihydrofolate reductase, thereby preventing the conversion of dihydrofolate to tetrahydrofolate. This inhibition blocks the formation of thymidine, some purines, methionine and glycine. Mechanisms of each antibiotics are summarized in Table 2.4.

2.8 Bacterial resistance to antimicrobial agents

Bacteria either have pre-existing resistance to drugs, or they develop resistance after exposure to antibiotics. Misuse and overuse of antibiotics have contributed greatly to the increase in resistant strains of bacteria. Often, when bacteria acquire resistance to a certain drug from a particular class (e.g., the penicillin), the bacteria also acquire resistance to all other drugs in that class. Some of the many mechanisms of resistance are summarized as follows (Lim and Web, 2005; Mcmanus, 1997).

1. The organism may acquire genes encoding enzymes, such as β-lactamases, that destroy the antibacterial agent before it can have an effect.

2. Bacteria may acquire efflux pumps that extrude the antibacterial agent from the cell before it can reach its target site and exert its effect.

3. Bacteria may acquire several genes for a metabolic pathway which ultimately

produces altered bacterial cell walls that no longer contain the binding site of the antimicrobial agent.

4. Bacteria may acquire mutations that limit access of antimicrobial agent to the intracellular target site via downregulation of porin genes.

Some examples of drugs and their resistance mechanisms are given in Table 2.5.

The mechanism of action of vancomycin is to prevent cross-linking of peptidoglycan by binding to D-Ala-D-Ala dipeptide of the muramyl peptide. As shown in Table 2.5, most Gram-positive bacteria acquire vancomycin resistance by changing the target molecule D-Ala-D-Ala to D-Ala-D-lactate, resulting in their inability to bind to vancomycin (Epstein, 1991). In case of *S. epidermidis*, it has been reported that *S. epidermidis* are often resistant to antibiotics, including penicillin (Georgopapadakou, 1993), amoxicillin (Kawano, Shimizu, Saitoh, Yagi, Saito and Okamoto, 1996), methicillin (Pierre, Williamson and Bornet Gutmann, 1990), oxacillin (Kiri, Archer and Climo, 2002) and linezolid (Almeida, Lincopan, Araujo and Mamizuka, 2012). Unfortunately, there is currently lack of scientific report according to their mechanism of drug resistance. Therefore, it would noteworthy to find the alternative agents or natural products to against this pathogen.

Table 2.4 Basic Mechanisms of Antibiotic Action (Murray, Rosenthal and Pfaller, 2005).

Antibiotic	Action
Disruption of Cell Wall	Binds PBPs and enzymes responsible for peptidoglycan synthesis
Penicillin	
Cephalosporin	
Cephamycin	
Carbapenem	
Monobactam	
β-lactam/β-lactamaseinhitor	Binds β-lactamases and prevents enzymatic inactivation of β-lactam
Vancomycin	Inhibits cross-linkage of peptidoglycan layers
Isoniazid	Inhibits mycolic acid synthesis
Ethionamide	
Ethambutol	Inhibits arabinogalactan synthesis
Cycloserine	Inhibits cross-linkage of peptidoglycan layers
Polymyxin	Inhibits bacterial membranes
Bacitracin	Inhibits bacterial cytoplasmic membrane and movement of peptidoglycan precursors
Inhibition of Protein Synthesis	Produces premature release of aberrant peptide chains from 30S ribosome
Aminoglycoside	
Tetracycline	Prevents polypeptide elongation at 30S ribosome
Oxazolidinone	Prevents initiation of protein synthesis at 50S ribosome

Table 2.4 Basic Mechanisms of Antibiotic Action (Continued).

Antibiotic	Action
Inhibition of Protein Synthesis	Prevents polypeptide elongation at 50S ribosome
Macrolide	
Clindamycin	
Streptogramins	
Inhibition of Nucleic Acid Synthesis	Bind α subunit of DNA gyrase
Quinilone	
Rifampin	Prevents transcription by binding DNA-dependent RNA polymerase
Rifabutin	
Metronidazole	Disrupts bacteria DNA (is cytotoxic compound)
Antimetabolite	Inhibits dihydropteroate synthase and disrupts folic acid synthesis
Sulfonamide	
Dapsone	Inhibits dihydropteroate synthase
Trimethoprim	Inhibits dihydrofolate reductase and disrupts folic acid synthesis

DNA, Deoxyribonucleic acid; PBPs, penicillin-binding protein; RNA, ribonucleic acid.

Table 2.5 Mechanism of resistance to antimicrobial agents (Luvira, 2006).

Antimicrobial agents	**Mode of Action**	**Resistance mechanisms**
β-lactams	Inhibit cell wall synthesis, Cell division	β- lactamases, altered penicillin binding protein, altered GNB outer-membrane porins, active efflux
Glycopeptides (vancomycin, cycloserine)	Inhibit cell wall division	Altered target site
Aminoglycosides	Inhibit protein synthesis (bind to30s ribosome)	Aminoglycoside-modifying enzyme, Decreased membrane permeability, active efflux
Macrolides	Inhibit protein synthesis (bind to 50s ribosome)	Altered target, enzymatic inactivation, active efflux
Tetracycline	Inhibit protein synthesis (bind to 30s ribosome)	Efflux, altered target, enzymatic inactivation, decreased permeability
Chloramphemcol	Inhibit protein synthesis (bind to 50s ribosome)	Chloramphenicol acetyltransferase, active efflux
Rifampin	Inhibits nucleic acid synthesis	Altered target, decreased permeability of membrane
Sulfonamides Trimethoprim	Inhibit folic acid synthesis	Altered target

CHAPTER III

MATERIALS AND METHODS

3.1 Plant material

The leaves of young *A. crassna* (2-3 year old) were collected from a cultivated field in Nakhon Ratchasima province, Thailand. The plant was identified by a botanist, Dr. Paul J. Grote, School of Biology, Suranaree University of Technology (SUT) and specimen of the plant has been kept at School of Pharmacology, SUT. The voucher specimen number is Pharm-Chu-005.

The leaves were oven dried at 50 °C, and then cut into small pieces. Dried leaves (24 g) were extracted in boiling water (400 ml) for 30 min twice. The pooled extracts were filtered and concentrated at 40 °C using a rotary evaporator under low pressure. The residue was freeze-dried in a lyophilizer. The extract with a total yield of 14.2% was stored at -20 °C until used.

3.2 Phytochemical screening

Phytochemical screening procedures were carried out according to the standard methods previously reported (De *et al*., 2010; Yadav and Agarwala, 2011). Qualitative phytochemical compositions of the crude extract of *A. crassna* leaves were determined for the presence of alkaloids, flavonoids, tannins, saponins and cardiac glycosides.

3.2.1 Test for alkaloids

Alkaloids were tested by Mayer's reagent (HgCl2, 13.6 g, KI, 5 g in 100 ml of distilled water), Dragendorff's reagent (bismuth nitrate, 8 g; nitric acid 30%, 12 ml; and potassium iodide, 27.2 g in 100 ml of distilled water), Hager's reagent (saturated aqueous solution of picric acid), Wagner's reagent (iodine crystal 1.27 g, potassium iodide 2.0 g in 100 ml of distilled water) and tannic acid (5% w/v). The formation of cream color precipitate in Mayer's test, reddish brown precipitate with Dragendorff's reagent and Wagner's reagent, yellow precipitate in Hager's test and buff precipitate with tannic acid solution were regarded as positive for the presence of alkaloids.

3.2.2 Test for flavonoids

Existence of flavonoids in the extract was examined using Shinoda test by adding 1 to 2 pieces of magnesium ribbon followed by a few drops of concentrated hydrochloric acid. Appearance of pink scarlet color indicated the presence of flavonoids.

3.2.3 Test for tannins

The presence of tannin was determined by ferric chloride test, gelatin test and vanillin hydrochloride test. The extract was dissolved with distilled water and added a few drop of ferric chloride solution ($FeCl_3$ 3%), 1% gelatin salt solution (1% gelatin containing 10% NaCl) or vanillin hydrochloride reagent (1:1 v/v of 1% vanillin in methanol and 8% HCl). The extract was considered to have tannins when blue green color, white and purple red precipitates were formed with ferric chloride test, gelatin test and vanillin hydrochloride test, respectively.

3.2.4 Test for saponins

The extract was added to 1 ml of distilled water, shaken vigorously to froth and then allowed to stand for 30 min. The formation of stable foam was taken as an indication for the presence of saponins.

3.2.5 Test for cardiac glycosides

The presence of unsaturated lactone ring was analysed by Kedde's test. Kedde's reagent (2% 3,5 dinitrobenzoic acid in methanol) was added to the extract followed by a few drops of 1 N KOH. Production of purple color indicated a positive result. Test for deoxy sugar was conducted by adding 1 ml of glacial acetic acid containing trace amount of ferric chloride. 1 ml of concentrated H_2SO_4 was carefully added by the side of test tube. A brown ring at the interface indicated a positive result. Liebermann's test was performed to examine steroid nucleus. A few drops of acetic anhydride were added to the extract followed by a few drops of concentrated sulfuric acid. A color change from violet to blue to green indicated the presence of steroid nucleus of glycoside. All of positive results from these three tests are required for the presence of cardiac glycoside.

3.3 Determination of total phenolic compounds

The amount of total phenolic compounds was measured by a method described by Matthaus (2002). In brief, 5 mg of the extract was dissolved in 1 ml of distilled water. A 100 μl aliquot of this mixture was added to 2 ml of 2% Na_2CO_3 followed by 100 μl of Folin-Ciocalteau reagent in methanol (1:1 v/v). After 30 min of incubation, the absorbance was measured at 750 nm. The concentration was

calculated using gallic acid as a standard. The results were expressed as milligrams gallic acid equivalents (GAE) per gram extract.

3.4 Determination of antioxidant activity

3.4.1 Scavenging effects on DPPH radicals

To measure antioxidant activity, the 2,2-diphenyl-1-picrylhydrazyl hydrate (DPPH) radical scavenging assay was carried out according to the procedure described previously (Bor *et al*., 2006). The crude extract (100 μl; final concentration range from 0-50 μg/ml) was added to 4.0 ml of 50 μM DPPH in methanolic solution and the final volume was adjusted to 5.0 ml with water. After vortexing, the mixture was incubated for 30 min in the dark at room temperature. The decrease in absorbance at 517 nm was measured using a spectrophotometer. Antioxidant activity was expressed as IC_{50}, which was defined as the concentration of the extract required to inhibit the formation of DPPH radicals by 50%.

3.4.2 2,2'-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid (ABTS) assay

ABTS radical scavenging activity of extract was carried out according to the procedure described previously (Re et al., 1999). ABTS radical cation ($ABTS^{\bullet+}$) was produced by the reaction between 5 ml of 14 mM ABTS and 5 ml of 4.9 mM potassium persulfate ($K_2S_2O_8$). The resulting solution was stored in the dark at room temperature for 16 hr. Before use, this solution was diluted with ethanol to give an absorbance of 0.700 ± 0.020 at 734 nm. The plant extract (50 μl) at various concentrations were added to 950 μl of $ABTS^+$ solution and mixed thoroughly. The reaction mixture was allowed to stand at room temperature for 6 min, the absorbance

was measured at 734 nm and compared to the standard butylated hydroxytoluene (BHT).

3.4.3 Ferric reducing antioxidant power (FRAP) assay

The FRAP assay was conducted according to Dordevic *et al*. (2010) with minor modification. Briefly, the FRAP reagent consists of 10 mM TPTZ (2,4,6-tripyridyl-striazine) in 40 mM HCl, 20 mM $FeCl_3$, and 300 mM acetate buffer (pH 3.6) in proportions of 1:1:10 (v/v/v). 50 μl of the sample was added to 1.5 ml of FRAP reagent (freshly prepared and warmed to 37 °C before use). The absorbance was measured at 593 nm using a UV spectrophotometer after 4 min of incubation. A standard curve was constructed using $FeSO_4$ solution. The results were expressed as μmol Fe^{2+}/mg dry weight of plant material. All measurements were carried out in triplicate and the mean values were calculated.

3.5 Acute toxicity in mice

Male and female mice (weighing 25-35 g) were used for acute toxicity. The acute toxicity performed in this experiment was conducted by following the OECD guidelines for Testing of Chemicals (2001). The *A. crassna* aqueous extract was prepared in the concentration of 200 mg/ml and 750 mg/ml by dissolving in distilled water for dosing group of 2,000 and 15,000 mg/kg body weight respectively, which was calculated based on their body weight after fasting. The mice were fasted overnight for 16 h prior to dosing (n = 10; 5 of each sex per group). Food was continuously withheld for 3-4 h after administration, whereas water was provided ad libitum. All mice were monitored for clinical signs of toxicity at 0.5, 1 and 3 h after oral administration of the extract and once daily thereafter for 14 days. The following

toxicity signs and symptoms were examined: skin, fur, eyes and mucous membranes evaluation, autonomic effects (e.g., salivation), central nervous system effects (tremors and convulsions), and changes in the level of activity, posture, strength and bizarre behavior. Weekly changes in body weight were examined. The animals were sacrificed on day 14 by lethal dose of sodium pentobarbital. The organs and tissues were examined macroscopically for toxicant-induced changes. Internal organ (heart, lung, liver, spleen, testes, ovaries and uterus) were removed and gross histopathological examinations were performed.

3.6 Antibacterial assays

3.6.1 Disc diffusion assay

The antibacterial activity of the crude extract was assayed against *S. epidermidis* (obtained from Thailand Institute of Scientific and Technological Research; TISTR 518) using disc diffusion method described by Rabe and Van Staden (1997). Briefly, 100 μl of bacteria (10^8 CFU/ml) was spread onto the Mueller-Hinton agar plate. The extract (2, 4 and 6 mg) was applied to filter paper discs (Whatman No. 1, 6 mm diameter) and then placed on the previously inoculated agar plate. After 24 h of incubation at 37 °C, clear inhibition zones around the discs indicated the presence of antibacterial activity. The assay was carried out in triplicate. Vancomycin (30 μg) was used as a positive control.

3.6.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Determination of the minimum inhibitory concentration (MIC) against *S. epidermidis* (104 CFU/ml) was conducted by a two-fold serial dilution method.

MIC was considered as the lowest concentrations of the agents that yielded no visible growth of microorganisms after 24 h of incubation at 37 °C. The MBC determination was carried out by subculturing 100 µl from each tube from the MIC assay onto fresh substance-free MH agar plates. The MBC was defined as the lowest concentration of agent that produced no growth of subcultures.

3.6.3 Scanning electron microscopy (SEM)

The SEM study was performed on *S. epidermidis* treated with MIC of the extract of *A. crassna* leaves. *S. epidermidis* was cultured to reach mid-log phase in mueller hinton broth (MHB) before use. Treated cells were prepared and the morphological changes were observed. The bacterial samples were washed five times with fresh media and then fixed with 2.5% glutaraldehyde in phosphate buffer (pH 7.2) at 4 °C for 1 h, washed three times with phosphate buffer for 10 min and fixed with 1% osmium tetraoxide for 2 h. This was followed by three washings in phosphate buffer for 10 min and subsequently dehydrated in a series of ethanol concentrations (30%, 50%, 70%, 90% and 95%), for 15 min each. The samples were subjected to 100% ethanol and CO_2 to achieve the critical point and then coated with gold ion in a pressure metallic chamber. At the end of the process, the samples were submitted for analysis by SEM.

3.6.4 Transmission electron microscopy (TEM)

Cellular damage of bacteria was examined using TEM. Bacterial cells treated with vehicle, vancomycin and the extract of *A. crassna* leaves were harvested after 24 h of incubation and fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, for 2 h. The cells were washed three times with 0.05 M phosphate buffer (pH 7.2) and postfixed for 2 h with 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.2) at

room temperature. The cells were washed twice in phosphate buffer. The cells were dehydrated through serial graded concentrations of ethanol (twice in 35, 70, 95 and 100% ethanol, respectively, for 15 min), then infiltrated and embedded in spurr's resin. Ultrathin sections were cut with a diamond knife using an ultramicrotome and then mounted on bare copper grids. They were stained with 2% uranyl acetate and lead citrate. Microscopy was performed with Tecnai G2 20-Twin at Suranaree University of Technology.

3.6.5 Biofilm assay

The inhibitory effect of the extract on biofilm formation was examined by microscopic analysis. A culture of S. epidermidis was prepared in tryptic soy broth (TSB) at 37 °C for 24 h. 30 µl aliquots of the culture were pipetted into each well of 24-well plates in the presence of 3 ml TSB and incubated at 37 °C for 24 h to form biofilm. Thereafter, medium was replaced with fresh medium containing the extract of *A. crassna* leaves or vancomycin. After incubation of another 24 h, the medium was removed and each well was gently washed three times with phosphate buffer saline (PBS). The inhibitory effect on biofilm formation was observed by phase contrast microscopy.

3.7 Determination of antihyperglycemic activity

3.7.1 Induction of diabetes

In this study, adult male Wistar-Albino rats (200-250 g) were followed in accordance with guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council of Thailand. The procedures of the experiment was performed in accordance with the advice of the Institutional Animal

Care and Use Committee, Suranaree University of Technology, Nakhon Ratchasima, Thailand.

Each animal were housed in a cage and fed with standard pellet diet and water. Prior to experimental treatments, animals were fasted overnight. Diabetes was induced by the intraperitoneal injection of STZ, dissolved in citrate buffer (0.1 M, pH 4.5). The first day after STZ administration (65 mg/kg), rats were given 5% glucose solution orally. Three days after the injection, the blood glucose levels were measured and the animals with blood glucose levels above 200 mg/dl were considered to have diabetes. Steady diabetes were confirmed by measuring blood glucose values before starting an experiment. Five animals were used for each group. The extracts were given orally using oral gastric gavages to the fasted animals. Blood samples were collected from the tip of tail.

3.7.2 Experiment design

Male Wister rats were divided into 5 groups and each group included 5 rats: Group I (NC) normal control rats received sterile water. Group II (DM); diabetic control rats received sterile water. Group III; diabetic rats treated with glibenclamide tablets 10 mg/kg; Group IV (DM+Extract); diabetic rats treated with the *A. crassna* extract 250 mg/kg; Group V (DM+Extract); diabetic rats treated with the *A. crassna* extract 1000 mg/kg.

3.7.3 Sample collection

Body weight and blood glucose analyses were done on 5th, 10th and 15^{th} day. Blood samples were collected from tail vein. Blood glucose levels were determined using Glucometer Accu-Chek Performa glucose test strips (Roche Diagnostics, USA).

3.7.4 Histological examinations

After taking blood samples, all animals were sacrificed and their pancreases were removed. The pancreases were cleaned with 0.85% NaCl solution and fixed with 10% neutral buffered formaldehyde. Then, tissues were dehydrated in serial ethanol solution (95%, 100%) and acetone. Tissue samples were cleaned with xylene. The tissues were infiltrated with molten paraffin at 65 ºC and embedded in paraffin block. Tissues were sectioned of 5 µm thickness. The sections were mounted onto slides using gelatin coating solution. The sections were dried at 56 ºC for 45 min in a hot air oven. Tissue sections were deparaffined in xylene. Tissue sections were hydrated with serial ethanol solution (100%, 95%). The sections were washed in running tap water. Sections were stained with haematoxylin for 10 min and washed in running tap water. The sections were stained with eosin for 10 min. Stained sections were dehydrated with serial ethanol solution (95%, 100%). The sections were immersed in acetone and xylene. The stained sections on the slides were covered with cover slip. The photomicrographs of each tissue section were observed under microscope (model CX31, Olympus, Tokyo, Japan).

3.8 Statistical analysis

All experimental results were expressed as means ± standard deviation and analyzed by one way ANOVA followed by Student-Newman-Keuls test. The results with p value < 0.05 were considered as statistically significant differences.

CHAPTER IV

RESULTS

4.1 Phytochemical screening

Figure 4.1 *A. crassna* leaves (A) and dried extract (B).

The results of the phytochemical analysis of the leaf of *A. crassna* are shown in Table 4.1. The results obtained showed the presence of flavonoids, tannins, saponins, cardiac glycosides and an absence of alkaloids.

4.2 The total phenolic compounds

Total phenolic compound of plant extract, using water as solvent is shown in Table 4.1. The result for total phenolics was expressed as gallic acid equivalent (mg GAE/g). The total phenolic of the leaf extract was 176.61±24.46 mg GAE/g extract (n=3).

Table 4.1 The phytochemical compounds and total phenolics content of the aqueous extract.

Phytochemical compounds	Presence	mg GAE/g extract
Tanins	+	ND
Flavonoids	+	ND
Saponins	+	ND
Cardiac glycosides	+	ND
Alkaloids	-	ND
Total phenolic	+	176.61±24.46

+: Present, -: Absent, ND: not determined

4.3 Antioxidant activity

The result of radical scavenging capacities which were determined by DPPH, ABTS and FRAP scavenging methods are shown in Table 4.2. The DPPH scavenging effect of *A. crassna* exhibited a potential free radical scavenging activity. Maximum scavenging activity (91.52%) was observed at 50 µg/ml concentration and the half inhibition concentration (IC_{50}) of *A. crassna* leaves extract and ascorbic acid were found to be 7.25±2.05 µg/ml and 1.33±0.08 µg/ml respectively. The results indicated that the aqueous extract of *A. crassna* decrease in the DPPH radical may be due to the scavenging ability of *A. crassna*. Free radical scavenging activity was increased with an increasing concentration.

The results of ABTS radical scavenging activity of the extract and standard (BHT) were present in Table 4.2. The scavenging activity of *A. crassna* leaves extract against ABTS radical was in a concentration dependent manner. The percentage inhibition of the extract (96.60%) was observed at 1,200 µg/ml concentration. The IC_{50} values of the extract and BHT were 218.93±29.77 µg/ml and 83.09±0.45 µg/ml, respectively.

Ferric reducing antioxidant power (FRAP) is shown in table 4.2. The FRAP value, expressed in µmol of Fe^{2+}/ mg dried extract, was found in *A. crassna* leaves extract at 1.18±0.07 µmol of Fe^{2+}/ mg dried extract.

Table 4.2 Radical scavenging activities and reducing power of the aqueous extract of *A. crassna* leaves.

	Methods		
	DPPH radical (IC_{50}: µg/ml)	**ABTS (IC_{50}: µg/ml)**	**FRAP (µmol Fe^{2+}/mg dried extract)**
A. crassna leaf extract	7.25 ± 2.05	218.93 ± 29.77	1.18 ± 0.07
Ascorbic acid	1.33 ± 0.08	-	-
Butylated hydroxytoluene (BHT)	-	83.09 ± 0.45	-

Values are expressed as means ± standard deviation (n=3).

4.4 Acute oral toxicity of *A. crassna* leaf extract in mice

The acute toxicity of aqueous *A. crassna* leaf extract in mice is summarized in Table 4.3. The results showed that with the doses of 2,000 and 15,000 mg/kg body weight of the extract, all treated mice did not exhibit abnormal signs of toxicity or

deaths. Normal increase in body weight and none of gross pathological lesions were observed on the control and treated mice.

4.5 Antibacteria activity of *A. crassna*

By disc diffusion assay, it was found that the extract can inhibit growth of *S. epidermidis* and *S. aureus* (Table 4.4) at 2, 4 and 6 mg. Whereas, vancomycin was used as a positive control. The results showed that the extract possessed less potency than vancomycin (30 µg). The extract did not inhibit the growth of *Pseudomonas aeruginosa* and *Bacillus subtilis.*

The MIC values of the extracts obtained using the micro-dilution method as shown in Table 4.5. The leaf extracts of *A. crassna* gave MIC and MBC values at 6 and 12 mg/ml against *S. epidermidis*, respectively, when compared with control cell after 24 h of incubation at 37 °C. The assay were carried out in triplicate.

Table 4.3 Acute oral toxicity of the extract of *A. crassna* leaves in mice.

treatment (mg/kg)	sex	Body weight (g)			Mortality	Symptoms Of toxicity	Gross pathology
		Day 1	Day 8	Day 15			
0		33.20±0.84	37.06±1.52	41.00±1.73	0	None	Normal
2000	Male	32.80±1.30	37.20±0.84	41.00±1.00	0	None	Normal
15000		34.40±1.14	39.80±0.84	43.20±0.84	0	None	Normal
0		28.40±1.67	30.80±2.17	34.00±1.58	0	None	Normal
2000	Female	28.00±1.22	30.00±1.22	33.20±1.64	0	None	Normal
15000		28.80±1.30	31.80±1.30	34.00±1.00	0	None	Normal

Values are expressed as means ± standard deviation (n=5); $p>0.05$ compared to control.

Table 4.4 Antibacterial activity of the aqueous extract of *A. crassna* leaves.

Microorganisms	Diameters of inhibitions zone (mm)			Positive control	
	A. crassna extract (mg)			Vancomycin	Kanamycin
	2	4	6	(30 µg)	(10 µg)
Gram positive bacteria					
Staphylococcus aureas	10.0±1.0	11.0±1.1	13.0±1.3	18.0±1.0	-
S. epidermidis	12.0±1.0	15.0±0.4	18.0±1.0	21.0±1.0	-
Bacillus subtilis	n.i.	n.i.	n.i.	24.0±0.57	-
Gram negative bacteria					
Pseudomonas aeruginosa	n.i.	n.i.	n.i.	-	20.0±0.3

n.i.; no inhibition zone, Data are means ± standard deviation (n=3).

Table 4.5 The MIC and MBC values of aqueous extracts against *S. epidermidis*.

S. epidermidis	***A. crassna*** **(mg/ml)**	**Vancomycin (µg/ml)**
MIC	6	1.5
MBC	12	3

4.6 Scanning Electron Microscopy (SEM)

S. epidermidis was treated with the extract at MIC (6 mg/ml) and incubated at 37 °C for 24 h. SEM analyses were performed and compared to untreated and vancomycin-treated groups. Control bacteria in the absence of the extract showed regular morphology (Figure 4.2A), whereas cells treated with vancomycin (Figure 4.2B) and the aqueous extract of *A. crassna* leaves (Figure 4.2C) appeared swollen and distorted after 24 h of incubation.

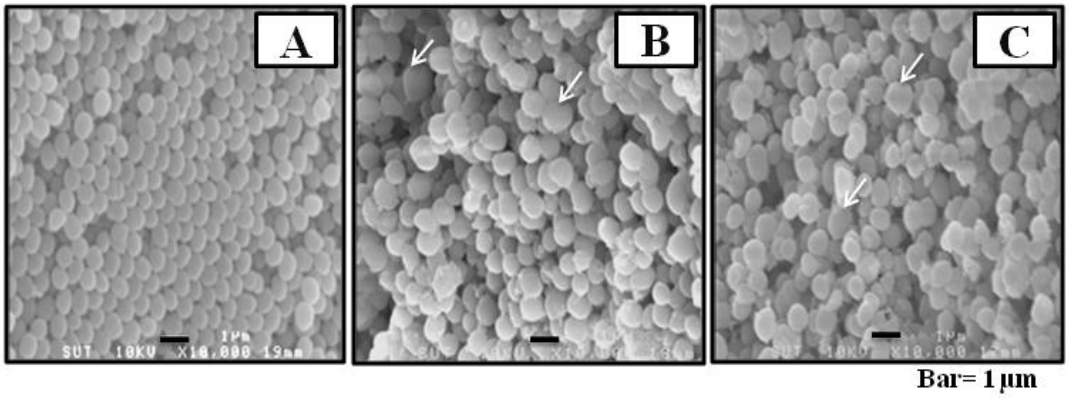

Figure 4.2 Scanning electron micrographs of *S. epidermidis* treated with *A. crassna* leaves extract. The swollen cells (arrows) were observed after treated for 24 h with vancomycin (B) and the extract (C) compared with regular shape of control (A). Enlargement: ×10000.

4.7 Observation of cellular damage by TEM

Electronmicroscopic investigations clearly showed that the bacterial cells treated with crude *A. crassna* leaves extract caused damage to the ultrastructure of *S. epidermidis* cells compared with untreated control. Figure 4.3 showed that cell wall and cytoplasmic membrane of *S. epidermidis* cell were damaged after the addition of the extract. Similarly, some of vancomycin treated bacterial cells showed morphological damages such as cell shape distortion, indicating that vancomycin was positive control and had effect on the cell wall and irregular morphology.

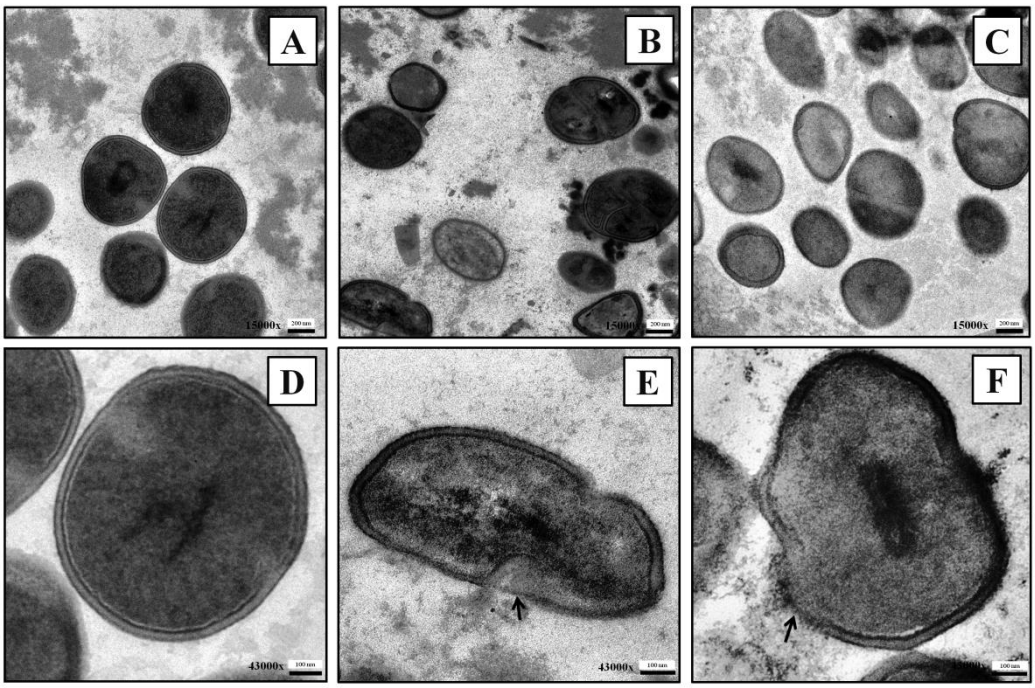

Figure 4.3 Transmission electron micrographs of *S. epidermidis* treated with *A. crassna* leaves extract. (A), (B) and (C) are overview of the untreated cells and cells treated with vancomycin and the extract, respectively (Enlargement: ×15000). Bacterial cells with irregular shape were seen in both treated groups. Damaged cell walls (arrows) were observed after 24 h of incubation with vancomycin (E) and the extract (F) compared with control (D). Enlargement: ×43000.

4.8 Effects of the extract on biofilm formation

Crystal violet staining of surface-attached cells by microtiter plate assay is a popular and convenient method for quantitative detection of bacterial biofilm formation; however direct microscopic observation is strongly recommended to confirm the data from staining analysis (Merritt, Kadouri and O'Toole, 2011). Due to the absorption of the *A. crassna* leaf extract at 595 nm, in the present study the effect on biofilm formation was investigated by microscopic analysis which gave more accurate information. The phase contrast microscopic images (Figure 4.4) showed that *S. epidermidis* formed vast empty regions on surface with a few clusters of cells and scattering of single cell when treated with the extract and vancomycin.

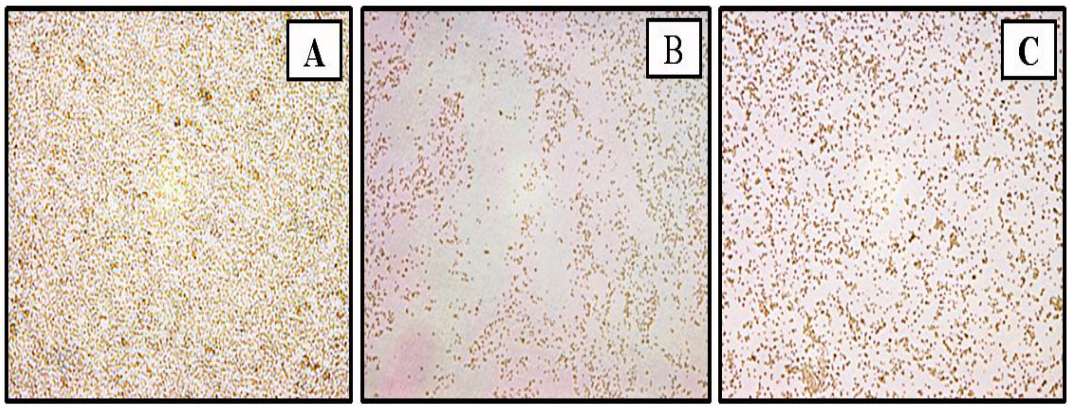

Figure 4.4 Microscopic images on biofilm formation of *S. epidermidis* treated with *A. crassna* leaf extract. Cells treated with vancomycin (B) or the extract (C) formed less biofilm compared to control sample (A). Enlargement: ×400.

4.9 Determination of antihyperglycemic activity

4.9.1 Effect of *A. crassna* extract on body weight

Body weights of rats in the 5 groups were shown in Figure 4.5. After administered with STZ, rat body weights in all groups except for the glibenclamide-teated group significantly decreased compared to normal control (0 day; before treatment). At the 5^{th}, 10^{th} and 15^{th} day of treatment, the body weights of glibenclamide rats, but not the *A. crassna* treated rats, significantly increased compared to diabetic-vehicle control group (p<0.05).

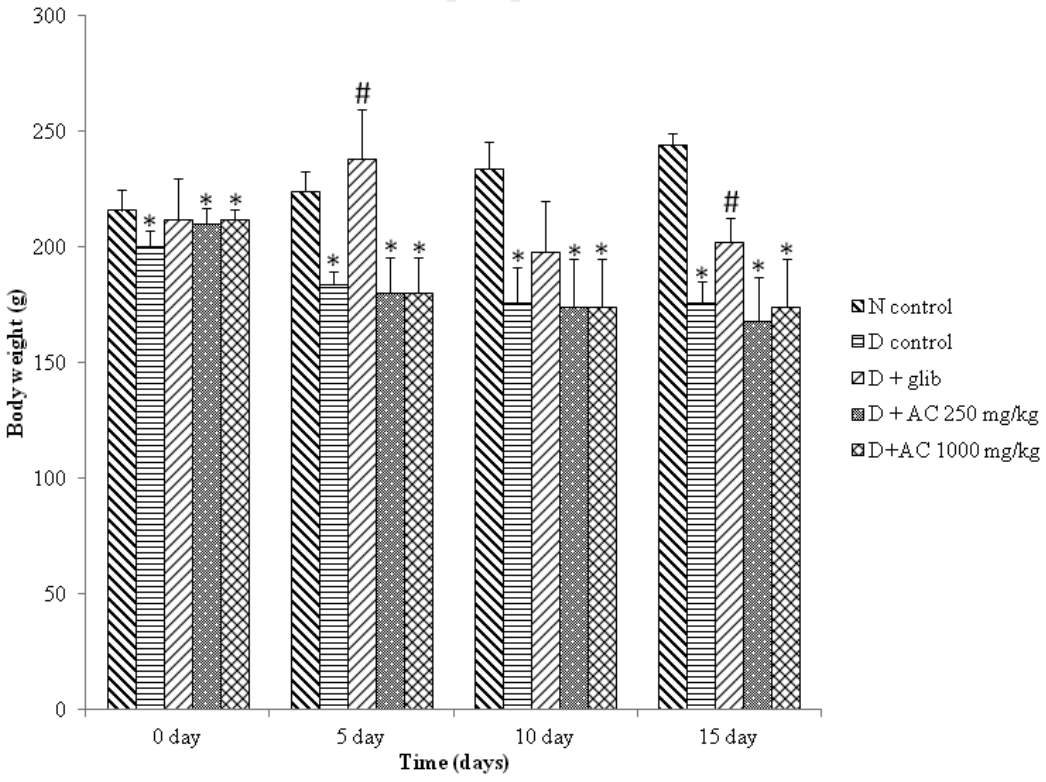

Figure 4.5 Body weight changes of rats, Values are mean ± SD (n=5). N group: normal control rats; D group: diabetic rats; D+glib group: glibenclamide treated diabetic rats; D+AC group: *A. crassna* treated diabetic rats. *p<0.05 statistically significant difference from normal control. #p<0.05 statistically significant difference from diabetic control.

4.9.2 Effect of *A. crassna* leaf extract on blood glucose in experimental groups

The mean blood glucose of the diabetic rats after STZ induction was significantly higher than that in normal control rats (Figure 4.6). After 10 and 15 days of treatment, blood glucose level of glibenclamide-treated diabetic rats were significantly (p<0.05) decreased compared with diabetic vehicle control rats. However, in accordance with its effect on body weight, the extract did not decrease blood glucose level of diabetic rats after the same duration of treatment even with high concentration (1000 mg/kg).

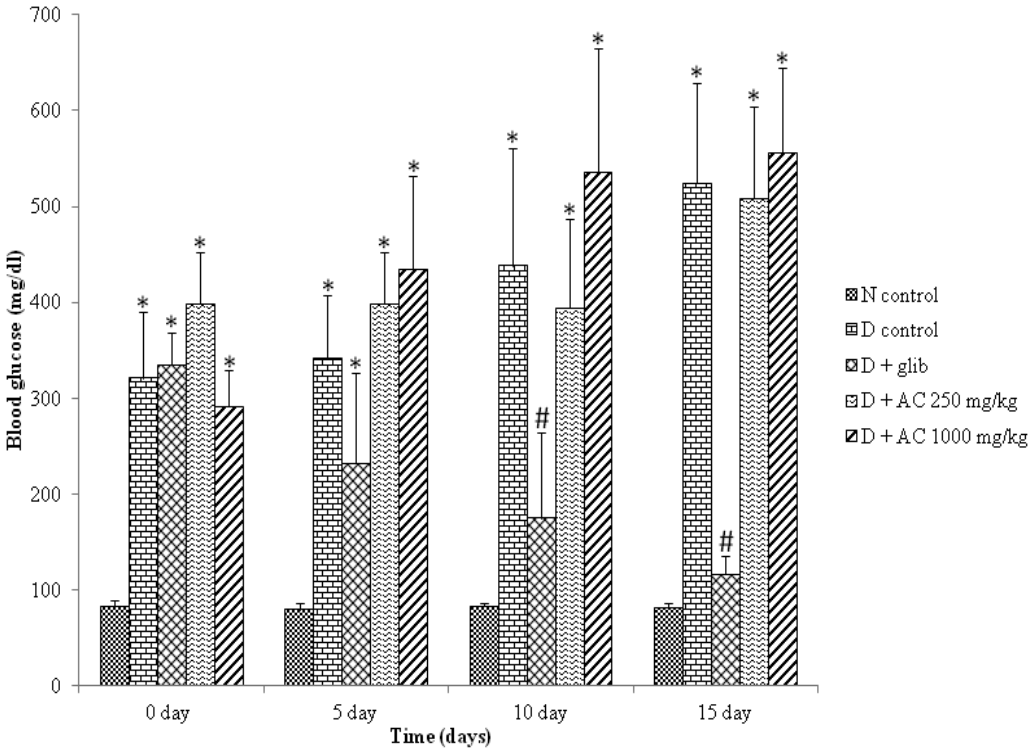

Figure 4.6 Changes of blood glucose of rats, Values are mean ± SD (n=5). N group: normal control rats; D group: diabetic rats; D+glib group: glibenclamide treated diabetic rats; D+AC group: *A. crassna* treated diabetic rats. *p<0.05 statistically significant difference from normal control. #p<0.05 statistically significant difference from diabetic.

4.9.3 Histopathological examination

Histopathological examination of pancreas exhibited normal appearances of islets of Langerhans in normal control rats (Figure 4.7A). Streptozotocin induced diabetes in rats by damaging the islets of Langerhans as shown in Figure 4.7B. This effect was reversed when animals were treated with glibenclamide for 15 days (Figures 4.7C). In contrast, the islet cells of diabetic rats receiving the extract of *A. crassna* leaves at 250 (Figure 4.7D) and 1,000 mg/kg (Figure 4.7E) appeared distorted and damage similar to those of diabetic vehicle control group (Figure 4.7B).

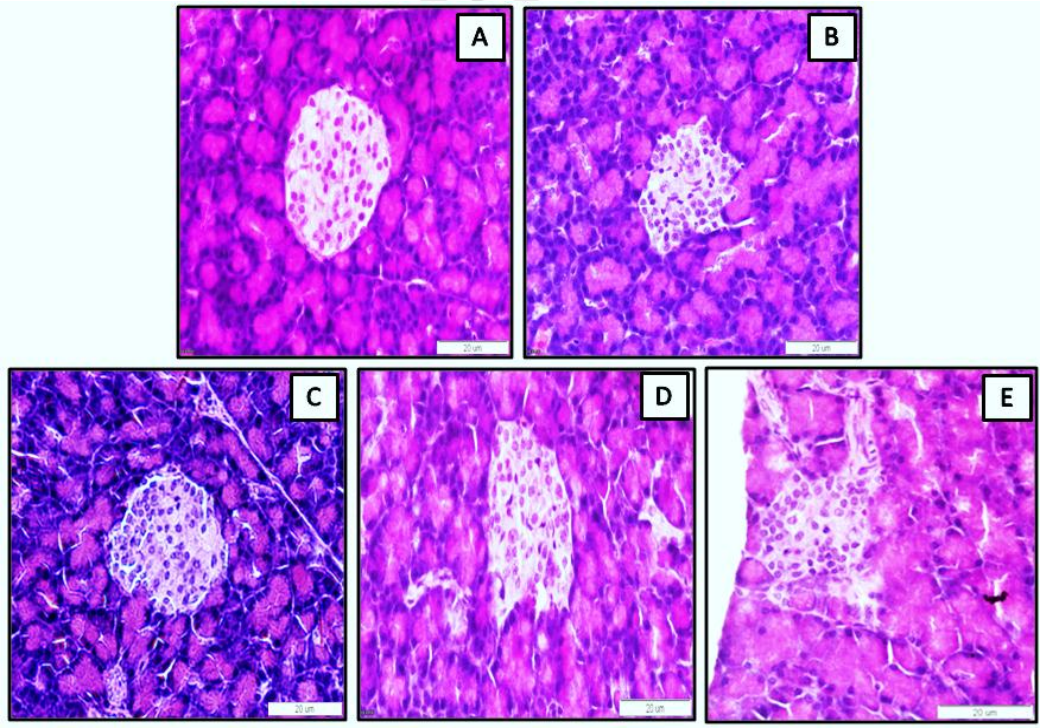

Figure 4.7 Histopathological observations of *A. crassna* and glibenclamide treated pancreas in streptozotocin induced diabetic rats stained by haematoxylin and eosin. (A) Normal control, (B) Diabetic control, (C) Diabetic+glibenclamide 10 mg/kg, (D) Diabetic+*A. crassna* 250 mg/kg, (E) Diabetic+*A. crassna* 1000 mg/kg. Enlargement: ×400.

CHAPTER V

DISCUSSION AND CONCLUSIONS

In general, type and level of biological activity exhibited by any plant material depends on many factors, including the plant part, geographical source, soil conditions, harvest time, moisture content, drying method, storage conditions and post-harvest processing. For example, the relatively high temperatures that can be generated during tissue grinding can denature chemical constituents and the extraction solvent, time period and temperature can affect the level and composition of secondary metabolites extracted from plant tissues (Wendakoon, 2012). Thus, phytochemical constituents of the extract from the same plant may be different from laboratory to laboratory. In this study, hot water was chosen as an extraction solvent to simulate the method used for healthy tea consumption. The aqueous extract is widely accepted for crude extraction of phytochemicals from medicinal plants especially for therapeutic applications. The aqueous extract of *A. crassna* obtained from this study consisted of tannins, flavonoids, saponins and cardiacglycosides. Alkaloids are theoretically lipophilic, therefore they were not detected.

A various phytochemical constituents are known to have beneficial importance in medicinal sciences (Mithraja, Antonisamy, Mahesh1, Paul and Jeeva, 2011; Abraham and Aeri, 2012). Each group of phytochemical compounds shows different biological effects. For example, flavonoids which are the major group of phenolic compounds are reported to have antimicrobial, antiviral, antioxidants and

spasmolytic activity. Tannins which are water-soluble polyphenols are also known to possess antimicrobial agents. The growth of many fungi, yeasts, bacteria and viruses was inhibited by tannins (Chung, 1998). Phytotherapeutically tannin-containing plants are used to treat nonspecific diarrhoea, inflammations of mouth and throat and slightly injured skins (Westendarp, 2006). There is evidence showing that saponins can be therapeutically used in hypercholesterolemia, hyperglycemia, antioxidant, anticancer, anti-inflammatory and weight loss (Hasan *et al.*, 2009; Maobe, Gatebe and Gitu Rotich, 2013). Moreover, it is a bioactive antibacterial agent of several plants (Mandala, Babub and Mandal, 2005; Manjunatha, 2006). Cardiac glycosides are naturally cardioactive drugs used in the treatment of congestive heart failure and cardiac arrhythmia (Newman, Yang, Pawlus and Block, 2008).

Phenolic compounds are constituted in one of the biggest and widely distributed groups of secondary metabolites in plants (Scalbert and Williamson, 2000). The phenolic compounds are very important constituents in plants because of their scavenging ability of their hydroxyl groups and preventing decomposition of hydroperoxides into free radicals (Maisuthisakul, Suttajit and Pongsawatmanit, 2007). It has been shown that phenolics are active in curing kidney and stomach problems as well as helpful as anti-inflammatory in action (Zhu, Phillipson, Greengra, Bowery and Cai, 1997). The total phenolic content in the extract in this study was 176.61 mg GAE/g which is considered as moderate level.

There are several lines of evidence showing positive correlation between total phenolic content and antioxidant properties (Velioglu,Mazza, Gao, Oomah., 1998; Moller, Lindberg, Aaltonen and Skibsted, 1999; Turkoglu, Duru, Mercan, Kivrak and Gezer, 2007). It is expected that the extract may have some beneficial antioxidant

activities similar to other plants. ABTS, DPPH and FRAP assays are widely used to determine the antioxidant capacity in plant extracts because of their simplicity, stability and accuracy (Reddy, Sreeramulu and Raghunath, 2010). The Folin-Ciocalteau method is a rapid and widely used assay to investigate the total phenolic content. This method is based on an oxidation-reduction reaction in which phenolic compounds are oxidized with simultaneous reduction of a phosphotungsten-phosphomolybdate complex in an alkaline medium, reacting blue (Gzella, Makuch and Matlawska, 2012). However, it is known that different phenolic compounds have different responses in the Folin-Ciocalteau method (Kahkonen *et al*., 1999). In recent years, phenolic compounds from natural sources have been the subject of interests in many scientific researches due to their positive effects on human health, attributed mainly to their antioxidant activities (Kojic *et al*., 2011).

DPPH assay is a stable free radical method. It is an easy, rapid and sensitive way to survey the antioxidant activity of a specific compound or plant extract (Koleva, Beek, Linssen, Groot and Evstatieva, 2002). The principle of DPPH method is based on the reduction of DPPH in the presence of a hydrogen donating antioxidant due to the formatiom of diphenyl picryl hydrazine (Rajan, Gokila, Jency, Brindha and Sujatha, 2011). The results indicated that the aqueous extract of *A. crassna* decrease in the DPPH radical may be due to the scavenging ability of *A. crassna*. Free radical scavenging activity was increased with an increasing concentration.

ABTS assay is base on the scavenging of light by ABTS radicals. ABTS radical cation has been often used in the evaluation of antioxidant activity of single compounds and complex mixtures of various origins (body fluids, foods, beverages, plant extracts) (Katalinic, Milos, Kulisic and Jukic, 2006). An antioxidant with an

ability to donate a hydrogen atom will quench the stable free radical, a process that is associated with a change in absorption. The relatively stable ABTS radical was green and it was quantified spectrophotometrically at 734 nm. The ABTS scavenging capacity of the extract was comparable with that of the standard BHT (Rajan, Gokila, Jency, Brindha and Sujatha, 2011).

Ferric reducing antioxidant power (FRAP) assay was used to evaluate the antioxidant potential of *A. crassna* leaves extract. Principally, FRAP assay treats the antioxidants in the sample as reductant in a redox-linked colorimetric reaction (Guo *et al*., 2003). This assay is relatively simple and easy to conduct. FRAP assay measures the reducing potential of antioxidant to react on ferric tripyridyltriazine (Fe^{3+}- TPTZ) complex and produce blue color of ferrous which can be detected at absorbance 593 nm (Benzie and Strain, 1996). Antioxidant compound which acts as reducing agent exert their effect by donating hydrogen atom to ferric complex and thus breaking the radical chain reaction. The higher the absorbance is, the higher is the antioxidant activity which is indicated by the high FRAP value.

As shown in appendix A1, maximum scavenging activity (91.52%) was observed at 50 µg/ml by DPPH method. The half inhibition concentration (IC_{50}) of *A. crassna* leaves extract and ascorbic acid were found to be 7.25 µg/ml and 1.33 µg/ml respectively (Table 4.2). The scavenging activity of *A. crassna* leaves extract against ABTS radical is in a concentration dependent manner. The percentage inhibition of the extract (96.60%) was observed at 1,200 µg/ml concentration (Appendix A2). The IC_{50} values of the extract and BHT were 218.93 µg/ml and 83.09 µg/ml, respectively. The FRAP value, expressed in µmol of Fe^{2+}/ mg dried extract, was found in *A. crassna* leaves extract at 1.175 µmol of Fe^{2+}/ mg dried extract. These results indicated

that the aqueous extract of *A. crassna* leaves exhibited substantial antioxidant activity in all of the methods used.

In addition to antioxidant activity, plant phenolic compounds have been shown to possess antimicrobial activities (Ayoola *et al*., 2008; Omogbai and Eze, 2010; Shan, Cai, Brooks and Corke, 2007), suggesting that the extract has the potential to possess antimicrobial activity. Recently, a group of researchers from Japan has reported antibacterial activities of the aqueous extract of *A. crassna* leaves against enteric bacteria, such as *S. aureus*, *Clostoridium difficile, Peptostreptococcus anaerobius* and *Bacteroides fragilis* (Kakino *et al*., 2012). Herein, the inhibitory effect on the Gram-positive bacteria *S. epidermidis* was observed by agar disc diffusion technique. This type of microorganism is one of coagulase-negative staphylococci which become increasingly recognized as pathogen of nosocomial infection, following ophthalmologic, neurologic and cardiothoracic surgery, in immunocompromised patients and in patients with prosthetic devices. This microbe was found susceptible to the extract with MIC of 6 mg/ml, which is in the range of MICs (4-8 mg/ml) against other bacterial strains reported by Kakino and colleagues (Kakino *et al*., 2012). It was further found that the extract exhibited bactericidal activity with MBC of 12 mg/ml.

The ability to form biofilm, a slimy layer with embedded microcolonies, is one of the most important and one of the most widespread virulence factors occurring in microbes. Biofilms grows easily on surfaces of artificial materials used for catheters and prosthetic devices (Stewart and Costerton, 2001) and it is estimated that biofilms are associated with about 65% of nosocomial infections. It has been suggested that biofilm formation is the main virulence mechanism of *S. epidermidis*

(Vuong and Otto, 2002). In addition to growth inhibitory effect, the extract was found to impede the production of slimy biofilm by *S. epidermidis* similar to vancomycin which has been shown to inhibit *S. epidermidis* biofilm formation (Aybar *et al*., 2012).

Crystal violet staining of surface-attached cells by microtiter plate assay is a popular and convenient method for quantitative detection of bacterial biofilm formation, however direct microscopic observation is strongly recommended to confirm the data from staining analysis (Merritt, Kadouri and O'Toole, 2011). Due to the absorption of *A. crassna* leaf extract at 595 nm, therefore in the present study the effect on biofilm formation was investigated by microscopic analysis which gave more accurate information. The phase contrast microscopic images (Fig 4.5) showed that *S. epidermidis* formed vast empty regions on surface with a few clusters of cells and scattering of single cell at the MICs of *A. crassna* extract. Vancomycin which has been shown to inhibit the production of slimy biofilm by *S. epidermidis* (Aybar *et al*., 2012) was used as positive control.

It is widely accepted that plants are good sources of novel antimicrobial agents. Screening of antimicrobial activities to find which type of bacteria is susceptible to plant extracts is useful, however the investigation of underlying mechanism is also crucial for drug development. To explore the possible antibacterial mechanism, the effect of the extract on morphological changes of *S. epidermidis* cells was studied by SEM and TEM. Vancomycin, an antibiotic which is widely known to damage bacterial cell wall of most Gram-positive bacteria, including staphylococci and enterococci, was used as positive control. As revealed by SEM, bacterial cells treated with aqueous extract of *A. crassna* leaves appeared swollen and distorted after

24 h of incubation. Moreover, as evident by TEM monographs, the extract caused rupture of bacterial cell wall and alteration of bacterial shape compared to control. It has been reported that biofilm formation process consists of two steps, of which the staphylococci first adhere to the foreign-body surface and then accumulate into a complex biofilm structure (Mack *et al*., 2006). The interaction between specific adhesions located on cell wall and extracellular matrix components deposit on the surface is essential for the primary attachment. The destruction of cell wall by the extract is likely to cause bacteria unable to grow and create primary biofilm architecture.

The disadvantage of this study is the lack of identification of the active ingredients which are responsible for the antibacterial effects. However, there are some documents explaining possibility action of phenolic compounds. The mechanisms of action of each phenolic againt various bacteria were quite complicated (Kalemba and Kunicka, 2003). However there are some documents that explain possibility mechanisms of action for their antimicrobial activities, such as degradation of cell wall, interaction with cellular composition and disrupt the cytoplasmic membrane of bacteria (Shan, Cai, Brooks and Corke, 2007). Several mechanisms of antimicrobial activity of tannins have been proposed including inhibition of extracellular microbial enzymes and metabolisms, deprivation of the substrates required for microbial growth and destabilization of cytoplasmic and plasma membranes (Pimia, Nohynek, Alakomi, Marja and Caldentey, 2005; Min *et al*., 2008). In addition, tannin compounds to cause the bacterial cells to disintegrate possibly results from their interference with the bacterial cell wall thereby inhibiting the microbial growth (Erasto, Moleta and Majinda, 2004; Viljoen *et al*., 2003). The

data obtained in this study provide additional indirect evidence that phenolic compounds may contribute the antibacterial effect of plant extract by damaging bacterial cell wall. The active ingredients responsible for this action need further investigation.

A number of chemicals or plant extracts have shown strong *in vitro* antimicrobial activities with low MIC, however not all of them can be used *in vivo* due to their high toxicity. Because the extract showed relatively high MICs, the acute toxicity of aqueous *A. crassna* leaf extract was investigated to assess its *in vivo* safety and the results showed that all mice did not exhibit abnormal signs of toxicity or deaths after receiving the extract even at high dose (15,000 mg/kg body weight).

It is claimed in the commercial advertisements of *A. crassna* herbal tea that it possesses an antidiabetic activity. One of the experiments in the present study was aimed to examined whether the aqueous extract produced hypoglycemic activity *in vivo*. STZ is selective pancreatic islet beta cell cytotoxicity. STZ is taken up by the β cells via the glucose transporter GLUT-2 and causes alkylation of DNA (Prasad, Kulshreshtha and Qureshi, 2009). Sulfonylureas are commonly used as a standard antidiabetic drug in STZ induced diabetes to compare the efficacy of variety of hypoglycemic drugs (Fernandes, Lagishetty, Panda and Naik, 2007; Arulmozhi, Mazumder, Lohidasan and Thakurdesai, 2010). In this study, glibenclamide in sulfonylurea group is used as a standard drug to compare the antidiabetic activity of *A. crassna*. The main mechanism of action of glibenclamide is the stimulation of insulin release and the inhibition of glucagon secretion (Suba *et al.*, 2004). The islets of Langerhans of the glibenclamide treated group was resumed to be normal structure

similar to those in the study by Prasad and co-workers (Prasad, Kulshreshtha and Qureshi, 2009). The results showed that blood glucose level did not decrease after 15 days of treatment when compared to glibenclamide-treated group. Decreased body weight were observed in both diabetic control and *A. crassna* treated group, thus indicating the extract lacked antidiabetic activity resulting in excessive breakdown of tissue proteins of experiment animals. Although, the extract consists of antioxidant activity, it did not show any protective effect against cytotoxicity of STZ on pancreas as seen in Figure 4.7. It is possible that the antioxidant compounds may not reach pancreatic cells which are the target organ.

Conclusion

This research was designed to investigate the preliminary phytochemical property, toxicity, antioxidant, antibacterial and antidiabetic activities of the aqueous *A. crassna* leaf extract. The results showed that flavonoids, saponins, tannins, cardiac glycosides were present. Total phenolic compound of the plant extract was 176.61 mg GAE/g using Folin-Ciocalteau assay. The results showed that the aqueous extract of *A. crassna* leaves exhibited quite strong antioxidant activities in all methods tested including DPPH, ABTS, FRAP assays. More importantly, the antibacterial activity and inhibitory effect on biofilm formation of the extract against *S. epidermidis* were demonstrated. The postulated underlying mechanism was disruption of bacterial cell wall. In addition, high dose of the extract showed the absence of acute oral toxicity in mice. The data also suggest that *A. crassna* may be a potential source for the discovery of new antibacterial agents against *S. epidermidis* and probably other Gram-positive bacteria as well.

In term of antidiabetic activity, the data obtained from this study suggested that the extract did not possess antihyperglycemic effect *in vivo* (STZ induce diabetic rats type I).

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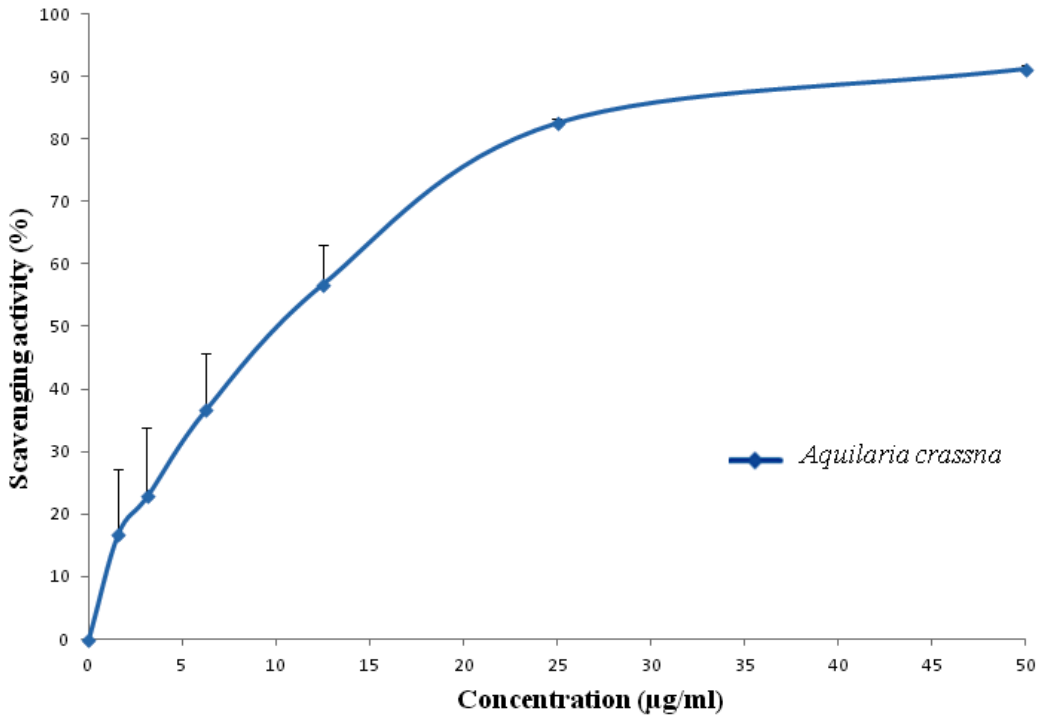
APPENDICES

APPENDIX A

ANTIOXIDANT ASSAY

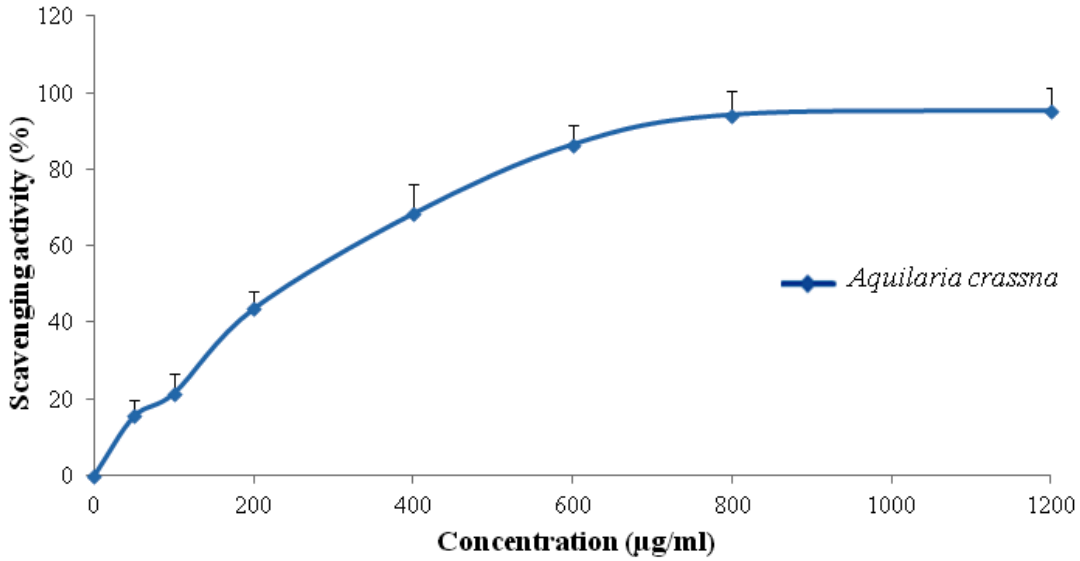
APPENDIX A1

DPPH assay



APPENDIX A2

ABTS assay

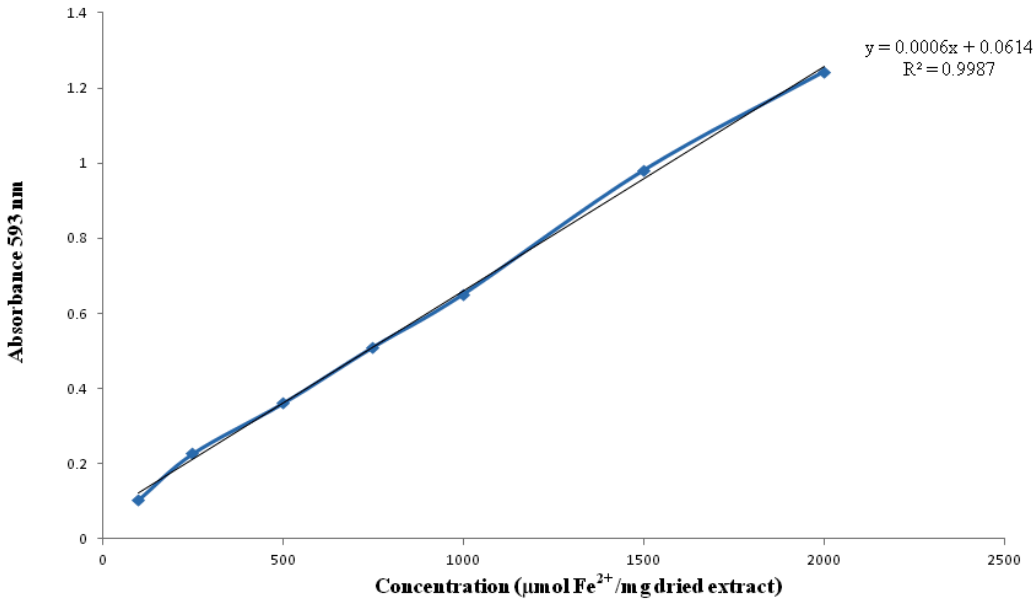


APPENDIX A3

FRAP assay

STANDARD CURVE

Standard $FeSO_4$



CURRICULUM VITAE

NAME: Miss Sirilak Kamonwannasit

DATE OF BIRTH: December 26, 1982

PLACE OF BIRTH: Sakaeo, Thailand

EDUCATION: Suan Dusit Rajabhat University, 2004, Bachelor of Science in Microbiology

Khon Kaen University, 2007, Master of Science in Pharmaceutical Chemistry and Natural Products

PUBLICATIONS:

Kamonwannasit, S., Phrompittayarat, W., Ingkaninan, K., Tanaka, H., and Putalun, W. (2008). Improvement of pseudojujubogenin glycosides production from regenerated *Bacopa monnieri* (L.) Wettst. and enhanced yield by elicitors. **Zeitschrift für Naturforschung C**. 63(11-12): 879-883.

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