สารออกฤทธิ์ต้านแบคทีเรียก่อโรคผิวหนังในสัตว์เศรษฐกิจจากสบู่ดำ *Jatropha curcas* Linn.

นางสาวสุทธิเดือน ชุณหกานต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ANTIBACTERIAL AGENTS FOR LIVESTOCK DERMATOSIS FROM *Jatropha curcas* linn.

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สุทธิเดือน ชุณหกานต์ : สารออกฤทธิ์ต้านแบคทีเรียก่อโรคผิวหนังในสัตว์เศรษฐกิจจากสบู่ดำ *Jatropha curcas* Linn. (ANTIBACTERIAL AGENTS FOR LIVESTOCK DERMATOSIS FROM *Jatropha curcas* Linn.) อาจารย์ที่ปรึกษา : ผศ.ดร. วรินทร ชวศิริ, อาจารย์ที่ปรึกษาร่วม : ผศ.ดร.ณกัญภัทร จินดา, 88 หน้า.

การทดสอบฤทธิ์ต้านแบคทีเรียก่อโรคผิวหนังเบื้องต้นจากส่วนสกัดต่างๆ ของสบู่ดำ ด้วยวิธี agar well diffusion พบว่า ส่วนสกัดไดคลอโรมีเทนจากใบ แสดงฤทธิ์ต้านเชื้อแบคทีเรียที่ดีทั้งแบคทีเรียแกรมบวกและแกรมลบ ได้แก่ *Staphylococcus auricularis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* และ *Aeromonas hydrophila* เมื่อใช้การทดสอบฤทธิ์ทางชีวภาพเป็นตัวชี้นำสามารถแยกของผสมเตียรอยด์ ของผสมแอลกอออล์โซ่ยาว และ 7,2′-dihydroxy-3′-methylflavone สารตัวหลังแสดงฤทธิ์ต้าน *P. aeruginosa* ที่ดี นอกจากนี้ส่วนสกัดเฮกเซนของน้ำมันและส่วนสกัดไดคลอโรมีเทนของน้ำยางแสดงฤทธิ์ต้านแบคทีเรียที่ดี จากการศึกษาฤทธิ์ทางชีวภาพของส่วนสกัดทั้งสองคาดว่าเกิดจากการออกฤทธิ์ร่วมกันของสารประกอบหลักและสารประกอบรอง

สาขาวิชา.....เทคโนโลยีชีวภาพ.....ลายมือชื่อนิสิต....สุทธิเดือน ชุณหกานต์.....
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ลายมือชื่ออาจารย์ที่ปรึกษาร่วม....ณกัญภัทร จินดา.....

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SUTTHIDUEAN CHUNHAKANT : ANTIBACTERIAL AGENTS FOR LIVESTOCK DERMATOSIS FROM *Jatropha curcas* Linn. THESIS ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., THESIS COADVISOR: ASST. PROF. NAKANYAPATTHRA JINDA, Ph.D., 88 pp.

The antibacterial activity screening for dermatotis disease from various extracts of *Jatropha curcas* using agar well diffusion method was conducted. The CH_2Cl_2 extract of the leaves revealed good antibacterial activity against gram positive and gram negative bacteria including *Staphylococcus auricularis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. Using biological activity as a guide, among isolated substances: a mixture of steroids, long chain alcohols and 7,2′-dihydroxy-3′-methylflavone, the latter was disclosed as the active principle revealing the highest activity against *P. aeruginosa*. Moreover, the hexane extract of oil and the CH_2Cl_2 extract of the latex displayed high antibacterial activity. According to the bioactivity results of these two extracts, the synergists of major and minor substances were expected.

Department.....Biotechnology.....Student's signature.....Sutthiduan Chunhakant.....
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CONTENT

Page

LIST OF TABLES

LIST OF FIGURES

Page

LIST OF SCHEMES

LIST OF ABBREVIATIONS

°C	degree Celsius
$CDCl_3$	deuterated chloroform
mm	millimeters
mL	milliliter (s)
^{13}C-NMR	carbon nuclear magnetic resonance
^{1}H-NMR	proton nuclear magnetic resonance
δ	chemical shift
RT	room temperature
µL	microlitre (s)
CH_2Cl_2	dichloromethane
G	gram (s)
h	hour (s)
GC	gas chromatography
ppm	part per million
NB	nutrient broth
NA	nutrient agar
min	minute (s)
MeOH	methanol
Hz	hertz
EtOAc	ethylacetate
TLC	Thin layer chromatography
SD	standard deviation
m.p.	melting point

CHAPTER I

INTRODUCTION

Plants have long provided mankind with a source of medicinal agents, with natural products once serving as the source of all drugs (Balandrin *et al*., 1993). Dependence on plants as the source of medicine is prevalent in developing countries where traditional medicine plays a major role in health care (Farnsworth, 1994; Srivastava *et al*., 1996). The rural population of a country is more disposed to traditional ways of treatment because of its easy availability and cheaper cost. Herbal therapy, although still an unwritten science, is well established in some cultures and traditions, and has become a way of life in almost the people in rural areas (Banquar, 1993). The several species of medicinal plants contain physiologically active principles, certain displaying anti-microbial properties. A large number of plant parts such as leaves, fruits, roots, stems have been used by many ethnic groups in the treatment of common maladies in both humans and domestic animals.

The treatment of livestock diseases using ethnoveterinary medicine traditional remedies is widely practiced by small scale livestock. The livestock have used these remedied in the rural communities for generations, however as modern medicines *i.e.* commercial pharmaceuticals came on the market as natural products waned and in some cases disappeared altogether (Guèye, 1999). The medicinal plants are more efficacious than pharmaceuticals for chronic pathologies. They are reputed to have no side effects and no withdrawal periods for consumption of meat from treated animals are needed since the plants are thought to be non-toxic. In general, ethnoveterinary products are used after conventional pharmaceutical medicines, for chronic cases, proved ineffective (Zafar *et al*., 2005). The livestock mostly use a range of plants remedies for the treatment of sores, warts and skin disease. Even though pharmacological industries have produced a number of new antibiotics in the last three decades, resistance to these drugs by microorganisms has increased.

Bacteria have the genetic ability to transmit and acquire resistance to drugs, which are utilized as therapeutic agents. The problem of microbial resistance is growing and the outlook for the use of antimicrobial drugs in the future is still uncertain. Therefore, actions must be taken to reduce this problem, for example, to control the use of antibiotic, develop research to better understand the genetic mechanisms of resistance, and to continue studying to develop new drugs, either synthetic or natural (Gislene *et al*., 2000).

1.1 Importance of livestock agricultural

Livestock production plays a major role in the life of farmers in developing countries. It provides food, income, employment and many other contributions to rural development. This region is vast in land, water, and vast amount of livestock and poultry population. With economies in many Asian countries growing at annual rates greater than 5-6% and a market potential of 2.8 billion people, the livestock and poultry industry growing faster than ever. Within the agriculture sector, livestock sub sector plays a vital role in economic development. But despite the increasing contribution of the livestock sector, which includes poultry sector, it has not yet achieved the level needed to provide sufficient meat for the growing population.

Increasing per capita expenditure on quality food products and growing demand for livestock and poultry products are characteristic and this region is full of surprises and business opportunities. Thailand has been in the news for past several years because of dramatic currency devaluation and stock market declines in the area. This has affected not only the international trade of livestock products but also the local livestock industries which depends on imported feed materials and other inputs. However, once these severely affected countries get on the road to economic recovery and income increase, there will be fastest growth in livestock and poultry production as well as consumption in these countries.

1.2 Pathogenic bacteria

A pathogen or infectious agent are a biological agent that causes disease or illness to its host. The term is most often used for agents that disrupt the normal physiology of a multi cellular animal or plant. However, pathogens can infect unicellular organisms from all of the biological kingdoms.

Pathogenic bacteria are bacteria that cause infectious disease. This article deals with animal pathogenic bacteria. Although the vast majority of bacteria are harmless or beneficial, a few bacteria are pathogenic. Each pathogenic species has a characteristic spectrum of interactions with its animal hosts. Some organisms, such as *Staphylococcus aureus* or *S. auricularis* can cause skin infections. Moreover, some genus, such as *Pseudomonas aeruginosa* is opportunistic pathogenic and cause disease mainly in animal suffering from immunosuppression.

1.2.1 *Staphylococcus aureus*

Taxonomy

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Genus: *Staphylococcus*

Species: *S. aureus*

Scientific name: *Staphylococcus aureus*

Characteristics

S. aureus is a gram positive coccus which appears as grape-like clusters when viewed through a microscope and has large, round, golden-yellow colonies, often with hemolyis, when grown on blood agar plates. The golden appearance is the etymological root of the bacteria's name: *aureus* means "golden" in Latin. *S. aureus* is a facultative anaerobe and opportunistic pathogen. *S. aureus* is catalase positive (meaning that it can produce the enzyme "catalase") and able to convert hydrogen peroxide (H_2O_2) to water and oxygen, which makes the catalase test useful to distinguish staphylococci from enterococci and streptococci. A large percentage of *S. aureus* can be differentiated from most other staphylococci by the coagulase test. *S. aureus* is primarily coagulase-positive (meaning that it can produce the enzyme "coagulase" that causes clot formation) while most other *Staphylococcus* species are coagulase-negative. However, while the majority of *S. aureus* are coagulase-positive, some may be atypical in that they do not produce coagulase. Incorrect identification of an isolate can impact implementation of effective treatment and control measures.

It is medically important to identify *S. aureus* correctly, as *S. aureus* is much more aggressive and likely to be antibiotic-resistant.

Pathogenesis

S. aureus also known as golden staph, is the most common cause of staph infections. It is a spherical bacterium, frequently living on the skin or in the nose of a person. *S. aureus* can cause a range of illnesses from minor skin infections Its incidence is from skin, soft tissue, respiratory, bone, joint, endovascular to wound infections.

1.2.2 *Pseudomonas aeruginosa*

Taxonomy

Domain: Bacteria

Kingdom: Eubacteria

Phylum: Proteobacteria

Class: Gamma Proteobacteria

Order: Pseudomonadales

Family: Pseudomonadaceae

Genus: *Pseudomonas*

Species: *aeruginosa*

Scientific name: *Pseudomonas aeruginosa*

Characteristics

Pseudomonas aeruginosa is a Gram-negative rod measuring 0.5 to 0.8 μm by 1.5 to 3.0 μm. Almost all strains are motile by means of a single polar flagellum. The bacterium is ubiquitous in soil and water, and on surfaces in contact with soil or water. Its metabolism is respiratory and never fermentative, but it will grow in the absence of O_2 if NO_3 is available as a respiratory electron acceptor.

The typical *Pseudomonas* bacterium in nature might be found in a bio film, attached to some surface or substrate, or in a plank tonic form, as a unicellular organism, actively swimming by means of its flagellum. *Pseudomonas* is one of the most vigorous, fast-swimming bacteria seen in hay infusions and pond water samples. In its natural habitat *P. aeruginosa* is not particularly distinctive as a pseudomonad, but it does have a combination of physiological traits that are noteworthy and may relate to its pathogenesis.

P. aeruginosa has very simple nutritional requirements. It is often observed "growing in distilled water", which is evidence of its minimal nutritional needs. In the laboratory, the simplest medium for growth of *Pseudomonas aeruginosa* consists of acetate as a source of carbon and ammonium sulfate as a source of nitrogen.

P. aeruginosa possesses the metabolic versatility for which pseudomonads are so renowned. Organic growth factors are not required, and it can use more than seventy-five organic compounds for growth.

Its optimum temperature for growth is 37 degrees, and it is able to grow at temperatures as high as 42 degrees. It is tolerant to a wide variety of physical conditions, including temperature. It is resistant to high concentrations of salts and dyes, weak antiseptics, and many commonly used antibiotics. *P. aeruginosa* has a predilection for growth in moist environments, which is probably a reflection of its natural existence in soil and water.

These natural properties of the bacterium undoubtedly contribute to its ecological success as an opportunistic pathogen. They also help explain the ubiquitous nature of the organism and its prominence as a nosocomial pathogen. *P. aeruginosa* strains produce two types of soluble pigments, the fluorescent pigment pyoverdin and the blue pigment pyocyanin. The latter is produced abundantly in media of low-iron content and functions in iron metabolism in the bacterium. Pyocyanin (from "pyocyaneus") refers to "blue pus", which is a characteristic of superlative infections caused by *P. aeruginosa.*

Pathogenesis

For an opportunistic pathogen such as *P. aeruginosa*, the disease process begins with some alteration or circumvention of normal host defenses. The pathogenesis of *Pseudomonas* infections is multifactorial, as suggested by the number and wide array of virulence determinants possessed by the bacterium. Multiple and diverse determinants of virulence are expected in the wide range of diseases caused, which include septicemia, urinary tract infections, pneumonia, chronic lung infections, endocarditis, dermatitis, and osteochondritis. Most *Pseudomonas* infections are both invasive and toxinogenic. The ultimate *Pseudomonas* infection may be seen as composed of three distinct stages: (1) bacterial attachment and colonization; (2) local invasion; (3) disseminated systemic disease. However, the disease process may stop at any stage. Particular bacterial determinants of virulence

mediate each of these stages and are ultimately responsible for the characteristic syndromes that accompany the disease.

1.2.3 *Aeromonas hydrophila*

Taxonomy

Domain: Bacteria

Kingdom: Proteobacteria

Phylum: Gamma proteobacteria

Class: Aeromonadale

Genus: *Aeromonas*

Species: *hydrophila*

Scientific name: *Aeromonas hydrophila*

Characteristics

Aeromonas hydrophila are gram negative straight rods with rounded ends (bacilli to coccibacilli shape). They usually grow from .3 to 1 micrometer in width, and 1 to 3 micrometers in length. *A. hydrophila* does not form endospores, and can grow in temperatures as low as four degrees Celsius. These bacteria are motile by a polar flagella.

A. hydrophila is a heterotrophic, mainly found in areas with a warm climate. This bacterium can also be found in fresh, salt, marine, estuarine, chlorinated, and un-chlorinated water. *A. hydrophila* can survive in aerobic and anaerobic environments. This bacterium can digest materials such as gelatin and hemoglobin. *A. hydrophila* was isolated from humans and animals until the 1950s. This bacterium is the most well known of the six species of *Aeromonas*. It is also very hard to kill, because it is a very resistant bacterium. *A. hydrophila* is resistant to chlorine, refrigeration or cold temperatures but in fact *A. hydrophila* has been known to survive in temperatures as low as four degrees Celsius.

Pathogenesis

The structure of *A. hydrophila*. it is very toxic to many organisms. When it enters the body of its victim, it travels through the bloodstream to the first available organ. It produces Aerolysin Cytotoxic Enterotoxin (ACT), a toxin that can cause tissue damage. It is known as a pathogenic bacterium. *A. hydrophila*, *A. caviae,* and *A. sobria* are all considered to be "opportunistic pathogens," meaning they only infect hosts with weakened immune responses. Though *A. hydrophila* is considered a

pathogenic bacterium, scientists have not been able to prove that it is the actual cause of some of the diseases it is associated with. It is believed that this bacterium aids in the infection of diseases, but do not cause the diseases themselves.

1.3 Livestock disease

Livestock like all other animals are affected by many diseases. Some diseases are caused by viruses, parasites, especially bacteria (internal and external) as well as by poisoning. Dermatitis diseases include bacteria organisms. The infectious disease such as mastitis which is the reaction of milk-secreting tissue to injury, is produced by physical force, chemicals introduced into the gland or most commonly from bacteria and their toxins. This disease is considered as serious disease particularly for dairy farmers and breaks out in high frequency. It gives them great loss, such as decrease of milk production of animals, degradation of milk quality, shortening of productive period of milk, hygienic problems due to the contamination of bacteria into milk and decrease of yield at processing for dairy products due to the increase of contamination of somatic cells in the milk.

The mastitis of livestock, cattle is a disease which causes inflammation of the mammary gland or udder due to the invasion of pathogenic bacteria, such as *S. aureus*, *K. pneumonia*, *E. coli*, *P. aeruginosa, P. pseudomallei*, *S. agalactiae*, *Streptococcus* spp., *Enterobacter* spp., from the opening of teat into udder (Morris, 2007).

Furthermore, metritis and endometritis are inflammations of the womb caused by bacterial infection. Metritis involves the endometrium, the underlying glandular tissues and the muscular layers. Endometritis involves only the endometrium and the underlying glandular tissues. Commonly, metritis is used to indicate both conditions. A large number of microorganisms have been implicated as causes of metritis. Bacteria have been cultured from uteri when metritis has been present. Microorganisms enter the uterus through several routes. Most commonly organisms, particularly bacteria, *C. pyogenes, P. aeruginosa, Staphylococcus* spp., *Clostridium* spp. *Streptococcus* spp. contaminate the uterus during calving or the early post calving period. The reproductive tract is very susceptible at this time, especially if trauma or lesions in the vagina or vulva are present, and the animal's natural defenses are lowered. Any assistance or manipulations performed during parturition can easily introduce organisms into the uterus (Arthur *et al*., 1951).

The diseases caused by the *Aeromonas* spp. as motile *Aeromonas* disease are ubiquitous of aquatic environment such as fresh and coastal water, sewage, and wastewater. Among the 14 species currently described in this genus, only five (*A. hydrophila*, *A. veronii, A. caviae, A. jandaei,* and *A. schubertii*) are recognized as animal pathogen. These emerging pathogens commonly produce different virulence factors such as cytotoxins and enterotoxins against humans and aquatic animals. The prevalence of motile *Aeromonas* may be more important than that of faecal coliforms.

Despite the evident involvement of motile *Aeromonas* strains in severe wound infections and occasionally gastritis in animal exposed to natural water in which the bacterium is present, relatively little information is available on the environment factors that simultaneously influence their survival in natural aquatic environment. Most available information is derived from fresh water experiments or from laboratory seawater studies with one environmental factor at a time controlling changes in *Aeromonas* spp. abundance. Thus, the density of *Aeromonas* spp. cells in fresh water has been found to be seasonally distributed, with a maximum occurring during the late summer and winter for temperate regions, respectively (Leblanc *et al*., 1981).

Skin and soft tissue infections, including wound infections, pyoderma and dermatitis. *P. aeruginosa* can cause a variety of skin infections, both localized and diffuse. The common predisposing factors are breakdown of the integument which may result from burns, trauma or dermatitis; high moisture conditions such as those found in the ear of swimmers and the toe webs of athletes, hikers and combat troops, in the perineal region and under diapers of infants, and on the skin of whirlpool and hot tub users. Individuals with AIDS are easily infected. *Pseudomonas* has also been implicated in folliculitis and unmanageable forms of acne vulgaris.

1.4 Antibiotic resistant

Resistant to antimicrobial agents is recognized at present as a major global public health problem. Infective diseases account for approximately one half of all deaths in tropical countries. Industry of livestock, despite the progress made in the understanding of microorganisms and their control, incidents of epidemics due to drug resistant microorganisms and the emergence of hitherto unknown disease causing microbes, pose enormous public health concerns (Iwu *et al.*, 1999).

Almost since the beginning of the antibiotic era, bacteria resistance has been seen as the major obstacle to successful treatment (Iwu *et al.*, 1999). Microbial resistance to antibiotics in the clinic emerged soon after their first use in the treatment of infectious disease, and continue to pose a significant challenge for the healthcare sector. Resistance has now firmly emerged as problem in the wilder community.

Recent reports have shown a marked increase in antibiotic resistance of food poisoning bacteria due to non rational and excessive use of antibiotics as therapeutics agents or as growth promoters in livestock. Another factor of resistance potentially lies in the use of antibiotic resistant genes as selection markers in genetically modified organisms (GMOs). The main safety issue of concern is the release of these resistant genes to sensitive organisms when these GMOs are introduced into in the environment.

Due to emergence of drug resistant bacteria, the search for new antibacterial compounds with improved activity is necessary (Harold and Health, 1992).

1.5 Plants as a potential source of antibiotics

The use of medicinal plants is widespread (Famsworth, 1994). The production of medicines and the pharmacological treatment of diseases began with the use of herbs (Tyler, 1997). Life saving and essential drug from medicinal plants were introduced into modern therapeutics several centuries ago. An antibiotic has been defined as a chemical compound derived from or produce by living organisms, which is capable, in small concentrations of inhibiting the growth of organisms. This definition limited antibiotics to substances produced by microorganisms but the definition could now be extended to include similar substances present in higher in plants. Plants have many ways of generating antibacterial compounds to protect them against pathogen (Balandrin *et al*., 1993).

1.6 *Jatropha curcas* Linn.

1.6.1 Botanical description

J. curcas (Figure 1.1) is a plant belonging to the family Euphorbiaceae. In Thailand, the general is called Sabu Dam. Other names are such as Sabu Hua Ted, Sahlod Dum, Sahlod Yai (Central), Ma Hua, Ma Hong (Northern) Ma Yoa, Sihlod, Mak Yoa (North-Easthern) Ma Kao and Hongted (Southern). (เต็ม สมิตินันท์, 2544).

Common name(s): Barbados nut, Black vomit nut, Curcas bean, Kukui haole, Physic nut, Purge nut, Purgeerboontjie, Purging nut tree

J. curcas is a small tree or shrub with spreading branches, stubby twigs and smooth gray bark which exudes whitish colored, watery, latex when cut with sometime reddish and gummy. Normally, it grows between 3-6 m in height.

Leaves: large green to pale-green leaves, deciduous, alternate to sub-opposite but apically crowded, ovate, acute to acuminate, basally cordate, 3-5 lobed with a spiral phyllotaxis in outline or sometimes entire cordate at the base as small stipules and undulating margins as 6-10 cm long, 6-35 cm broad.

Figure 1.1 The different parts of *Jatropha curcas* Linn.

Flowers: several to many in greenish cymes, yellowish, bell-shaped, 5 sepals, broadly deltoid. The petiole length ranges between 6 to 23 mm. The inflorescence is formed in the leaf axil. Flowers are formed terminally, individually, with male flowers containing 10 stamens, 5 united at the base only, 5 united into a column. Female flowers borne singly, with elliptic 3 celled triovulates, many ovaries with 3 spreading bifurcate stigmata.

Fruits: Fruits are produced in winter when the shrub is leafless, or it may produce several crops during the year if soil moisture is good and temperatures are sufficiently high. Each inflorescence yields a bunch of approximately 10 or more

ovoid fruits. A three, bi-valved cocci is formed after the seeds mature and the fleshy exocarp dries.

Seeds: The seeds become mature when the capsule changes from green to yellow, after 2-4 months from fertilization. The blackish, thin shelled seeds are oblong and resemble small castor seeds.

Habitat and distribution: The plant is resistant to a high degree of aridity and as such does not compete with food crops. The plant is originating in Central America and mainly grown in Asia and Africa. It is believed to be introduced to Thailand by the Portuguese.

1.6.2 Medicinal uses

J. curcas is more available because the use as ornamental garden plants, a living fence post as hedges and as a purgative. Mexicans grow the shrub as a host for the lac insect. The stem and twigs are used to arrest bleeding, toothache, eye inflammation and wound healing. The leaf decoction is used a lactagogue, rubefacient, sub-purgative and as poultices to external ulcers which were taken internally for jaundice. A mixture of the leaf decoction and lime juice is used for fevers, convulsions and as an anthelmintic. The leaves are used as a fish poison and to fumigate for bedbugs. In India, pounded leaves are applied near horses' eyes to repel flies.

Styptic latex is used to cure piles and burns as an antiseptic. The latex is used on carious teeth and to help teeth come through in children. The mixture of the latex and salt is employed as a toothbrush and mouthwash. The root decoction is used for the treatment of gonorrhea. The powdered root bark is utilized as addressing for wounds and sores. An infusion of the root is indicated for rheumatism, dyspepsia, diarrhea and incontinence. The root pulp was reported to mix with xylopia fruits for the treatment of dysentery. The root ashes are used as a salt substitute. The seed is employed as a strong purgative which is applied externally in cases of itch, herpes and rubefacient. The poisonous nuts mixed with palm oil to kill rats. The oil seed has been used to make soap, candles, illumination, adulteration of olive oil and making Turkey red oil.

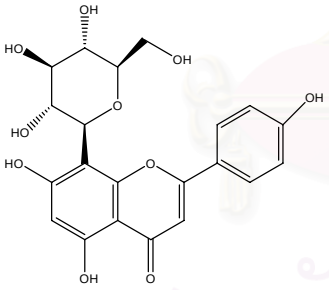
1.6.3 Literature Reviews of *Jatropha* species

In Thailand, 5 species belonging to *Jatropha* genus could be found. Those included *J. curcas* (สบู่ดำ), *J. gossypifolia* (สบู่แดง), *J. integgerima* (ปัตตาเวีย), *J. podagrica* (หนุมานนั่งแท่น), and *J. multifida* (มะละกอฝรั่ง, ฝิ่นต้น) (นันทวรรณ บุณยะประภัศร, 2542).

Chemical constituents as medicinal uses and pharmacological properties of various *Jatropha* species have been overviewed. The active compounds demonstrated antimicrobial activity from *J. curcas* were collected as presented in Table 1.1 and the structures of these compounds are exhibited in Figure 1.2.

Table 1.1 Antibacterial agents of compound from *J. curcas*

Compound	Plant parts	Reference
C-glycosylflavonoids vitexin	leave	Subramanlan *et al*., 1971
deoxypreussomerins palmarumycins JC1 palmarumycins JC2	stem	Rabindranath *et al*., 2004



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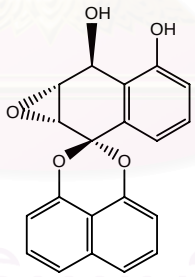
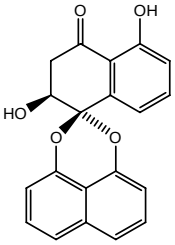


palmarumycins JC1



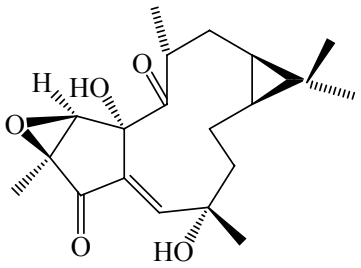
palmarumycins JC2

Figure 1.2 Antibacterial agents isolated from *J. curcas*.

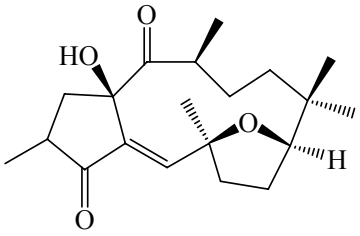
The screening of antibacterial activity of plant extracts and plant products from other *Jatropha* species has continuously been reported. The compounds possessing antibacterial activity isolated from *Jatropha* species are summarized in Table 1.2 and the structures are exhibited in Figure 1.3.

Table 1.2 Antibacterial agents from *Jatropha* species.

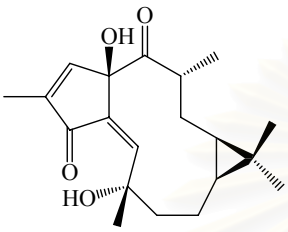
Compound	Plant parts	Source	Reference
Diterpenoids japodagrin japodagrone	root	*J. podagrica*	Aiyelaagbe *et al*., 2000
macro cyclic diterpene jatrophenone	whole plant	*J. gossypifolia*	Ravindranath *et al*., 2003
Rhamnofolane diterpene 2-*epi*-jatrogrossidione	root, leave	*J. gaumeri*	Roberto, *et al*., 2004
a penta-substituted pyridine 2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester	rhizome	*J. elliptica*	Marquez *et al*., 2005
Diterpenoids 4*Z*-jatrogrosidentadion 15-*Epi*-4Z–jatrogrossidentadion 2-Hydroxyisojatrogrossidion 2-*Epi*-hydroxyisojatrogrossidion	whole plant	*J. podagrica*	Olapeju *et al*., 2007



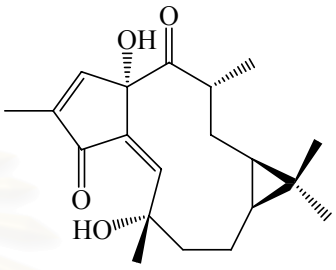
Japodagrin



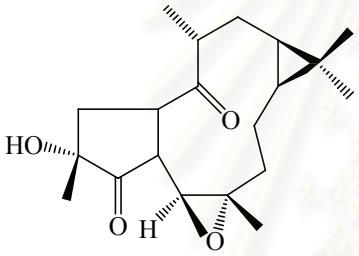
Japodagrone



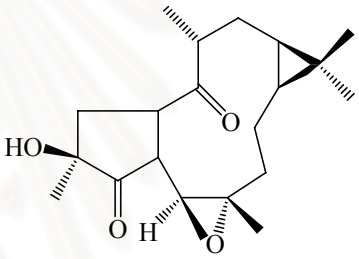
4Z-Jatrogrosidentadion



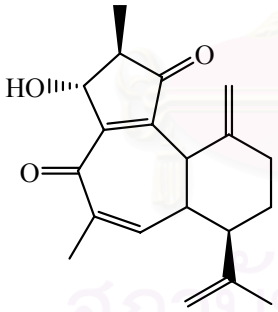
15-Epi-4Z–jatrogrossidentadion



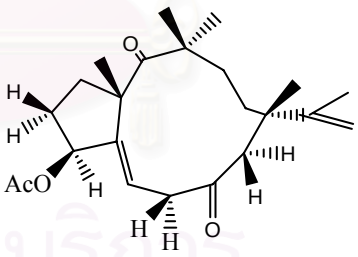
2-Hydroxyisojatrogrossidion



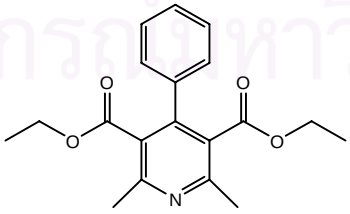
2-Epihydroxyisojatrogrossidion



2-*epi*-jatrogrossidione



Jatrophenone



2,6-dimethyl-4-phenyl-pyridine-3,5-dicarboxylic acid diethyl ester

Figure 1.3 Antibacterial agents from *Jatropha* species

In addition, previous chemical investigations of *Jatropha* species have been studied without biological activity reported. The compounds are accumulated as presented in Table 1.3 and the structures are exhibited in Figure 1.4

Table 1.3 Chemical constituents in *Jatropha* species

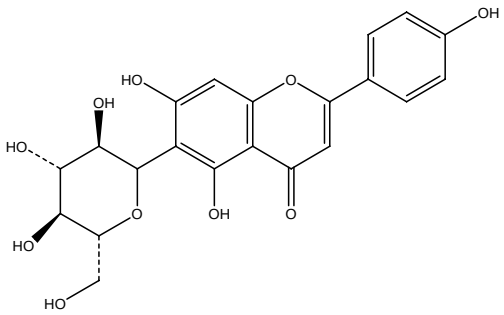
Compound	Plant parts	Source	Reference
C-glycosylflavonoids Isovitexin	leave	*J. curcas*	Subramanlan *et al*., 1971
lignan 2-piperonyllidene-3-verytryl-3R-γ-butylrol-acetone	stem, root, seed	*J. gossypifolia*	Chatterjee *et al*.,1981
lignan gadain	stem, root, seed	*J. gossypifolia*	Banerji *et al*., 1984
diterpene epoxytrione citlalitrione riolozatrione β-sitosterol jatropholone B	root	*J. dioica*	Villarreal *et al*., 1988
a cyclic nonapeptide podacycline A **a cyclic heptapeptide** podacycline B	latex	*J. podagrica*	Van den Berg *et al*., 1996
lignan jatrodien	stem	*J. gossypifolia*	Biswanath *et al*., 1996
a complex of 5-hydroxypyrrolidin-2-one and pyrimidine-2,4-dione	leave	*J. curcas*	Staubmann *et al*., 1999
a minor coumarino-lignoid propacin	whole plant	*J. gossypifolia*	Biswanath D. and Biswanath V., 2001
shikonin dimethylacrylshikonin acetylshikonin	seed, branch, root	*J. curcas*, *J. glandulifera*	Robert, D. and Watson A., 2003

Table 1.3 (cont.)

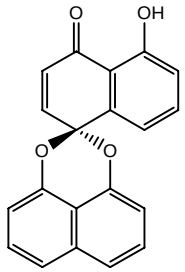
Compound	Plant parts	Source	Reference
Rhamnofolane diterpenes integerrimene **rhamnofolane endoperoxide** 2-epicaniojane caniojane 1,11-bisepicaniojane	root	*J. integerrima*	Sutthivaiyakit *et al*., 2003
lathyrane diterpene 15-*epi*-4*E*-jatrogrossidentadione	root leave	*J. gaume*ri	Roberto, *et al*., 2004
deoxypreussomerins palmarumycins CP1	stem	*J. curcas*	Ravindranath *et al*., 2004
heudelotinone *epi*-isojatrogrossidione 2α-hydroxy-*epi*-isojatrogrossidione 2-methyanthraquinone tetradecyl-*E*-ferulate 2-methoxyantraquinone scopaletin tomentin curcasones A—D jatropholones A and B (+)-Jatrophol Curcalathyranes A and B	aerial parts	*J. curcas*	Ravindranath *et al*., 2004
catechin polymers catechin catechin-7-*O*-β-glucopyranoside proanthocyanidin B-3	stem	*J. macrantha*	Angelyne *et al*., 2006

Table 1.3 (cont.)

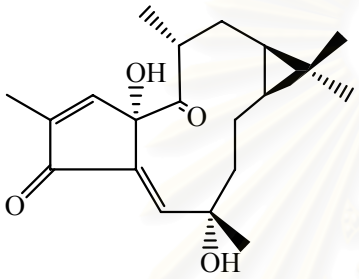
Compound	Plant parts	Source	Reference
heudelotinone *epi*-isojatrogrossidione 2α-hydroxy-*epi*-isojatrogrossidione 2-methyanthraquinone tetradecyl-*E*-ferulate 2-methoxyantraquinone scopaletin tomentin curcasones A—D jatropholones A and B (+)-Jatrophol Curcalathyranes A and B	aerial parts	*J. curcas*	Ravindranath *et al*., 2004
catechin polymers catechin catechin-7-*O*-β-glucopyranoside proanthocyanidin B-3	stem	*J. macrantha*	Angelyne *et al*., 2006



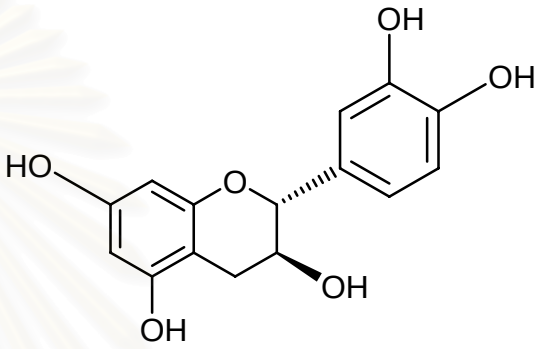
isovitexin



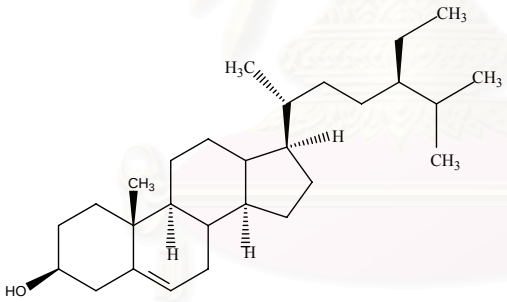
palmarumycins CP1



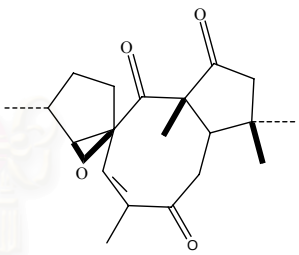
15-*epi*-4*E*-jatrogrossidentadione



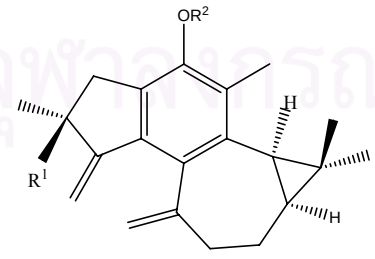
catechin



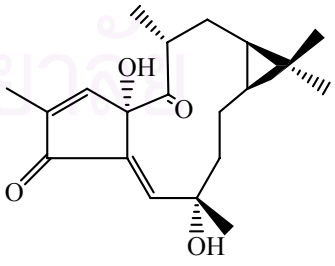
β-sitosterol



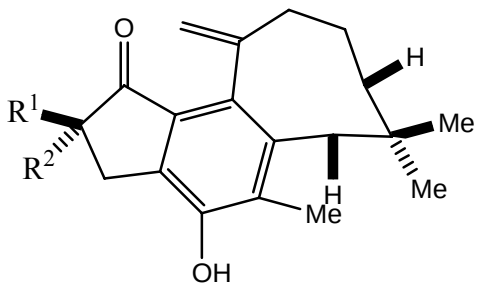
citlalitrione



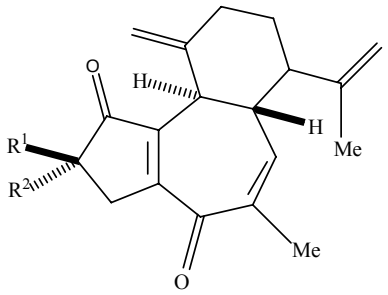
jatropholone B



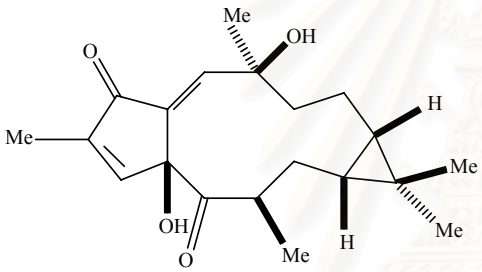
15-*epi*-4*E*-jatrogrossidentadione



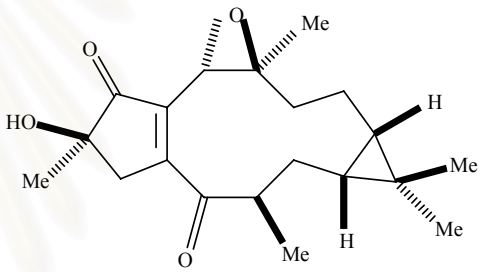
	R^1	R^2
Jatropholones A	H	Me
Jatropholones B	Me	H



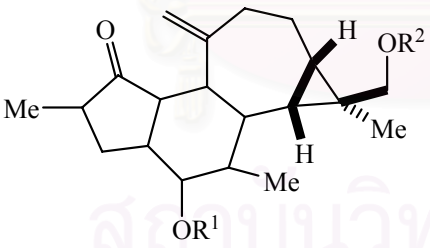
	R^1	R^2
Curcusones A	Me	H
Curcusones B	H	Me
Curcusones C	Me	OH
Curcusones D	OH	Me



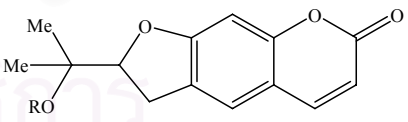
curcalathyranes A



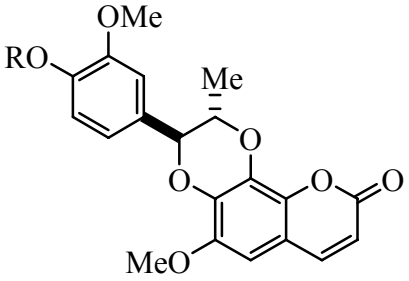
curcalathyranes B



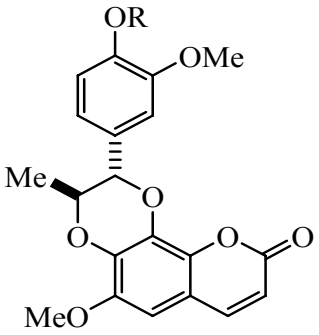
	R^1	R^2
(+)-jatrophol	H	H
5-methoxyjatrophol	Me	H
5-acetyljatrophol	COMe	H
5,8-diacetyljatrophol	COMe	COMe



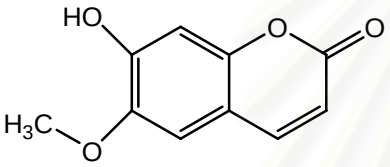
	R
(+)-marmesin	H
marmesin acetate	COMe



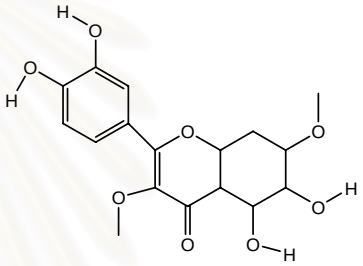
	R
propacin	H
propasin methylether	Me
propacin acetate	COMe



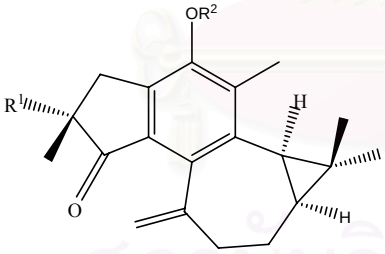
	R
jatrophin	H
jatrophin acetate	COMe



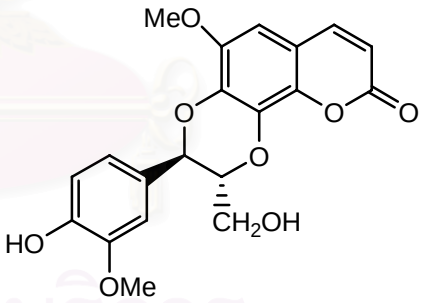
scopaletin



tomentin



Jatropholones A



cleomiscosin A

Figure 1.4 Chemical constituents of *Jatropha* species.

According to Berg *et al*. (1996), two novel cyclic peptides, podacyclines A and B were isolated from the latex of *Jatropha podagrica*. Podacycline A is a cyclic nonapeptide with the sequence Gly1-Leu2-Leu3-Gly4-Ala5-Val6-Trp7-Ala8-Gly9-Gly1 and the sequence of podacycline B, a cyclic heptapeptide, was determined to be Phe1-Ala2-Gly3-Thr4-Ile5-Phe6-Gly7-Phe1. The amino acid residues of both compounds were *L*-configuration.

Based upon the literature surveys, the MeOH extract of fresh sap of *J. curcas* showed antifungal activity (Faria *et al*., 2006, Agbelusi *et al*., 2007). The crude extracts of leaves displayed anti-diabetic activity in streptozotocin-induced diabetic rats (David *et al*., 1994). The water extract of branches showed anti-viral activity by inhibition of the HIV-induced cytopathic effects (Matsuse *et al*., 1999). The substance derived from the isolation of endophytic fungi from the latex possess antitumor and antifungal activities (Haiyan *et al*., 2005). The MeOH extract of the root exhibited significant anti-inflammatory activity against formalin-induced rat paw edema (Mujumdar A.M. and Misar A. V., 2004). The extract of *J. tanjorensis* expresses weak activity as antioxidant and cytoprotective activities (Iwalewa *et al*., 2005).

The toxic properties of various parts of *Jatropa* spp. have also been investigated. The fruit of *J.curcas* showed the fertility regulatory effect by oral administration of different extracts to pregnant rats (Goonasekera *et al*., 1995). The seed displayed the toxic effects in mice (Adam S.E.I., 1974, Aguye *et al*., 1986). Additionally, the MeOH extracts of the leaves of *J. curcas* and the roots of *J. elliptica* demonstrated cytotoxicity with human cell line HL60 (Faria *et al*., 2006). *Jatropha* seed oil was toxic after oral administration to rats and albino mice, producing severe diarrhea, bloodshot eyes and inflammation of the gastro-intestinal tract. (Gandhi *et al*., 1995). The aqueous and MeOH extracts of the seed oil showed the highest toxicity against snails transmitting *S. mansoni* and *S. haematobium* in snail hosts and larvae of schistosomes transmitting (Rug M. and Ruppel A., 2000), and insecticidal activity of the extracts exhibited 100% mortality on mosquito larvae as *O. triseriatus* (Kambou *et al*., 2008). *Jatropha* seed oil was evaluated for anti-ovipositional activity and long-term protective ability of treated cowpeas against the seed beetle *C. maculatus* (Adebowale, K.O. and Adedire, C.O., 2006). The bioactive compounds isolated from *Jatropha* species are shown in Table 1.4 and Figure 1.5.

Table 1.4 The bioactive compound of *Jatropha* species

Compound	Plant parts	Source	Biological activity	Reference
lactam jatropham (5-hydroxy-4-methyl-3-pyrrolin-2-one)	leave stem flower fruit	*J. macrorhiza*	tumor-inhibitory against P388 lymphocytic leukemia	Wiedhopf *et al*., 1973
diterpene jatrophatrione **triterpene** acetylaleuritolic acid	roots	*J. macrorhiza*	antitumor activity in the P-388 lymphocytic leukemia system	Torrance *et al*., 1976, 1977.
phorbol ester 12-deoxy-16-hydroxy phorbol	the seed oil	*J. curcas*	irritant activity	Adolf *et al*., 1984
phorbol ester 3H-12-*O*-tetradecano-ylphorbol-13-acetate	the seed oil	*J. curcas*	skin tumor promoters in a two-stage mouse carcinogenesis	Horiuchi *et al*., 1987
phorbol ester a macrocyclic dicar-boxylic acid diester) 13,16-diester of 12-deoxy-16-hydroxy phorbol	the seed oil	*J. curcas*	tumor-promoting activity	Hirota *et al*., 1988
a proteolytic enzyme curcain	latex	*J curcas*	inhibition activity of human complement and proliferation of human T-cells	Nath *et al*.,1991

Table 1.4 (cont.)

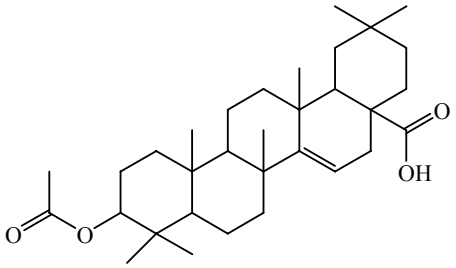
Compound	Plant parts	Source	Biological activity	Reference
a novel cyclic octapeptide curcacycline A	latex	*J. curcas*	inhibition of classical pathway activity of human complement and proliferation of human T-cells	Van Den Berg *et al*., 1995
phorbol esters 4-β-phorbol-13-decanoate	seed	*J. curcas*	molluscicidal activity against both *B. glabrata* and *O. hupensis*	Liu *et al*., 1997
jatrophone a mixture of jatro-pholones A and B	rhizome	*J. elliptica*	Molluscicidal activity against the snail *B. glabrata*	Santos *et al*., 1999
cyclooctapeptides chevalierins A chevalierins B **cyclononapeptide** chevalierin C	latex	*J. chevalieri*	antimalarial activities	Baraguey *et al*., 1998
diterpene curcusone B	latex	*J. curcas*	anti- metastatic activity which metastatic process was investigated against 4 human cancer cell lines	Caterine *et al*., 1995 Muangman *et al*., 2005
cyclicheptapeptide Mahafacyclin B	latex	*J. mahafalensis*	antimalarial activity	Baraguey *et al.,* 2000

Table 1.4 (cont.)

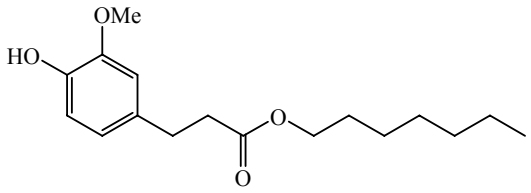
Compound	Plant parts	Source	Biological activity	Reference
curcin	seed	*J. curcas*	antitumor activity	Juan *et al*., 2003
β-sitosterol triterpenes α-amyrin, β-amyrin taraxasterol	leaves	*J. gaumeri*	antioxidant activity	Roberto *et al*., 2004
a ferulic acid ester n-heptyl ferulate 8-hydroxy-6,7-dimethoxy coumarin acetylaleuritolic acid γ-sitosterol	stem and roots	*J. podagrica*	cytotoxic towards the HeLa (cervical carcinoma) cell line	Ee *et al*., 2005
β-1,3-glucanase	seed	*J. curcas*	antifungal	Wei *et al*., 2005
cylic heptapeptides integerrimide A integerrimide B	latex	*J. integerrima*	a inhibition of neurite outgrowth of E7 chicken spinal cord neurons as well as an antiproliferation	Conrad *et al*., 2007
polyphenols - biflavone di-*C*-glycosides jatrophenol I jatrophenol II jatrophenol III	leaves	*J. multifida*	antianalgesic activity anti-inflammatory activity	Moharram *et al*.,2007

Table 1.4 (cont.)

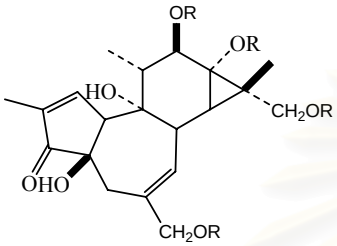
Compound	Plant parts	Source	Biological activity	Reference
biflavones phenolic compounds apigenin 7-*O*-β-D-neohespredoside ferulic acid quercetin vicenin-II isoorientin vitexin luteolin.	leaves	*J. multifida*	antianalgesic activity anti-inflammatory activity	Moharram *et al*.,2007

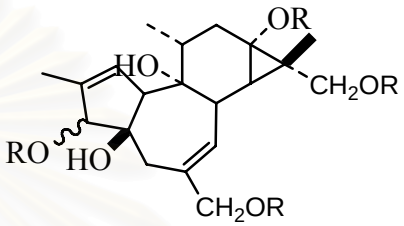


Acetylaleuritolic acid

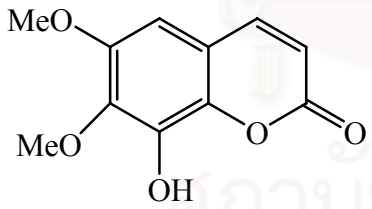


n-heptyl ferulate

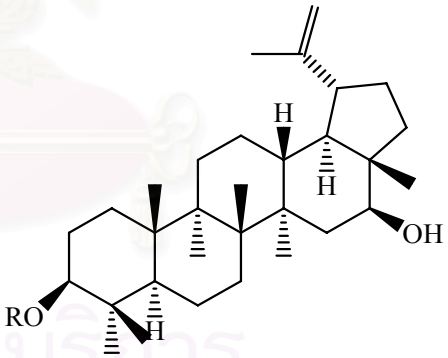




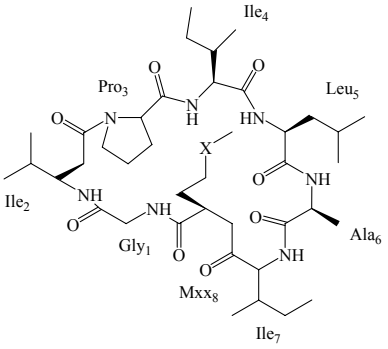
	R
16-hydroxyphorbol	H
12,13,16,20-tetraacetate	COMe
3-deoxo-12-deoxy-3ξ,16-dihydroxyphorbol	H
3,13,16,20-tetraacetate	COMe

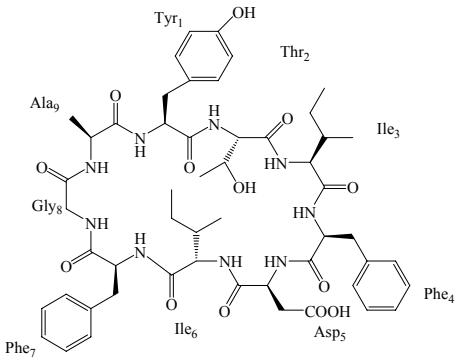


8-hydroxy-6,7-dimethoxycoumarin

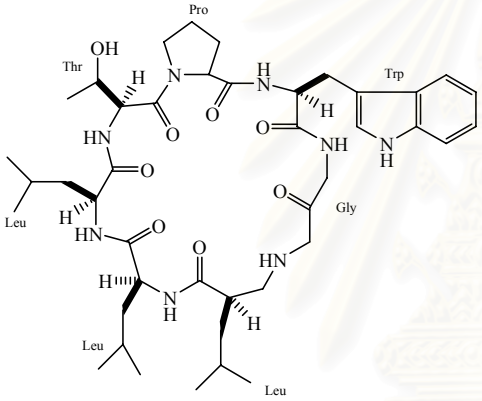


calenduladiol

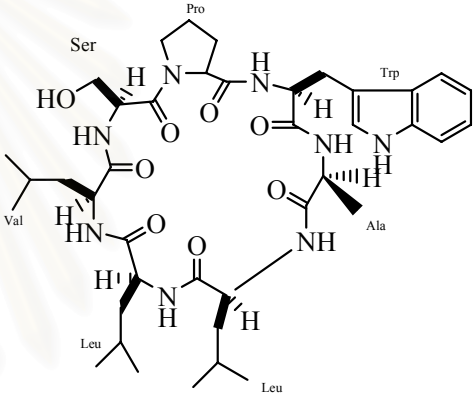




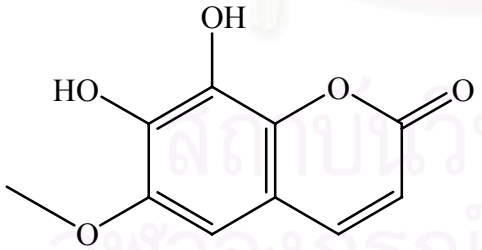
	Mxx_8	
chevalierins A	Met (X:S)	chevalierins C
chevalierins B	Mso (X:S=O)	



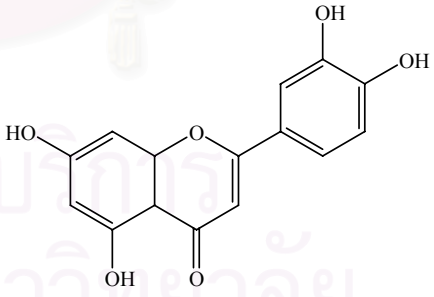
integerrimide A



integerrimide B



fraxetin



luteolin

Figure 1.5 The bioactive compound of *Jatropha* species

The seed oil showed fatty acid composition which rich oleic acids and linoleic acids 72-84% (Banerji *et al*., 1985). Triacylglycenol was the dominant lipid species while the major triacyglycerol was 1,2-Dioleoyl-3-linoleoyl-rac-glycerol. Linolenic acid was the dominant fatty acid in the oil. Ten seterols and thirteen tritepene alcohol

was identified in the unsaponifiable fraction of the oil. The development a techniquie for biodiesel production from crude seed oil for a high content of free fatty acid (Hanny J.B. and Shizuko H., 2008). In Tanzania, researcher developing biofuels from an oil-seed by Strategic Niche Management method (Janske V.E. and Henny R., 2008).

The deoiled *J. curcas* seed cake is substrate for enzyme production by solid-state fermentation (SSF) can produced proteases and lipases with a solvent tolerant strain of *Pseudomonas aeruginosa* PseA strain for utilization biodiesel production (Nilkamal *et al*., 2008). The seed meal of *J. curcas* was investigated lectin activity in a non toxic variety using latex agglutination and haemagglutination assays (Eroarome *et al*., 1998) and investigated the coagulant activity that whole latex reduced the clotting time of human blood (Omolaja *et al*., 2003). The latex of *J. multifida* was studied isolates and characterizes the anti-complement constituents present in the treatment of infected wounds which inhibition of classical pathway (CP) complement activity in human serum, a polymer was isolated which could be characterized as a proanthocyanidins (Kosasi *et al*., 1989). The MeOH and lipophilic extracts of aerial parts of *J. curcas* showed antiplasmodial activity which against *P. falciparum* (Inga *et al*., 2002).

J. curcas and *J. glandulifera* were utilized as strong purple colorant obtained from the seeds. The dark purple color could be obtained from the oil extracts of trunks, branches, and roots of many *Jatropha* species. The colorant was identified as in a naphtaquinone class.

1.7 The goal of this research

Up to the present time, antibacterial activity of *J. curcas* has been little reported. Therefore, the purpose of this investigation is to explore for antibacterial agents from *J. curcas*. The result from this investigation may serve as an additional information on the phytochemistry which could be a valuable lead in the field of pharmaceuticals and/or agrochemicals.

CHAPTER II

Materials and Method

2.1 Materials

2.1.1 Plant materials

Various parts of *Jatropha curcas* (Mukdaharn 20 breeding), namely leave, branch, latex and seed were collected in July 2006 from Suwan Wajokkasikit Field Crops Research Station, Nakhon Ratchasima province, Thailand.

2.1.2 Chemicals and equipment

All solvents used in this research were commercial grade and purified by distillation prior to use. Thin layer chromatography (TLC) was performed on precoated Merck silica gel 60 F_{254} plates (layer thickness 0.25 mm) was used for compound separation. Adsorbents such as silica gel 60 Merck 7729 and 7734 were used for quick column chromatography and open column chromatography, respectively. The blood agar medium and the nutrient broth (Himedia®) were used for antibacterial test. Horse blood was obtained from The Thai Red Cross Society.

2.1.3 Spectroscopic analysis

The ^{1}H and ^{13}C nuclear magnetic resonance spectra were determined with a Varian nuclear magnetic resonance spectrometer model Mercury 400. Chemical shifts were reported in ppm scale using the chemical shift of the solvent as the reference signal. The Fourier Transform-infrared spectra (FT-IR) were recorded on Nicolet Impact 410 FT-IR spectrometer. Melting points were determined with a Fisher-Johns melting point apparatus and are uncorrected. GC analysis was carried out on a Shimadzu GC-9A instrument equipped with flame ionization detector with N_2 as a carrier gas.

2.1.4 Microbial cultures and growth conditions

Four microorganisms selected for antibacterial activity were kindly provided by Dr. Nakanyapatthara Jinda, Department of Biotechnology, Agro-Industry Faculty, Kasetsart University. These include the gram positive bacteria *Staphylococcus aureus* (JKU1466) and *Staphylococcus auricularis* (JKU931), and gram negative bacteria *Pseudomonas aeruginosa* (JKU30) obtained from the wound of martitis in milk cattle and *Aeromonas hydrophila* (NJKU1321). The bacteria strains were enriched in nutrient broth at 37°C for 24 h and were maintained on respective nutrient agar slant s at 4°C. Before using, the mentioned bacterial strains were recovered from nutrient agar slants by inoculating into nutrient broth (NB) and incubated at 37°C in a water bath shaker (Memmert WB22, Germany) at 150 strokes/min for 24 h.

2.2 Method

2.2.1 Preliminary preparation of the extracts

In a screening program, different parts of plant material were air-dried at RT and powdered. About 30 g of powdered leave and that of branch were macerated by 200 mL of CH_2Cl_2 at RT for 3 days and filtered through Whatman No. 4 filter paper. The residue was subsequently extracted with 200 mL of MeOH for 3 days at RT and filtered. The filtrates were concentrated to dryness under vacuum with rotary evaporator to obtain crude extracts.

Jatropha seeds were cracked, the shells were carefully removed and the kernels thus obtained were used for oil extraction. About 30 g of powdered kernel was macerated by 200 mL of hexane at RT for 3 days and filtered. The filtrates were concentrated to dryness with rotary evaporator to obtain hexane crude extract, called kernel oil. The residue was subsequently extracted with 200 mL of CH_2Cl_2 and 200 mL of MeOH, respectively for 3 days at RT and filtered. The filtrates were concentrated to dryness with rotary evaporator to obtain the CH_2Cl_2 and MeOH crude extracts.

The latex from freshly cut stems of *Jatropha curcas* was extracted with hexane. The hexane soluble part was concentrated by rotary evaporator to afford the

crude hexane extract. The residue was further extracted with CH_2Cl_2 to afford the CH_2Cl_2 extract.

All crude extracts from various parts of *Jatropha curcas* were preliminary tested for antibacterial activity. The crude extract which displayed high antibacterial activity would be selected for larger scale extraction for isolation of bioactive compounds.

2.2.2 Screening for antimicrobial activity

The assessment of antibacterial activity of crude extracts or pure compounds was determined by agar well-diffusion assay method, agar serial dilution method and agar disc diffusion method. Each experiment was performed in triplicate.

2.2.2.1 Agar well-diffusion method

Cultured plates were employed for an agar well diffusion assay (Natarajan *et al*., 2005). Each of culture strains was prepared by pour plate method. A loopful of the bacterial culture from the slant was inoculated in the nutrient broth and incubated at 37°C, 150 strokes/for 24 h. The bacterial culture with OD 660 of 0.5 was required for the antibacterial activity.

100 μL of each inoculum was poured into each petri dish aseptically, added 25 mL of the malting sterilized nutrient agar to each dish. After thorough mixing, the warm agar was quickly poured into an empty steriled petri dish. Once the agar medium was cooled, it was solidified at RT. Four equidistant wells of 8 mm in diameter were then punched into 4 mm thicken agar using sterilized standard cork borer of 8 mm inner-diameter. 200 μL of 100 μg/mL of each crude extract and negative controls were carefully pipetted into each well. The crude extracts were tested for 4 wells per plate. The results were recorded by measuring the diameter of the inhibition zones around the wells after incubating at 37°C for 24 h. The negative controls were prepared using an organic solvent.

2.2.2.2 Agar serial dilution method

Antibacterial activity was carried out by agar serial dilution method (Francis Bacon, 2005). The extracts with the significant antibacterial activity were

tested by agar well diffusion method. The results were confirmed by checking with agar serial dilution method as described below.

A loopful of the bacterial culture from the slant was inoculated in the nutrient broth and incubated at 37°C, 150 strokes/for 24 h. The bacterial culture with OD 660 of 0.5 was used. The fresh nutrient broth 25 mL was seeded with a loopful of the bacterial culture and 200 µL of 100 µg/mL of crude extract incubated at 37°C, 150 strokes/min for 24 h.

The first dilution was achieved by adding 1 mL of this culture to 9 mL of steriled water. The second dilution was obtained by adding 1 mL of the first dilution to 9 mL of steriled water. The process was repeated until the tenth dilution was gained. A set of tubes containing only seeded broth was kept as control and a suitable solvent as another control was maintained.

100 µL of the 10^{-7}-10^{-9} dilution was pipetted into petri dish aseptically, added 25 mL of the malting sterilized nutrient agar to each dish. After thorough mixing, the warm agar was quickly poured into an empty steriled petri dish. Once the agar medium cools, the agar with culture was solidified, which then incubated at 37°C for 24 h. Bacteria colonies would develop both within the medium and on its surface. The number of bacteria colonies that appeared on medium agar was counted and reported in concentration of bacteria colonies forming units (CFUs) per mL. The percentage of growth inhibition was determined.

%inhibition = (NC-NS) / D

%inhibition = number of bacteria

NC = number of colonies counted on a plate of the control

NS = number of colonies counted on a plate of the sample

D = dilution factor

2.2.2.3 Agar disc diffusion method

Agar disc diffusion method was employed and filter paper disks (6 mm in diameter) were used (Natarajan *et al*., 2005). 25 mL of the malting sterilized nutrient agar was poured to each petri dish aseptically. After thorough pouring, the agar was allowed to set and solidified at RT. A loopful of the bacterial culture from the slant was inoculated in the nutrient broth and incubated at 37°C, 150 strokes/for

24 h. The bacterial culture at OD 660 of 0.5 was used. 100 µL of the bacteria culture was pipetted onto the top of agar plate. Bacteria culture was picked up on a steriled L-shape glass, and was moved lightly along the agar surface. 10 µL of 100 µg/mL of the crude extract or pure compound was dissolved in an appropriate solvent into steriled filter paper discs. A mixture of antibiotic drug 100 ppm of cipofoxaxin and norfoxacin 10 µl was used as a positive control. Negative controls were performed using paper discs loaded with 10 µL of organic solvents. The clear zones were recorded by measuring the diameter of the inhibition zones plus diameter of the paper discs after incubating at 37°C for 24 h.

2.2.3 Data analysis

The obtained results were presented as mean±SD. The SPSS statistical program (Version 14.0) was used for data analysis. Analysis of Variance (ANOVA) and LSD (Least significant difference) post hoc tests were used to determine any significant differences between the tested variables of inhibition zones and crude extracts. All significant tests were performed at $P \leq 0.05$.

CHAPTER III

Results and Discussion

This research focuses on the search of antibacterial agents that could be utilized in livestock from *J. curcas* which has never been addressed. The outcome from this investigation may serve as additional information on the phytochemistry of *J. curcas* which could be a valuable lead in the field of pharmaceuticals and agrochemicals.

3.1 Extraction

Various parts including leave, branch, latex and the kernel from the seed of *J. curcas* were separately extracted followed the procedure described in Chapter II. The results of the extraction are presented in Table 3.1.

Table 3.1 The results of extraction of *J. curcas*

Plant parts	Weight of plant (g)	Solvent	Weight (g), (% w/w)
leave	33.33	CH_2Cl_2	1.30 (3.90%)
		MeOH	1.12 (3.36%)
branch	34.56	CH_2Cl_2	0.34 (0.98%)
		MeOH	0.21 (0.61%)
latex	34.25	hexane	0.31 (0.91%)
		CH_2Cl_2	0.23 (0.71%)
kernel	34.31	hexane	0.44 (1.28%)
		CH_2Cl_2	0.35 (1.03%)
		MeOH	0.12 (0.35%)

The leaves and branches of *J. curcas* were extracted with CH_2Cl_2 and MeOH, respectively while the latex of this plant was extracted with hexane and CH_2Cl_2.

The kernel oil was obtained from the extraction of kernel with *n*-hexane, CH_2Cl_2 and MeOH, respectively. The results of extraction of each plant part are shown in Table 3.1. From this table, the highest amount was obtained from the CH_2Cl_2 extract of the leave (1.30 g, 3.90 %), while the lowest yield (0.12 g, 0.35%) was attained from the MeOH extract of the kernel.

All crude extracts from various parts of *J. curcas* were preliminary tested for antibacterial activity. The crude extracts which displayed high antibacterial activity would be selected for larger scale extraction for isolation of bioactive compounds.

3.2 Preliminary study on the antibacterial activity screening of the crude extracts of *J. curcas*

The antibacterial activity screening of various crude extracts of *J. curcas* was carried out by agar well diffusion method followed the procedure described in Chapter II. The microorganisms employed in the assay were *S. aureus*, *S. auricularis*, *A. hydrophila* and *P. aeruginosa* that caused infectious diseases in livestock. Motile Aeromonas caused by *Aeromonas sp*., *Pseudomonas sp*. and pathogenic bacteria of the animal digestive tract and mastitis most commonly from *Staphylococcus sp.* and their toxins were among those examples. These diseases were considered as serious disease particularly for dairy farmers and breaks out in high frequency.

The results of antibacterial activity of various parts of *J. curcas* are presented in Table 3.2.

Table 3.2 Antibacterial activity of the crude extracts of *J. curcas*.

Plant parts	**Solvent**	**Diameter of inhibition zone (mm)**			
		S. auricularis	***S. aureus***	***A.hydrophila***	***P.aeruginosa***
leave	CH_2Cl_2	3.27±1.57	1.73±0.36	2.32±0.71	2.14±0.66
	MeOH	1.87±0.73	2.18±0.43	2.37±0.65	2.02±0.66
branch	CH_2Cl_2	2.20±0.80	2.58±0.75	4.05±1.30	2.39±0.56
	MeOH	1.66±0.47	2.40±0.87	2.38±0.70	2.48±0.60
latex	hexane	2.78±0.87	1.82±0.68	2.84±0.96	1.78±0.79
	CH_2Cl_2	0.99±0.26	1.86±1.30	1.24±1.38	1.17±0.96
kernel oil	hexane	2.30±0.78	0.47±0.60	0.29±0.29	1.46±0.54
	CH_2Cl_2	0.61±0.19	1.24±0.33	1.00±0.30	1.26±0.20
	MeOH	1.01±0.14	1.34±0.45	1.85±1.15	1.61±0.20

From the antibacterial activity of the CH_2Cl_2 extract of the leaves inhibited both gram positive and gram negative bacteria, *P. aeruginosa* and *S. auricularis*; moderate inhibition against *A. hydrophila* and least activity against *S. aureus*. Besides, the MeOH extract of the leaves showed moderate inhibition against both gram positive and gram negative bacteria. The CH_2Cl_2 extract of the branch exhibited significantly antibacterial activity against *A. hydrophila*.

More activities could be observed from the hexane extract of kernel oil, especially against *S. auricularis.* In the case of oil cake, the extract showed less inhibition against both gram positive and gram negative bacteria. The CH_2Cl_2 extract of latex revealed less inhibition against both gram positive and gram negative bacteria except for *A. hydrophila* (Figure 3.1).

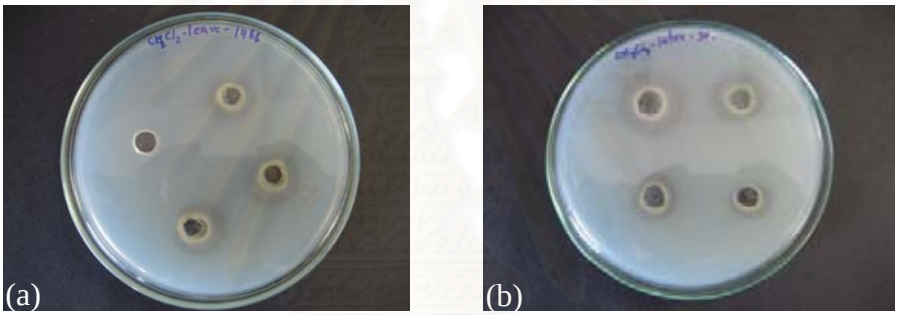
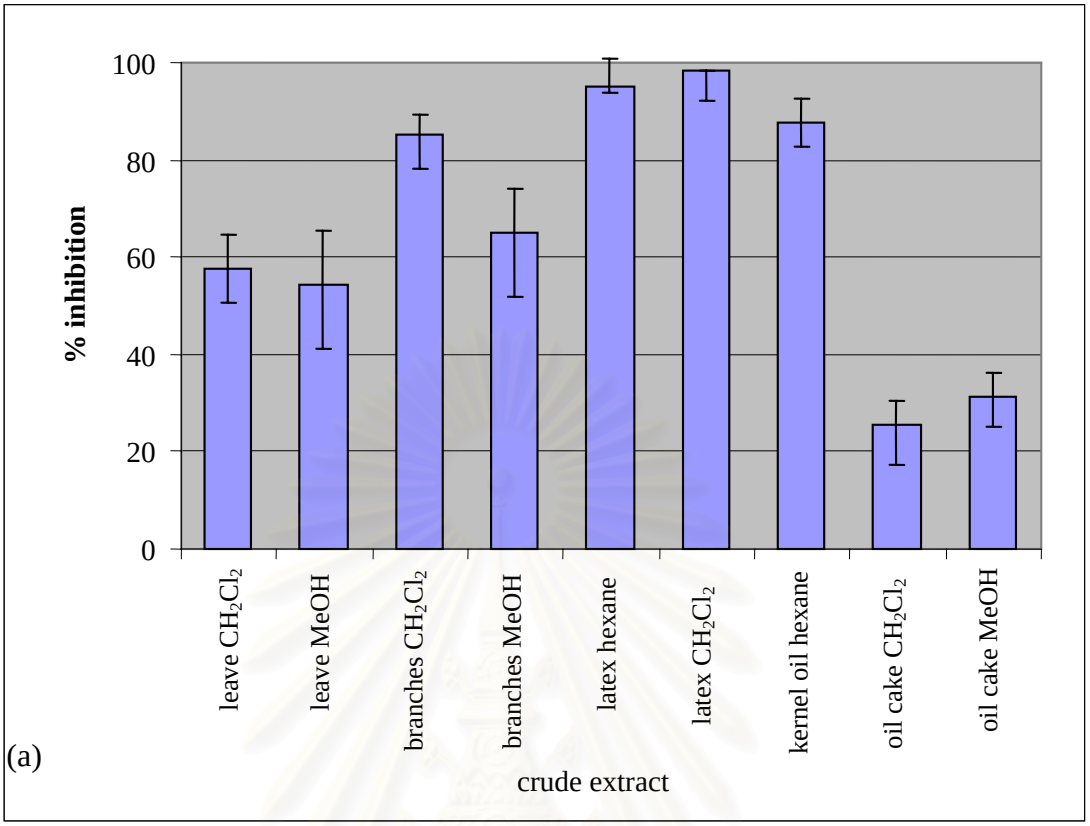
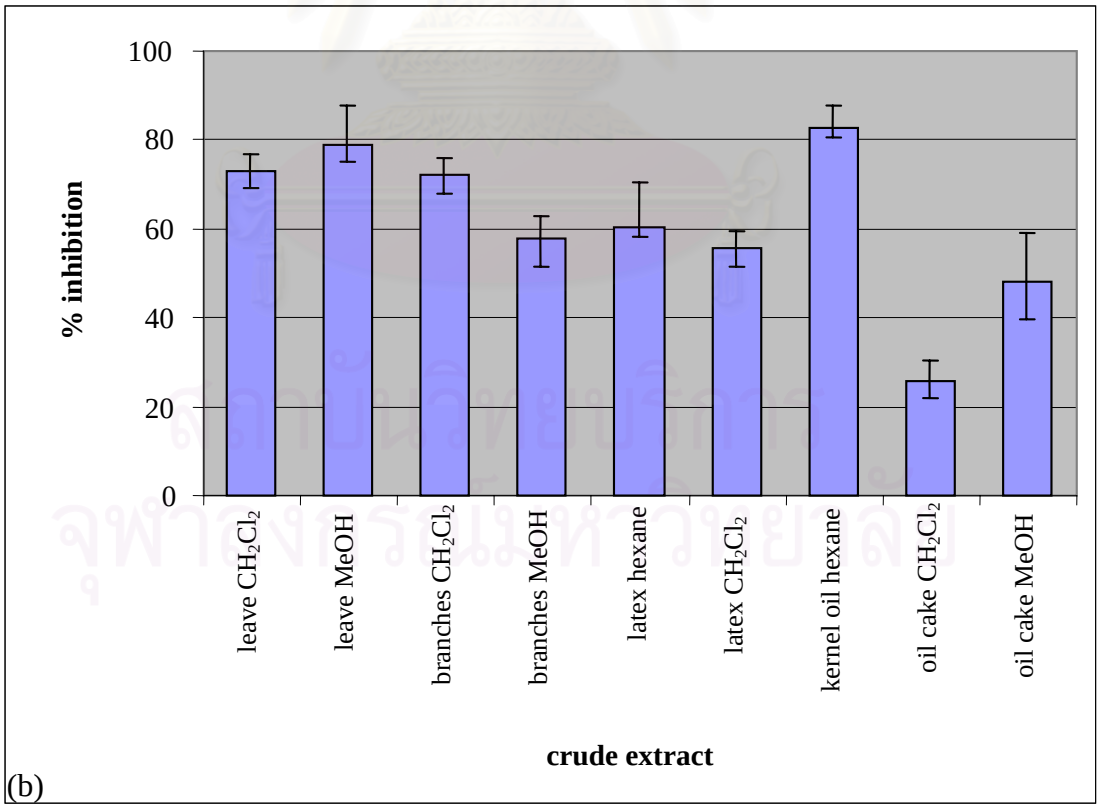


Figure 3.1 Inhibition zone of (a) the CH_2Cl_2 extract of the leave against *S. auricularis* (b) the CH_2Cl_2 extract of latex against *P. aeruginosa.*

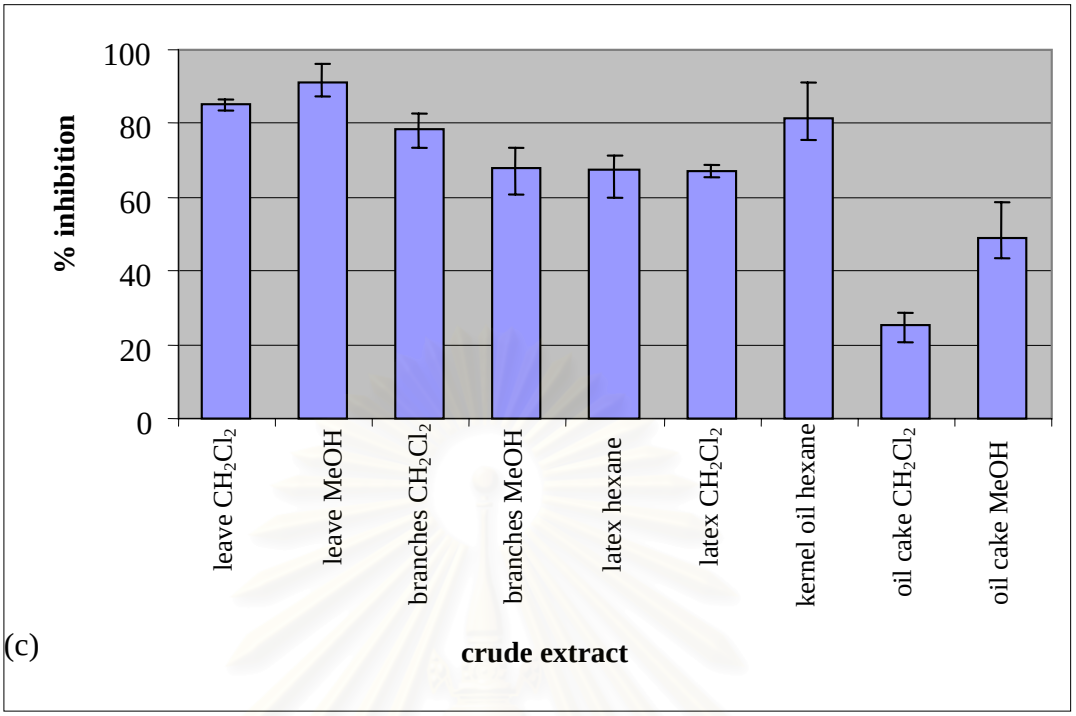
The extracts that revealed significant antibacterial activities were tested by agar well diffusion method. The results were confirmed by checking with agar serial dilution method. The results of these experiments are presented in Figure 3.2.


100
80
60
40
20
0
% inhibition
leave CH_2Cl_2
leave MeOH
branches CH_2Cl_2
branches MeOH
latex hexane
latex CH_2Cl_2
kernel oil hexane
oil cake CH_2Cl_2
oil cake MeOH
crude extract

(a)


100
80
60
40
20
0
% inhibition
leave CH_2Cl_2
leave MeOH
branches CH_2Cl_2
branches MeOH
latex hexane
latex CH_2Cl_2
kernel oil hexane
oil cake CH_2Cl_2
oil cake MeOH
crude extract

(b)



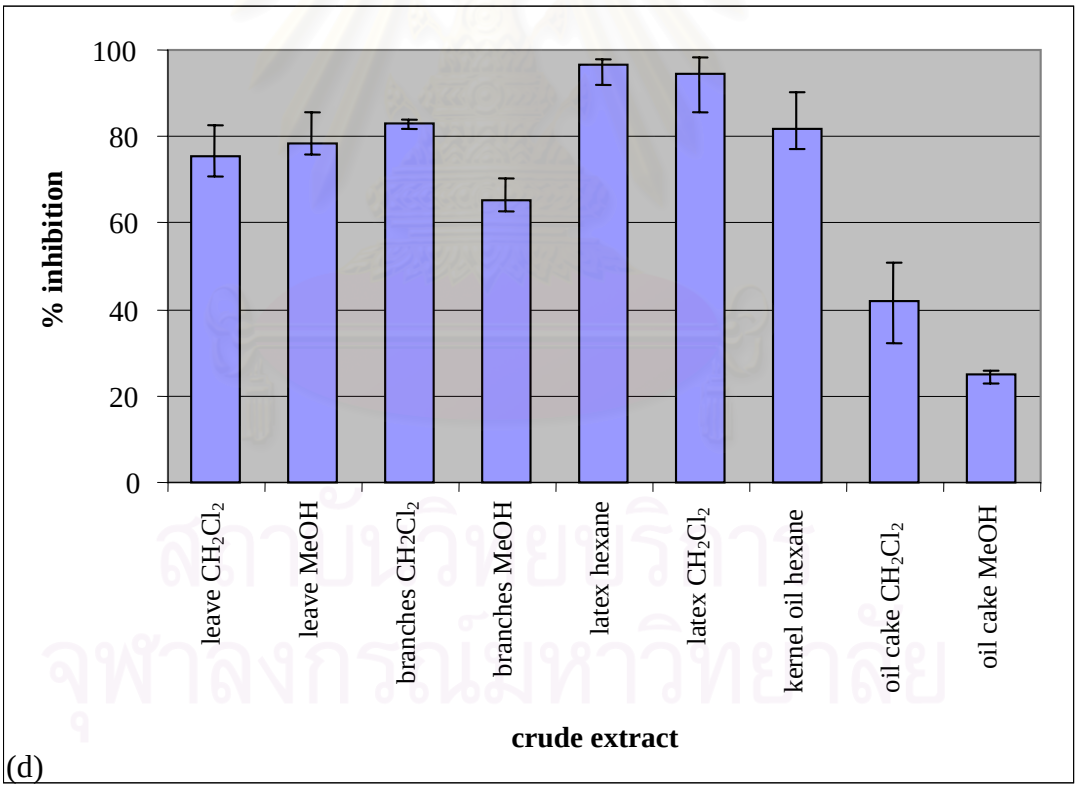


Figure 3.2 % Inhibition of the crude extracts of various parts of *J. curcas* at concentration of 100 ppm against (a) *A. hydrophila* (b) *P. aeruginosa* (c) *S. auricularis* and (d) *S. aureus*.

The CH_2Cl_2 and MeOH extracts of the leaves expressed more inhibition against pathogenic bacteria except for *A. hydrophila*, while the CH_2Cl_2 extract of the branches showed higher inhibition activity than that of the MeOH extract.

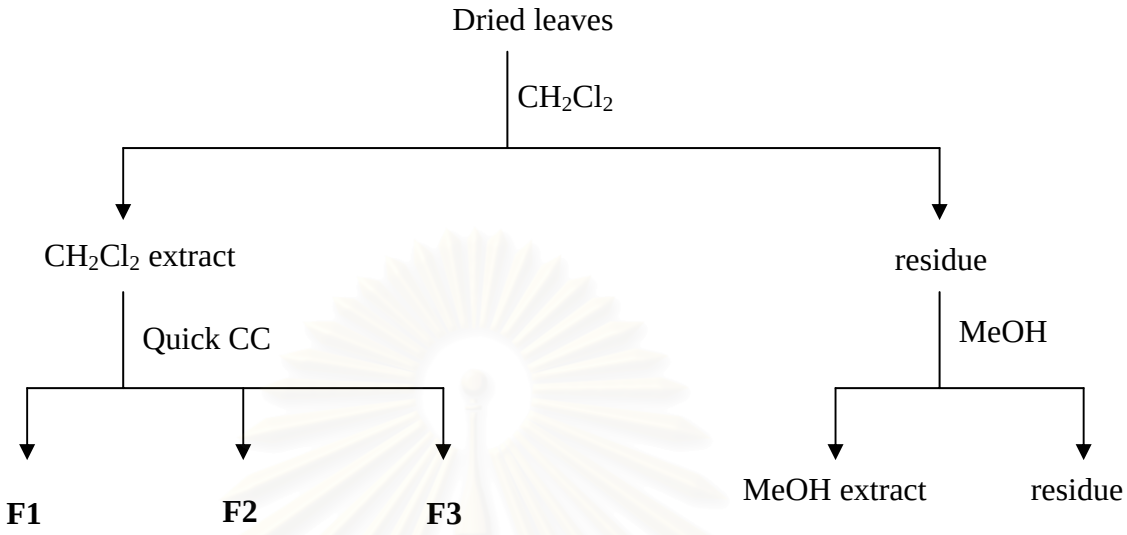
On the other hand, more inhibition activity of the hexane extract of kernel oil was observed for all bacteria especially *P. aeruginosa*, whereas the extract from oil cake revealed less inhibition both gram positive and gram negative organisms. The CH_2Cl_2 and hexane extracts of latex displayed strong inhibition against *A. hydrophila* and *S. aureus*.

It should be noted here that this work is the pioneer study to demonstrate antibacterial activity of *J. curcas*. These preliminary results implied the presence of biologically active compounds in almost of the parts of this plant (Figure 3.1).

Part I: Bioactive compounds from the leaves of *J. curcas*

3.3 The separation of the CH_2Cl_2 extract

The CH_2Cl_2 extract of the leaves was selected since it exhibited strong antibacterial activity. The dried leaves (6.0 kg) were powdered and extracted by maceration with CH_2Cl_2 (3 times, 3 days each). Then the solvent was evaporated to dryness, the CH_2Cl_2 extract as brown viscous solid 250.0 g (4.17% w/w) was attained. The marc was subsequently extracted with MeOH. The MeOH extract was concentrated to yield 152.6 g (2.54% w/w). The CH_2Cl_2 extract (240.0 g) was separated on silica gel quick column. The column was first eluted with hexane, then with a gradient solvent with increasing polarity, EtOAc and MeOH. Each eluent was collected according to a spot on TLC and similar fractions were combined to gain three fractions, **F1-F3**. Each fraction was subjected to antibacterial activity test by agar serial dilution method. The results of the isolation are presented in Scheme 3.1 and Table 3.3.



Scheme 3.1 The separation scheme of the CH_2Cl_2 extract of the leaves of *J. curcas*

Table 3.3 The results of the isolation of the CH_2Cl_2 extract of the leaves by silica gel quick column

Fraction No.	Solvent system	Appearance	Weights (g)
F1	100%hexane-15% EtOAc/hexane	orange wax	11.24
F2	20-80%EtOAc/hexane-100%EtOAc	brown sticky	120.00
F3	5-20%MeOH/EtOAc	brown sticky	44.29

From Table 3.3, the highest amount of the separates was obtained in **F2** (120.0 g, 48%w/w) and the lowest yield (11.24 g, 4.5%w/w) was attained in **F1**. All sub-fractions were subjected to antibacterial activity.

3.3.1 Antibacterial activity test of sub-fractions separated from the CH_2Cl_2 extract

The sub-fractions obtained from the separation of the CH_2Cl_2 extract were subjected to antibacterial activity test using agar serial dilution compared with those obtained from the CH_2Cl_2 extract. The results are presented in Figure 3.2.

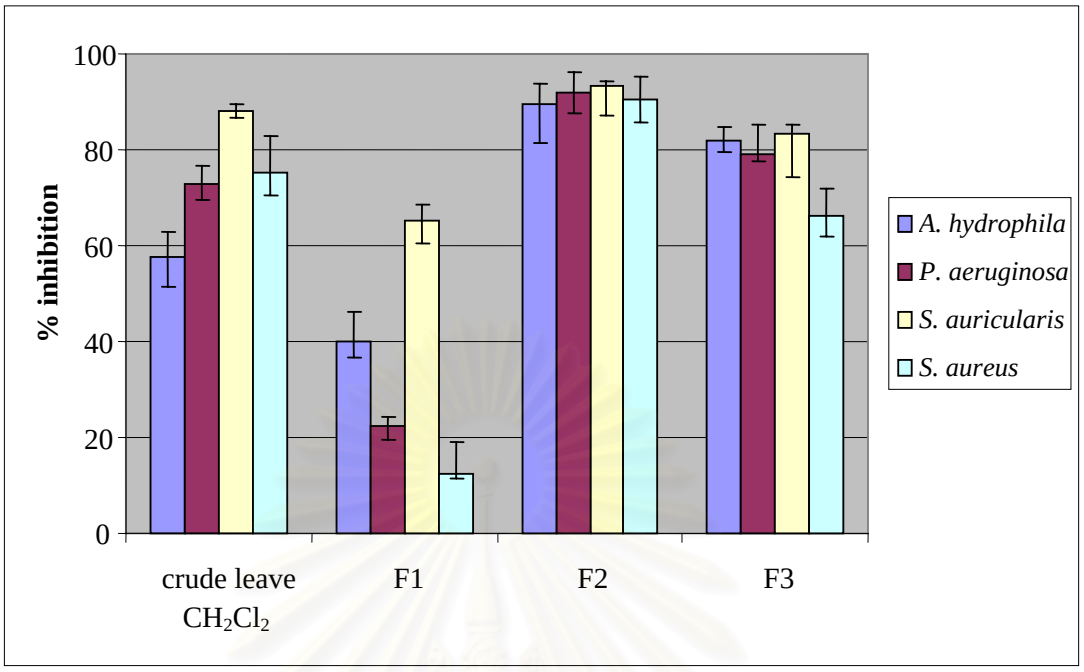
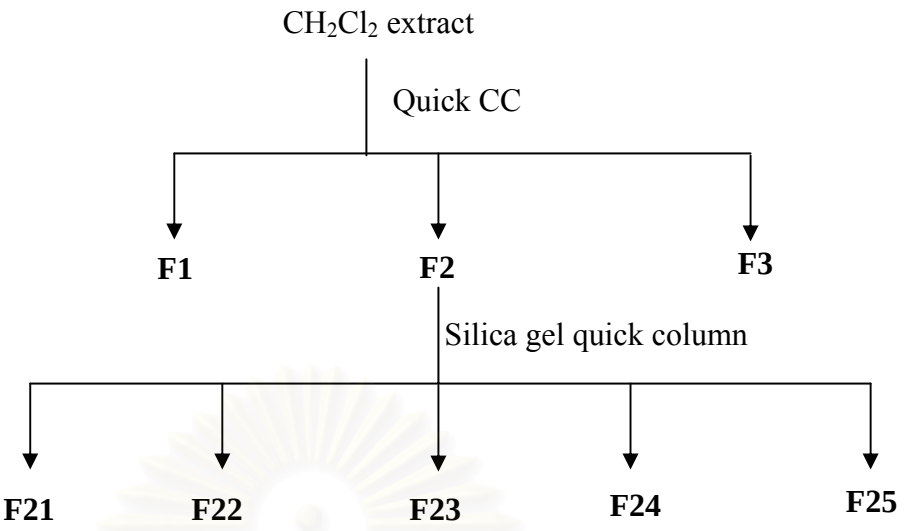


Figure 3.3 %Inhibition of the CH_2Cl_2 extract and its separated fractions

Fraction 2 (**F2**) showed higher inhibition activity against all bacteria strains than those of fractions 1 and 3 (**F1** and **F3**). %Inhibitions against *S. aureus*, *A. hydrophila* and *P. aeruginosa* of **F2** were in addition higher than those of the CH_2Cl_2 extract, while %inhibition against *S. auricularis* was similar to that of the CH_2Cl_2 extract. For **F1**, %inhibition against *S. auricularis* could be observed more than that against *S. aureus*, *A. hydrophila* and *P. aeruginosa*. **F3** exhibited higher %inhibition against *A. hydrophila* compared with the original CH_2Cl_2 extract, whereas **F3** revealed less inhibition against *S. aureus* than the crude extract. The CH_2Cl_2 extract and **F3** expressed the same level of inhibition against *S. auricularis* and *P. aeruginosa*. According to the obtained results, **F2** was selected for further separation.

3.4 The separation of F2

Stemmed from the aforementioned results, **F2** exhibited high antibacterial activity, thus it was further separated on silica gel quick column. The results of the isolation and antibacterial activity test are presented in Scheme 3.2 and Table 3.4.



Scheme 3.2 The separation of **F2**.

Table 3.4 The separation of **F2** by silica gel quick column

Fraction No.	Solvent system	Appearance	Weights (g)
F21	100%hexane-10% EtOAc/hexane	dark sticky crude	63.67
F22	20-40%EtOAc/hexane	brown sticky crude	23.39
F23	60-80%EtOAc/hexane	brown sticky crude	26.30
F24	100%EtOAc	brown sticky crude	16.34
F25	5-20%MeOH/ EtOAc	brown sticky crude	8.43

Based on TLC patterns of the separated fractions, five sub-fractions were obtained from the separation of **F2**. All sub-fractions were subjected for antibacterial activity test.

3.4.1 Antibacterial activity test of F2 and its separated fractions

F2 and its five separated fractions (**F21-F25**) were tested for their antibacterial activity using agar serial dilution. The results are presented in Figure 3.4.

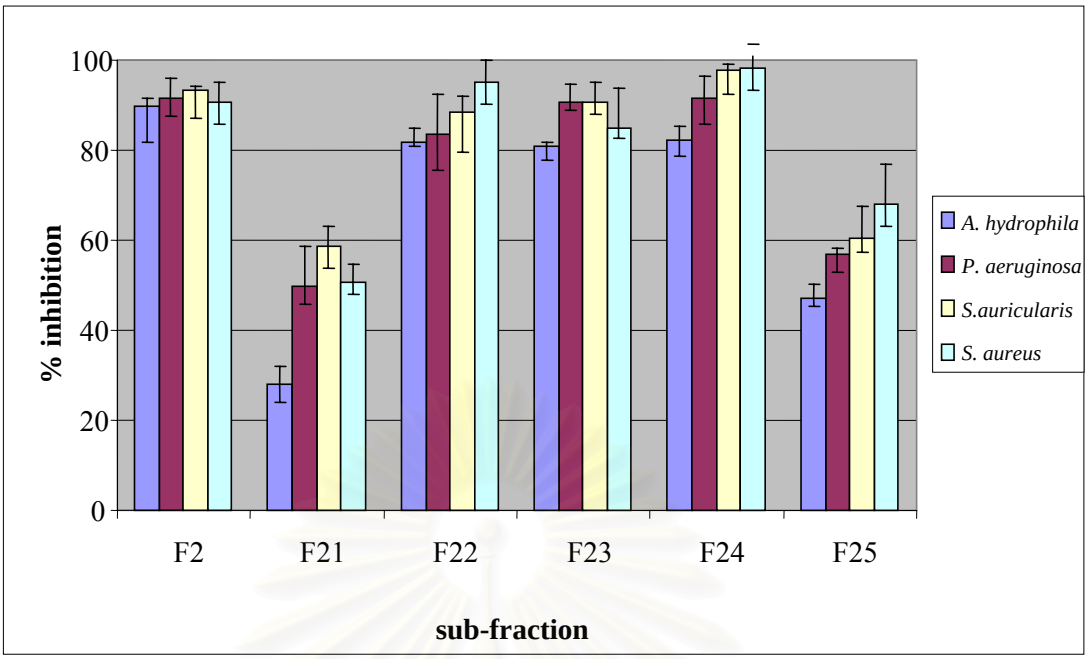
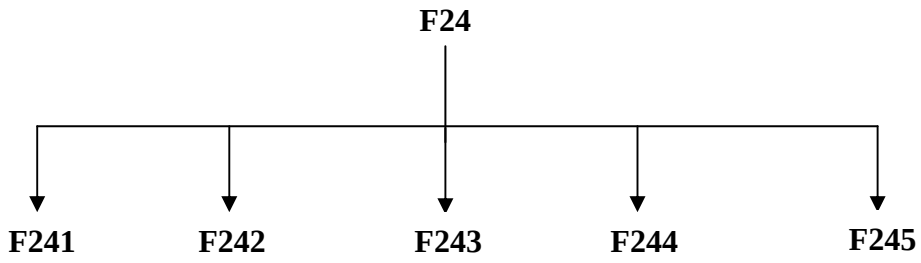


Figure 3.4 %Inhibition of **F2** and its sub-fractions

According to the results presented above, **F2** exhibited the significant inhibition against all pathogenic bacteria including gram positive, *S. aureus* and *S. auricularis,* and gram negative bacteria, *A. hydrophila* and *P. aeruginosa*. The separation of **F2** yielded 5 sub-fractions, **F21-F25**. **F22-F24** revealed better inhibitory activity than the rests. Especially, **F24** demonstrated the ability to inhibit *P. aeruginosa* prevailing to other sub-fractions, or even **F2**. Thus, this fraction was selected for further separation.

3.5 The separation of F24

The further separation of **F24** was conducted following bioassay guided with the aim to search for bioactive compounds possessing antibacterial activity. The results of isolation and antibacterial activity test are presented in Scheme 3.3 and Table 3.5.



Scheme 3.3 The separation of **F24**.

Table 3.5 The separation of **F24** by silica gel column

Sub-fraction No.	Solvent system	Appearance	Weights (g)
F241	100%hexane-5% EtOAc/hexane	brown sticky crude	2.39
F242	10-20%EtOAc/hexane	brown sticky crude	8.41
F243	40-60%EtOAc/hexane	brown sticky crude	1.33
F244	80%EtOAc/hexane-100%EtOAc	brown sticky crude	3.70
F245	5-20%MeOH/ EtOAc	brown sticky crude	1.84

The separation of **F24** which showed good inhibitory activity against all bacteria furnished 5 sub-fractions, **F241-F245**. **F243** was obtained in the highest yield (8.41 g, 51.47% w/w). All sub-fractions were subjected to the antibacterial activity test.

3.5.1 Antibacterial activity test of F24 and its separated fractions

All isolated sub-fractions, **F241-F245** and original **F24** were antibacterial assayed using agar serial dilution. The results of antibacterial activity test are presented in Figure 3.5.

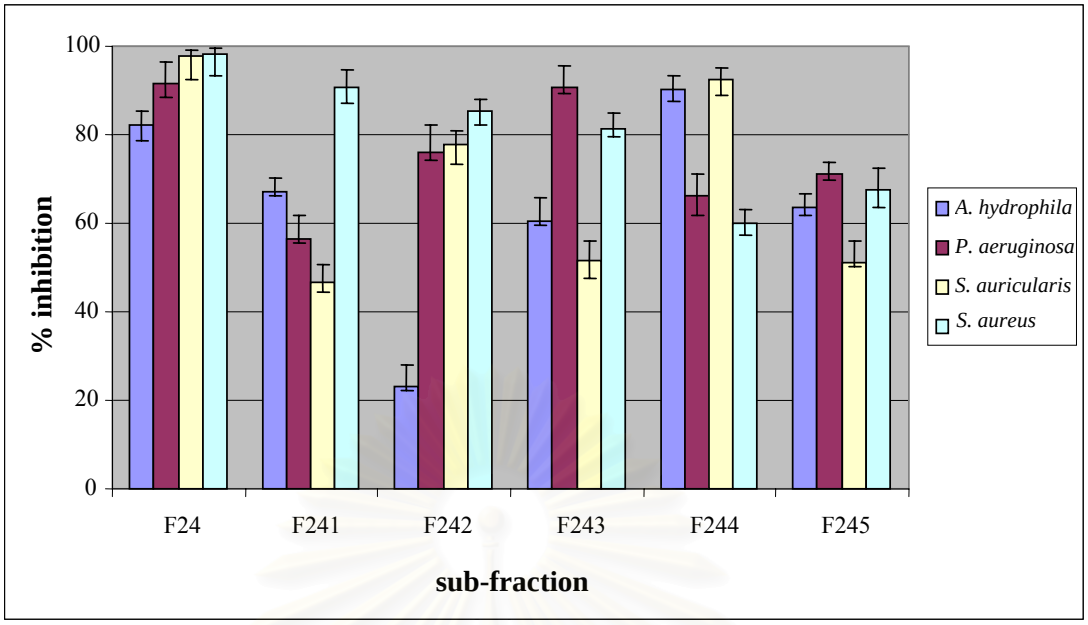
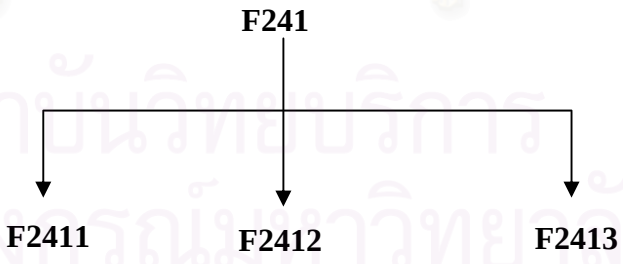


Figure 3.5 %Inhibition of **F24** and its sub-fractions

From Fig 3.5, **F241** revealed the highest antibacterial activity against *S. aureus*, thus it was rationalized for further exploration, while **F243** markedly displayed the antibacterial activity against *P. aeruginosa*. In addition, **F244** displayed strong activity against both gram positive, *S. auricularis* and gram negative, *A. hydrophila*. Thus, these three sub-fractions were carried on for further purification.

F241 was further purified by silica gel column resulting in three sub-fractions, **F2411-F2413**. The results are presented in Scheme 3.4 and Table 3.6.



Scheme 3.4 The separation of **F241**.

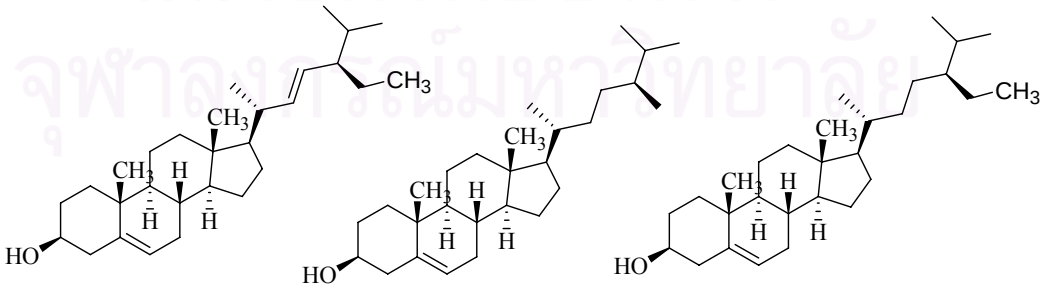
Table 3.6 The separation of **F241** by silica gel column

Sub-fraction No.	Solvent system	Appearance	Weights (g)
F2411	100%hexane-20%EtOAc/hexane	white solid	0.92
F2412	40%EtOAc/hexane	white solid	0.70
F2413	60%EtOAc/hexane - 100%EtOAc	brown sticky crude	0.77

According to Table 3.6, **F2411** was while solid. The recrystallization of this solid with hexane and MeOH yielded white needle, designated as mixture **1**, 0.80 g (33.47% yield based on **F24**), m.p.130-132°C. This substance was soluble in $CHCl_3$, CH_2Cl_2 and EtOAc and then analyzed its purity by GC. The GC chromatogram (Figure 3.6) showed three peaks at R_t 16.04, 16.44 and 17.32 min. Compared with a standard mixture of steroids, these components could be analyzed as β-sitosterol (12%), campesterol (14%) and stigmasterol (74%), respectively.

According to the spectroscopic data, the ^{1}H-NMR spectrum of mixture **1** (Figure 3.7) showed the signal at δ_H 5.04-5.02 (m), 5.12-5.18 (m) and 5.35 (m) which could be assigned to three olefinic protons. Another signal at δ_H 3.40 was attributable to the proton connecting to a hydroxyl group. The signals between δ_H 0.68-2.35 (m) are the signals of methylene and methyl groups in a molecule.

According to the obtained results, it could be conclude that this substance contained stigmasterol as a major component mixed with β-sitosterol and campesterol.



Stigmasterol Campesterol β-sitosterol

Mixture **1**

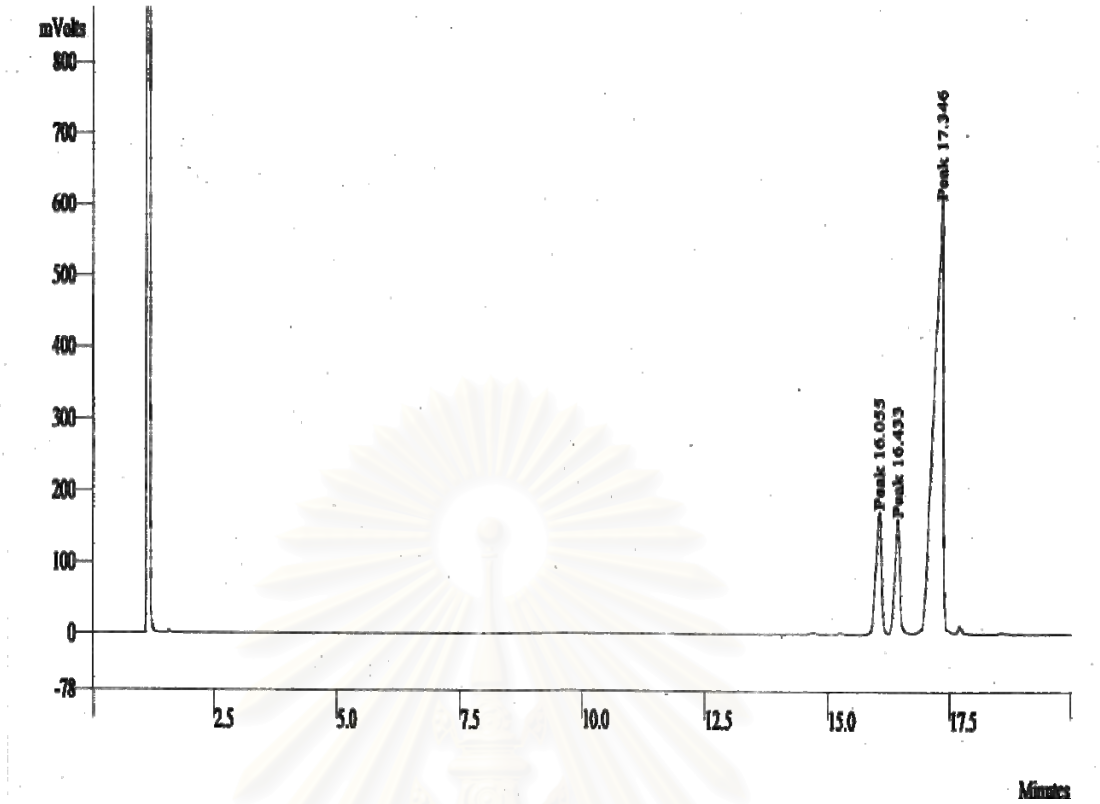


Figure 3.6 The GC chromatogram of **F2411**

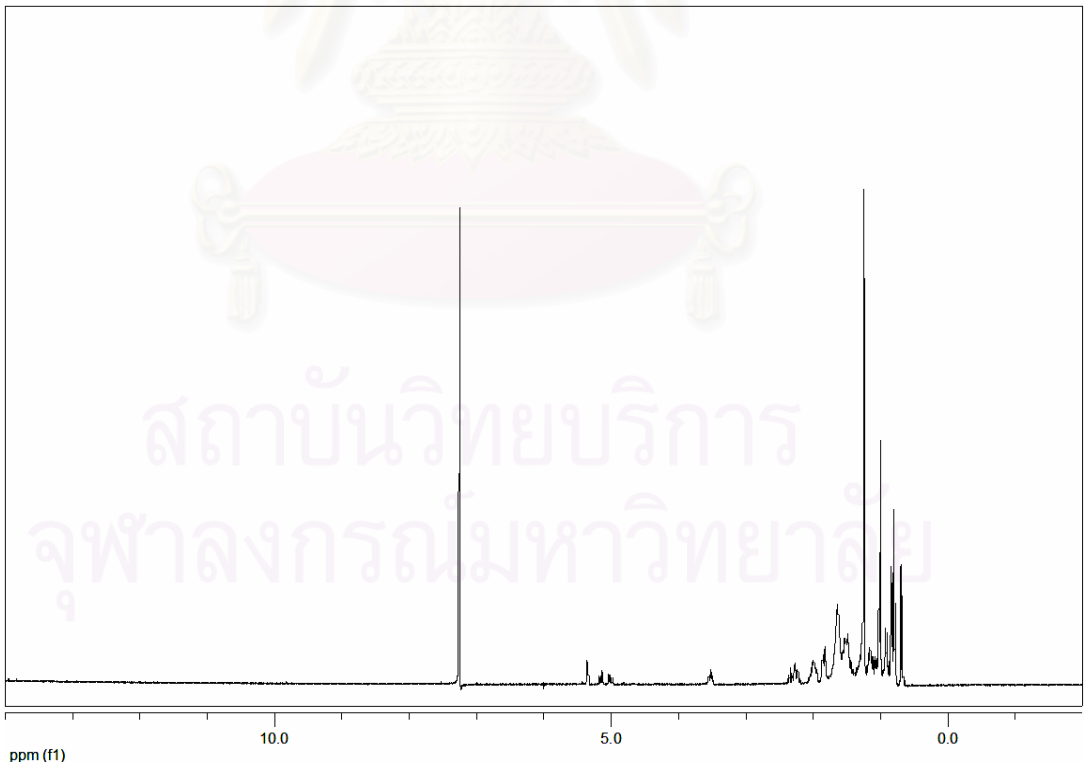


Figure 3.7 The ^{1}H-NMR spectrum of mixture **1**

F2412 was attained as white solid. After purification by recrystallization with hexane and acetone, substance **2** 0.5 g (21% yield), was obtained with m.p. 79-80°C. The ^{1}H-NMR spectrum (Figure 3.8) showed a high intensity signal at δ_H 1.27 suggesting several interlinking of methylene groups in a molecule. The signal of the methylene group connecting to a hydroxyl group was detected at δ_H 3.50. The signal of a methyl group exhibited a high intensity signal at δ_H 0.89. It was thus considered that this substance contained a hydroxyl group. This postulation was confirmed by the IR spectrum (Figure 3.9) which displayed C-H stretching at 2900 cm^{-1} and O-H stretching at 3200 cm^{-1}. On the basis of the spectroscopic data, the structure of substance **2** was identified as a mixture of long chain alcohols.

$$H_3C-(CH_2)_n-CH_2-OH$$

Long chain alcohols

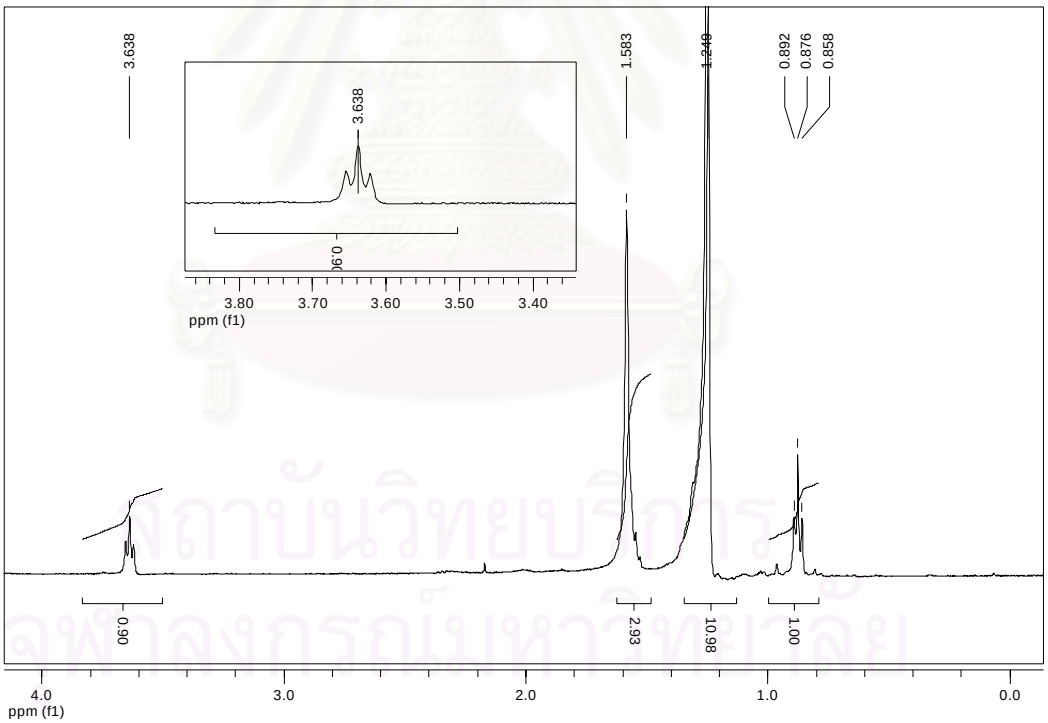


Figure 3.8 The ^{1}H-NMR spectrum of substance **2**

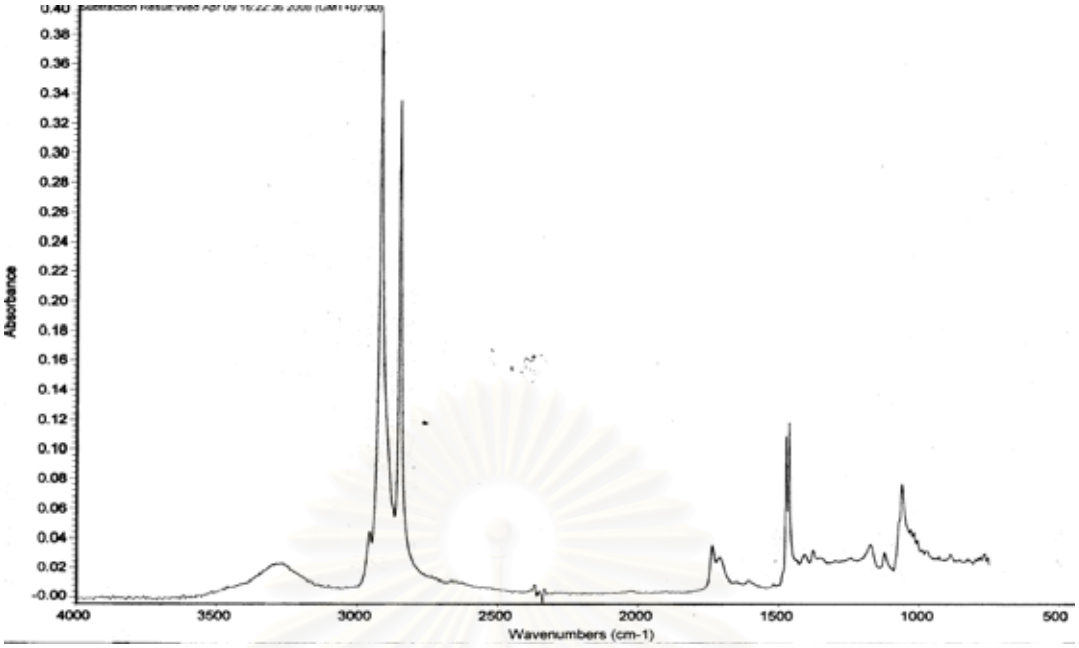


Figure 3.9 The IR spectrum of substance **2**

F2413 0.77 g was further purified by silica gel column eluting with CH_2Cl_2. The attempt to purify this sub-fraction was nonetheless not successful. **F2413** still remained four in-separatable spots on TLC.

F241, two isolated substances from **F24** and **F2413 (**mixture **1** and substance **2**) were subjected for antibacterial assay against *S. aureus*. The antibacterial activity results are presented in Figure 3.10 and Table 3.7.

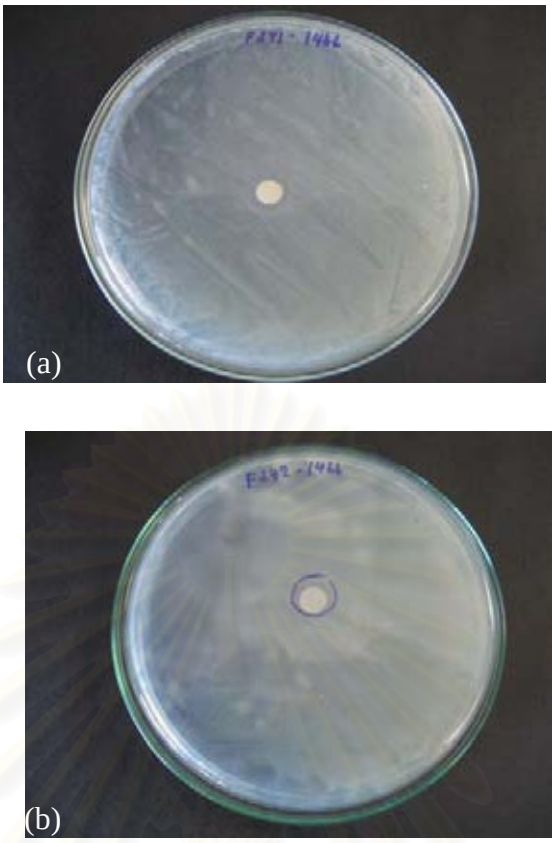


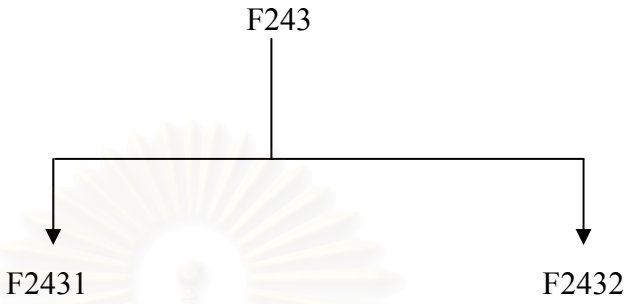
Figure 3.10 Inhibition zone of (a) **F241** (b) **F242** against *S. aureus*.

Table 3.7 The antibacterial activity of **F241**, mixture **1**, substance **2** and **F2413** against *S. aureus* by agar disc diffusion method.

sub-fractions	**Diameter of inhibition zone (mm)**
	S. aureus
F241	11
mixture **1**	7
substance **2**	8
F2413	7
negative control	no clear zone

The attained results revealed that all isolated substances were slightly active against *S. aureus.* **F241**, the original crude still exhibited better activity. This may imply that the synergist phenomena may involve in this particular case.

F243 (Figure 3.5) expressed antibacterial activity against *P. aeruginosa.* Therefore, it may contain bioactive compounds. The purification of the active **F243** by silica gel column eluted by EtOAc yielded two fractions, **F2431-F2432**. **F2431** was further purified by silica gel column.



Scheme 3.5 The separation of **F243**.

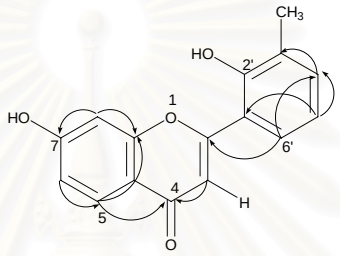
F2431 was attained as white powder 0.04 g (0.48% based on **F243**) after recrystallization with a mixture of hexane-acetone, white powder was achieved.

The ^{1}H-MNR spectrum (Figure 3.11) showed a sharp one-proton singlet at δ_H 6.83 (H-3), correlated with the carbon at 107.6 ppm (C-3) in the HMQC spectrum, ascribed to H-3 of an flavone moiety. The characteristic resonance of an aromatic proton was observed at δ_H 7.98 (*s*, H-8). Four doublet resonances at δ_H 7.51 (J = 7.7 Hz), 7.92 (J = 7.20 Hz), 7.63 (J = 7.40 Hz) and 8.30 (J = 7.90 Hz) were in agreement with four aromatic protons at H-4′ H-6′, H-6 and H-5 positions, respectively. The doublet of doublet signal of one aromatic proton (H-5′) were observed at δ_H 7.46 (J = 7.50 and 7.80 Hz), indicating the presence of *othro- and meta*-disubstituted aromatic ring. The presence of one methyl group was suggested from the singlet signal at δ_H 1.73.

The ^{13}C NMR showed the resonance of a carbonyl carbon of ketone group at δ_C 178.5 (C-4). Four carbon signals at 133.8 (C-2), 156.3 (C-9), 163.0 (C-7) and 163.4 (C-2′) were ascribed to four aromatic carbons, connected to oxygen atom. Seven sp^2 carbon signals at 107.6, 107.6, 125.2, 125.7, 126.3, 126.3 and 129.0 were attributed to C-3, C-8, C-5′, C-4′, C-6′, C-5 and C-6.

The arrangement of these substituents on skeleton was revealed by analysis of HMBC data. From the HMBC spectrum, one proton signal at δ_H 8.30 (H-5) of an aromatic ring showed key cross-peaks with the downfield signal of a quaternary

aromatic carbon at δ_C 156.3 (C-9) and a carbonyl group at δ_C 178.5 (C-4). The signals at δ_C 156.3 (C-9) and 163.0 (C-7) also correlated with the aromatic proton signal at δ_H 7.98 (H-8). The correlation of the aromatic proton signal at δ_H 7.63 (H-6) to the aromatic carbon signal at δ_C 126.3 (C-5) was detected. The correlations from δ_H 7.51 (H-4′) to δ_C 131.6 (C-3′) and from δ_H 7.46 (H-5′) to δ_C 123.9 (C-1′) and 125.7 (C-4′) were observed. The correlation of δ_H 7.92 (H-6′) to δ_C 125.7 (C-4′) and 178.5 (C-4) indicated that one aromatic ring was fused to C-2. The methyl moiety was placed at C-7 as the methyl proton at δ_H 1.73 correlated to C- 3′ (131.6 ppm) of the flavone nucleus in the HMBC spectrum.



HMBC correlations of compound **3**

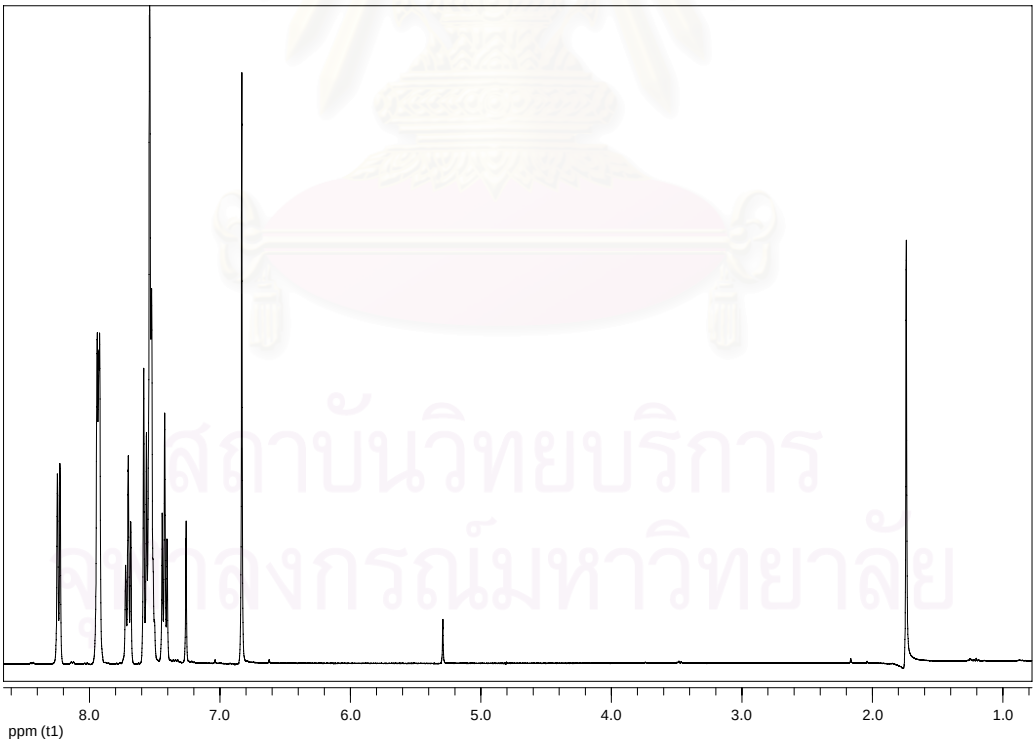
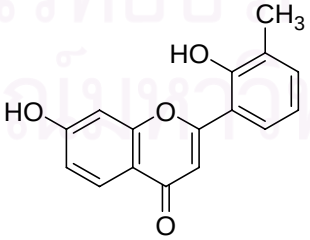


Figure 3.11 The ^{1}H-NMR spectrum of compound **3**

Table 3.8 The ^{1}H-MNR and ^{13}C-NMR chemical assignments of compound **3**

Position	δ_H (*J* in Hz)	δ_C	HMBC
1	-	-	-
2	-	133.8	-
3	-	107.6	-
4	-	178.5	-
5	8.30 (d, *J* = 7.9)	126.3	C-4, C--9
6	7.63(d, *J* = 7.4)	129.0	C-5
7	-	163.0	-
8	7.98 (s, *J* = 7.3)	107.6	C-7, C-9
9	-	156.3	-
10	-	107.6	-
1′	-	123.9	-
2′	-	163.4	-
3′	-	131.6	-
4′	7.51 (d, *J* = 7.7)	125.7	C-3′
5′	7.46 (dd, *J* = 7.8, 7.5)	125.2	C-1′, C-4′
6′	7.92 (d, *J* = 7.2)	126.3	C-4, C-4′
CH_3	1.73 (s)	-	-

The assignment of the linkage was confirmed by comparison with that of previous report. The structure of compound **3** was elucidated as 7,2′-dihydroxy-3′-methylflavone (Park, *et al.*, 2006).



7,2′-dihydroxy-3′-methylflavone

Compound **3** and **F2432** were assayed using blood agar disc diffusion at 10 µg/disc of 100 ppm. Two antibiotics: ciprofloxacin and norfoxacin were used as a positive control. The results of antibacterial activity of compound **3** and **F2432** are presented in Figure 3.14.

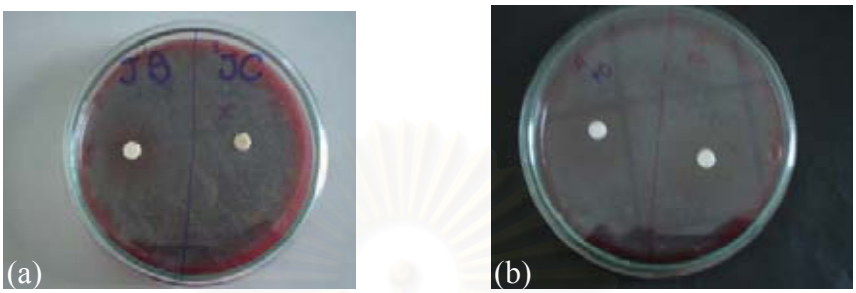
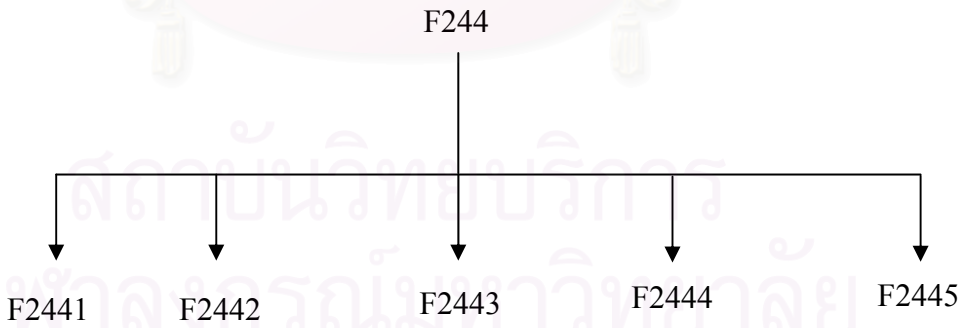


Figure 3.14 The inhibition zone of (a) compound **3** and **F2432** (b) positive control

The clear zone of a positive control and that of compound **3** were 17 and 14 mm, respectively. **F2432** did not show the inhibition while compound **3** was active against *A. hydrophila* with inhibition zone of 10 mm.

Finally, **F244** revealed a complete inhibition against gram positive, *S. auricularis* and gram negative, *A. hydrophila*. This fraction was then purified to accomplish 5 fractions as the results tabulated in Scheme 3.6 and Table 3.9. The antibacterial activity of **F244** and its sub-fractions were presented in Table 3.10.



Scheme 3.6 The separation of **F244**.

Table 3.9 The results of isolation of **F244** by silica gel column

Sub-fraction No.	Appearance	Weights (g)
F2441	brown sticky crude	0.40
F2442	brown sticky crude	0.50
F2443	brown sticky crude	0.80
F2444	brown sticky crude	0.70
F2445	brown sticky crude	0.03

The antibacterial activity of **F244** exhibited the complete inhibition against both gram positive and gram negative bacteria, *S. auricularis* and *A. hydrophila*.

Table 3.10 The antibacterial activity of **F244** against *S. auricularis* and *A. hydrophila* by agar disc diffusion method.

Fraction No.	Diameter of inhibition zone (mm)	
	S. auricularis	***A. hydrophila***
F244	15	14
F2441	9	9
F2442	12	8
F2443	8	10
F2444	8	13
F2445	8	6.5

F2442 showed better activity against *S. auricularis*, but less activity against *A. hydrophila*. **F2444** revealed high antibacterial activity against *A. hydrophila* while exhibited less activity against *S. auricularis*. However, **F2441**, **F2443** and **F2445** gave slightly inhibition results against all bacteria tested.

This preliminary screening test of various extracts of *J. curcas* for antibacterial activity indicated that most extracts of this plant were highly active. This supported its use in remedies for livestock disease. The screening in both assays (agar well diffusion and serial dilution method) revealed that the inhibitory activity was observed from the CH_2Cl_2 extract of the leaves as similarly addressed by Kisangau, *et al*. (2007). The high activity against *P. aeruginosa* and two gram positive bacteria, *S.*

auricularis and *S. aureus* and moderate activity against *A. hydrophila* (Aiyelagbe *et al*., 2000, Posangi *et al*., 2001) was also observed. The antibacterial activity of the CH_2Cl_2 extract of the leaves was high that it was decided to proceed with isolation.

The isolated compound, 7,2′-dihydroxy-3′-methylflavone exhibited high antibacterial activity against *P. aeruginosa* and moderate activity against *A. hydrophila* but no observable activity against gram positive bacteria. This compound displayed an inhibitory activity against gram negative bacteria was unexpected. Ravindranath *et al*. addressed in 2004 that the extract of this plant displayed high activity against gram positive. The activity was believed to derive from lipopolysaccharide layer along with proteins and phospholipids as major components in the outer surface of gram negative bacteria (Burn, 1988). Access of this compound to the peptidoglycan layer of cell wall was hindered by the outer lipopolysaccharide layer. These results were also consistent with other previous reports (Roberto *et al*., 2004, Jager, 2003 and Marquez *et al*., 2005) on this plant against gram negative bacteria. Thus, this plant may be a source providing bioactive compounds that could improve the treatment of infections caused by this bacteria.

F241 had the ability to inhibit the growth of *S. aureus*, a major cause of many skin disease (Mona, 1997) as similarly reported by Agbelusi *et al.* (2007). The isolation of **F241** yielded 3 sub-fractions. **F241** was slightly active against *S. aureus* which was possibly due to the synergistic action.

F244 exhibited inhibitory activity against gram positive, *S. auricularis* and gram negative, *A. hydrophila*, another livestock pathogen (Rojas *et al*., 2006). The isolation of **F2441** gave 5 sub-fractions. Among them, **F2442** revealed good inhibitory activity against *S. auricularis* and low activity against *A. hydrophila* which was found to be opposed to **F2444**. The antibacterial activity of **F244** may be derived from **F2441** being synergized with **F2444**. However, the active principle could not be identified owing to the limit amount of this sub-fraction.

Part II: Antibacterial activity of the kernel oils of *J. curcas*

According to the previous result, the kernel oil exhibited good antibacterial activity (80% inhibition) against both gram positive and gram negative bacteria. The kernel oil (150.0 g) was thus subjected to the separation using silica gel column eluting with hexane, a mixture of CH_2Cl_2-hexane, CH_2Cl_2, a mixture of CH_2Cl_2-MeOH and MeOH, consecutively to achieve four fractions (**O1-4**). The results of isolation of kernel oil are presented in Table 3.11.

Table 3.11 The isolation of kernel oil

Fraction No.	Solvent system	Appearance	Weights (g)
O1	100%hexane	white wax	0.02
O2	5-40%CH_2Cl_2/hexane	clear yellow oil	22.63
O3	80% CH_2Cl_2/hexane-100% CH_2Cl_2	clear oil	47.01
O4	10-20%MeOH/ CH_2Cl_2	gum orange oil	68.72

The investigation of antibacterial activity of **O2-O4** exhibited slightly inhibitory activity against both gram positive and gram negative bacteria, while **O1** displayed no activity against all pathogenic bacteria (Figure 3.15). This outcome clearly suggested the synergistic effect between major and minor components.

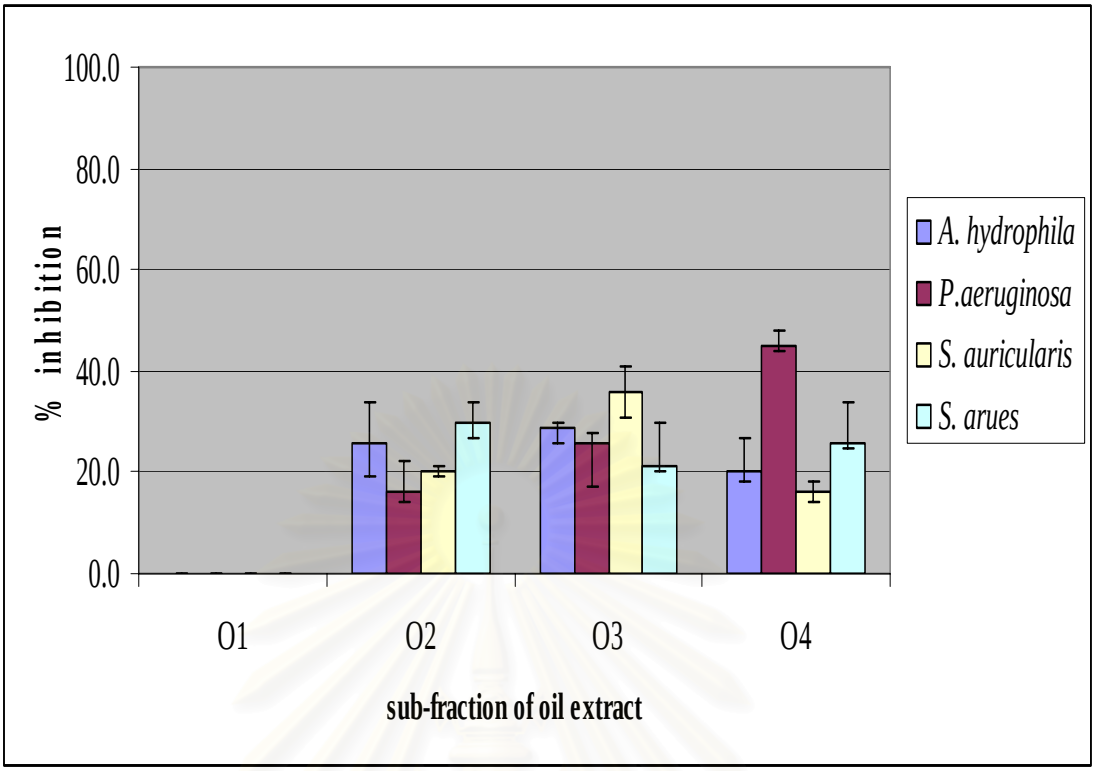


Figure 3.15 %Inhibition of sub-fraction of kernel oil extract

The kernel oil was analyzed for fatty acid content. The fatty acid composition of the oil was identified by GC after methylation (Figure 3.16). The oil extract was composed of most unsaturated fatty acids: oleic acid (46%), linoleic acid (31%), and saturated fatty acids as palmitic acid (14%) and stearic acid (7%). Other minor fatty acid components were eicosenoic (0.07%), palmioleic (0.72%), caproic (0.24%) and lauric acids (0.08%).

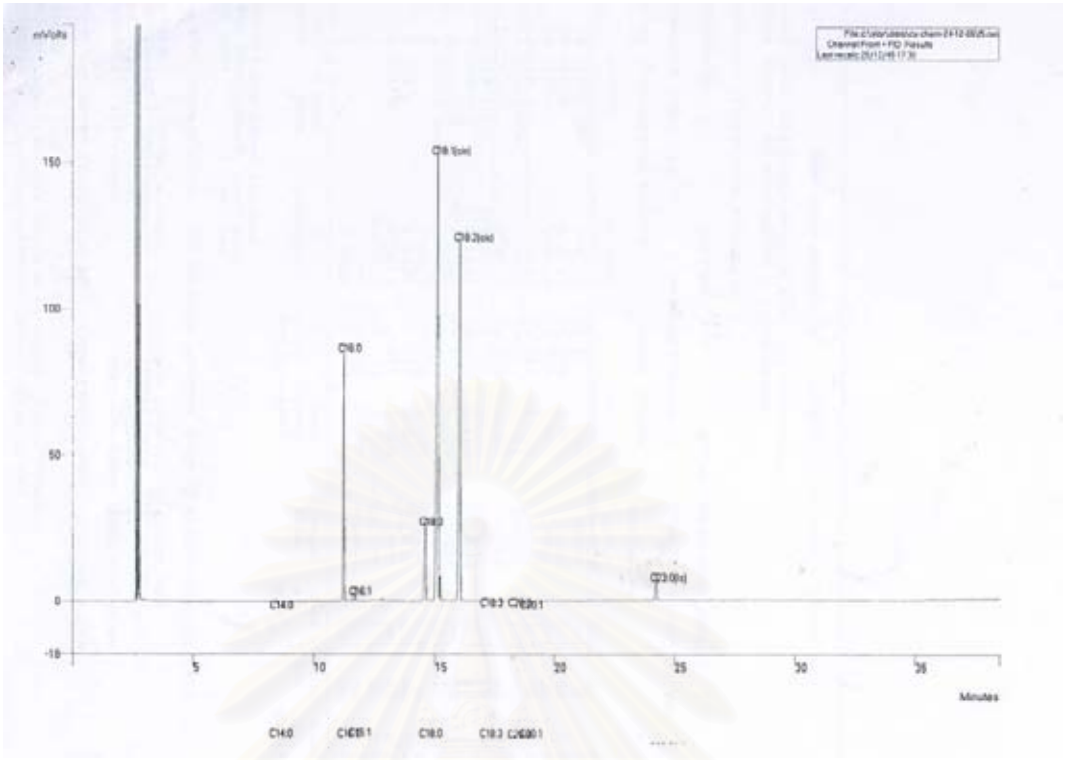


Figure 3.15 The GC chromatogram of kernel oil

The evaluation of antibacterial activity of pure fatty acids including palmitic, stearic, oleic and linoleic acids were conducted. All tested compounds did not express activity against pathogenic bacteria selected. This outcome suggested that the active principles responsible for antibacterial activity be present as minor component(s) or may need to be present together to display the synergist effect.

Further study on the kernel oil extract was continued by performing basic hydrolysis. A solution of 10% ethanolic KOH (20.0 mL) was added to oil extract (1.0 g) and the mixture was heated under refluxing for 8 h. Evaporation of EtOH gave a dryness crude which was further extracted with a mixture of Et_2O and H_2O (50 mL each) for three times. The combined Et_2O was dried over anhydrous Na_2SO_4. Evaporation of the solvent furnished yellow oil (**OL1**). The aqueous layer was acidified with conc HCl and extracted with Et_2O to afford a dryness substance (**OL2**). The inhibitory activity results of **OL1-2** are presented in Figure 3.17.

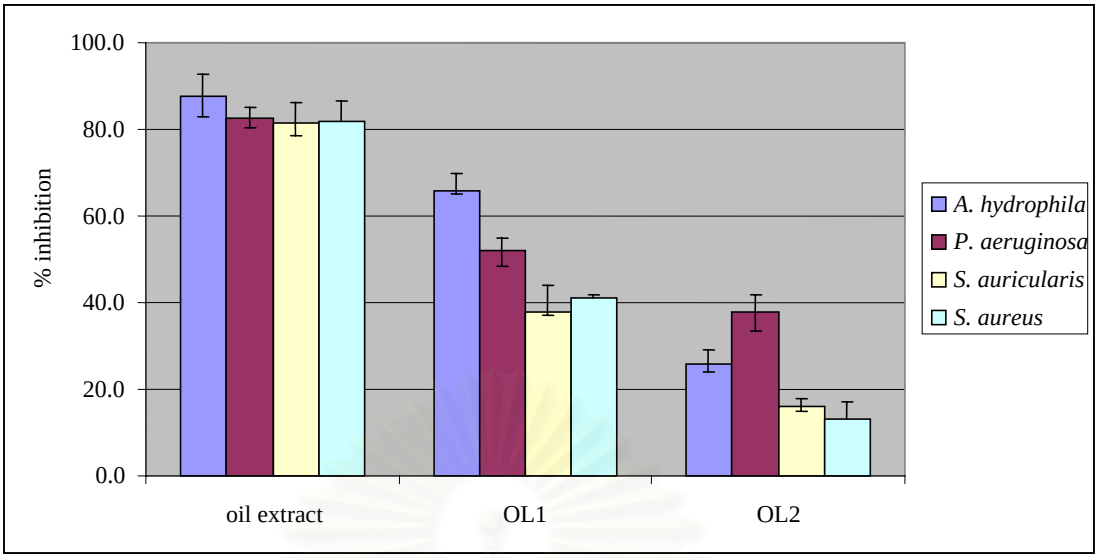


Figure 3.16 %Inhibition of oil extract and separated fractions **OL1-2**

The results of antibacterial activity of **OL1-2** expressed interesting results against gram positive and gram negative bacteria. **OL1** was effectively inhibited the growth of *A. hydrophila*. Thus, **OL1** was an indication of possible antibacterial compounds such as phorbol alcohol (Makkar *et al.*, 1997). **OL2**, a fatty acid part, displayed low inhibition against all pathogenic bacteria. This result was in good agreement with the aforementioned outcome.

The effect of the hexane extract of the kernel oils from *J. curcas* against all pathogenic bacteria (Kokwaro, 1993) was confirmed. The isolation of the hexane extract using bioassay guided yielded **O2-4** which displayed low inhibitory activity against all bacteria, whereas **O1** exhibited no activity against both gram positive and gram negative bacteria as opposed to the preliminary screening. The hexane extract may be an indication that the active principles against bacteria were the major toxic principle as phorbol ester available in *J. curcas*.

Part III: Antibacterial activity of the latex of *J. curcas*

According to the antibacterial activity of the latex of *J. curcas*, the results indicated a preliminary antibacterial activity of the latex extract of this plant. The isolation of the CH_2Cl_2 extract from silica gel column furnished 3 fractions. Further separation of the CH_2Cl_2 extract of latex (100.0 g) was conducted following the bioassay guided results. The results of isolation are presented in Table 3.12.

Table 3.12 The results of the isolation of CH_2Cl_2 extract of latex

Fraction No	Solvent system	Appearance	Weights (g)
L1	100%hexane-20%EtOAc/hexane	brown sticky	21.2
L2	40-80%EtOAc/hexane	brown sticky	46.9
L3	100% EtOAc -20%MeOH/ EtOAc	brown sticky	31.9

The investigation of antibacterial activity of each fraction showed that **L1** did not inhibition both gram positive and gram negative bacteria, while **L2** and **L3** displayed poor inhibitory activity against both gram positive and gram negative pathogenic bacteria (Figure 3.14).

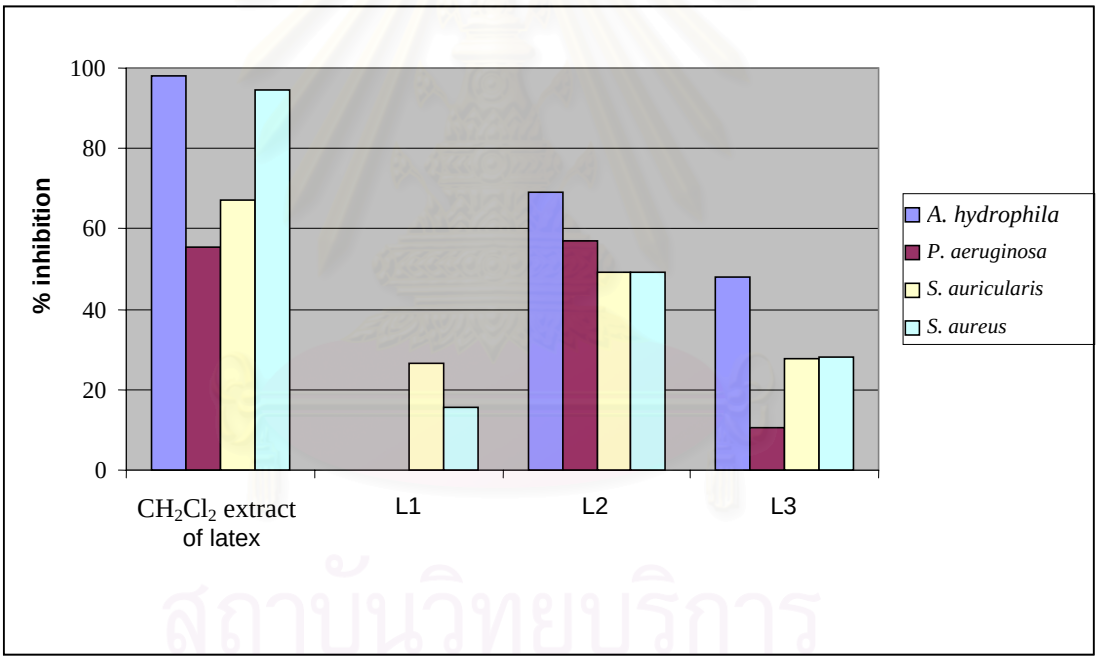


Figure 3.14 %Inhibition of sub-fraction of the CH_2Cl_2 extract of latex

From this result, the CH_2Cl_2 extract of latex displayed high antibacterial activity against both gram positive and gram negative bacteria. **L2** revealed moderate antibacterial activity against *A. hydrophila*, while **L1** displayed less antibacterial activity than **L3** against gram negative bacteria, *A. hydrophila* and *P. aeruginosa*.

The ability of **L1-3** to inhibit the growth of all gram positive and gram negative bacteria clearly display the necessity of being a combination of substances. This observation could possibly explain by the synergic effect between one or more active compounds.

From aforementioned obtained results, the CH_2Cl_2 extract of the leaves exhibited good antibacterial activity. Using bioassay guided, the isolation of 7,2′-dihydroxy-3′-methylflavone was achieved and could be confirmed its antibacterial activity as the active principle in this extract against two gram-negative bacteria, *P. aeruginosa* and *A. hydrophila*. This was the first time to report this particular compound responsible for antibacterial from this plant. In spite of the many advances in antibacterial agents may reduce the risk of infection from bacteria and produce antiseptic products.

CHAPTER IV

Conclusion

During the course of this research, various extracts of *Jatropha curcas* were tested for antibacterial activity against both gram negative and gram positive bacteria using well diffusion and serial dilution methods. The results from the well diffusion method showed that the CH_2Cl_2 extract of the leaves, that of latex and the hexane extract of kernel were capable of inhibiting the growth of pathogenic bacteria. Of these, the CH_2Cl_2 extract of the leaves displayed the strongest antibacterial activity both gram negative and gram positive bacteria.

Using the antibacterial activity as a bioassay-guided fractionation, 7,2′-dihydroxy-3′-methylflavone could be isolated as an active principle from the CH_2Cl_2 extract of the leaves. This compound strongly revealed as antibacterial properties against *P. aeruginosa* and slightly against *A. hydrophila*.

The oil extract of the kernel was also positive for antibacterial activity against both gram positive and gram negative; however, the separation of the oil into fractions diminished the activity. This may derive from the synergetic effect between each sub-fraction of oil. The results obtained from the hexane extract of the latex could also be observed in this similar trend.

Agar diffusion method and agar serial dilution method are two methods utilized as an bioassay and was found to be a very effective technique for antibacterial study. Using bioassay as a guide clearly supports the concept of searching for bioactive compounds. The isolation of flavone from the CH_2Cl_2 extract of the leaves from this study would open the research opportunity to study on the structure-antibacterial activity relationship of flavones. The more active and selective compounds against antibacterial should eventually discovered. To search new antibacterial activity from combination of each oil fraction and formulate the oil for antibacterial ingredient use in industry of livestock.

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APPENDIX

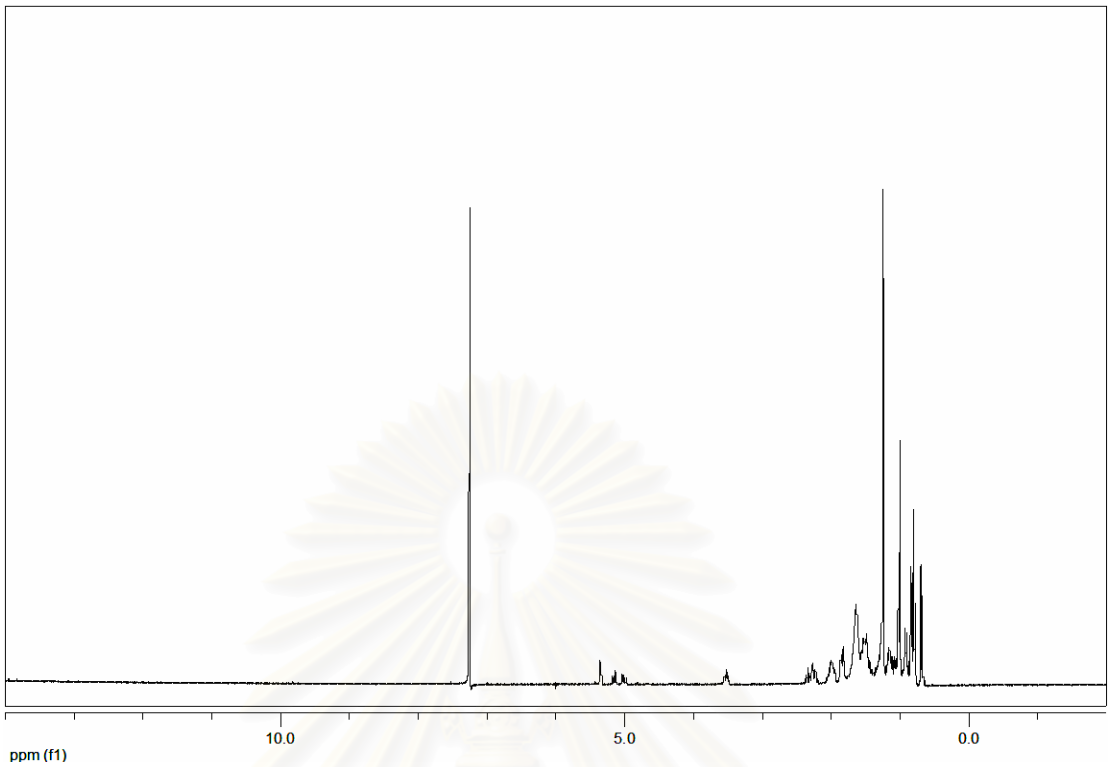

Figure A-1 The ^{1}H-NMR spectrum of mixture **1**

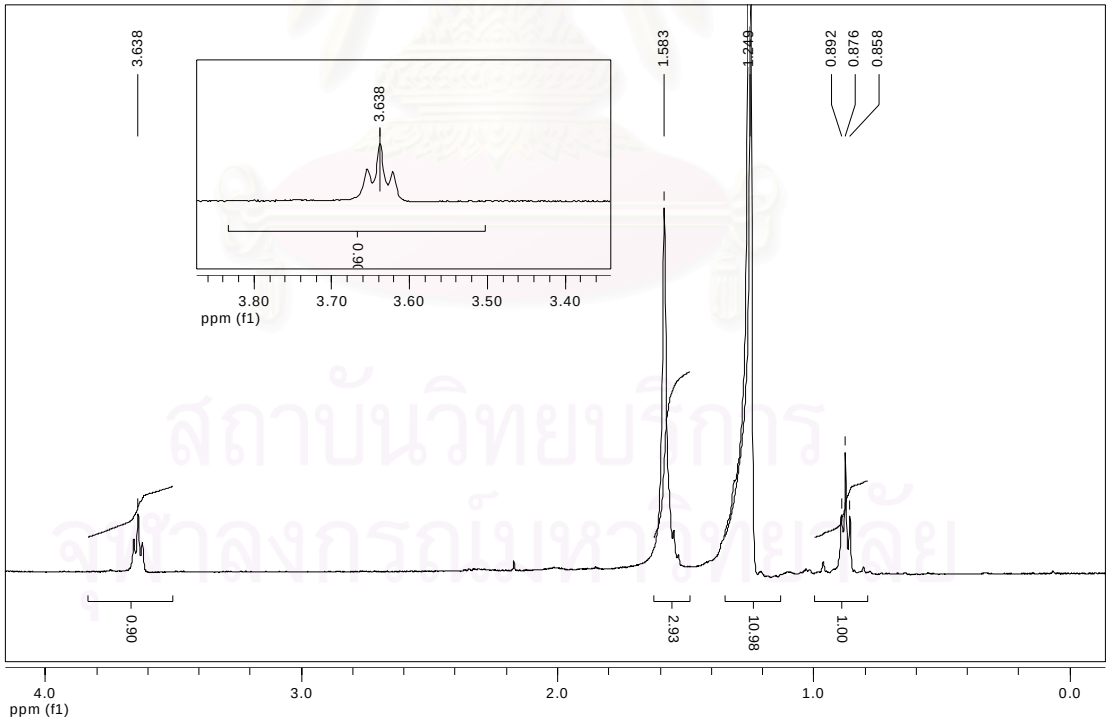

Figure B-1 The ^{1}H-NMR spectrum of substance **1**

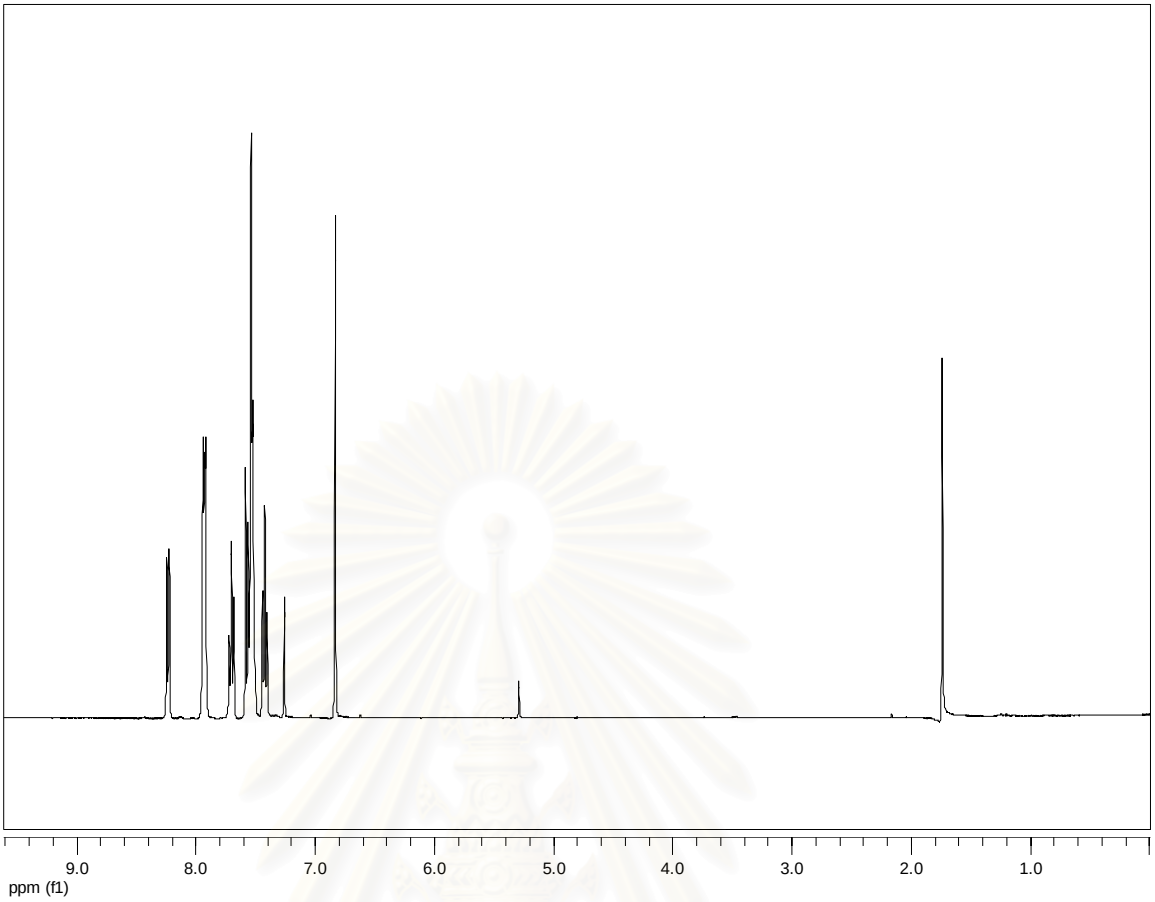

Figure C-1 The ^{1}H-NMR spectrum of compound **3**

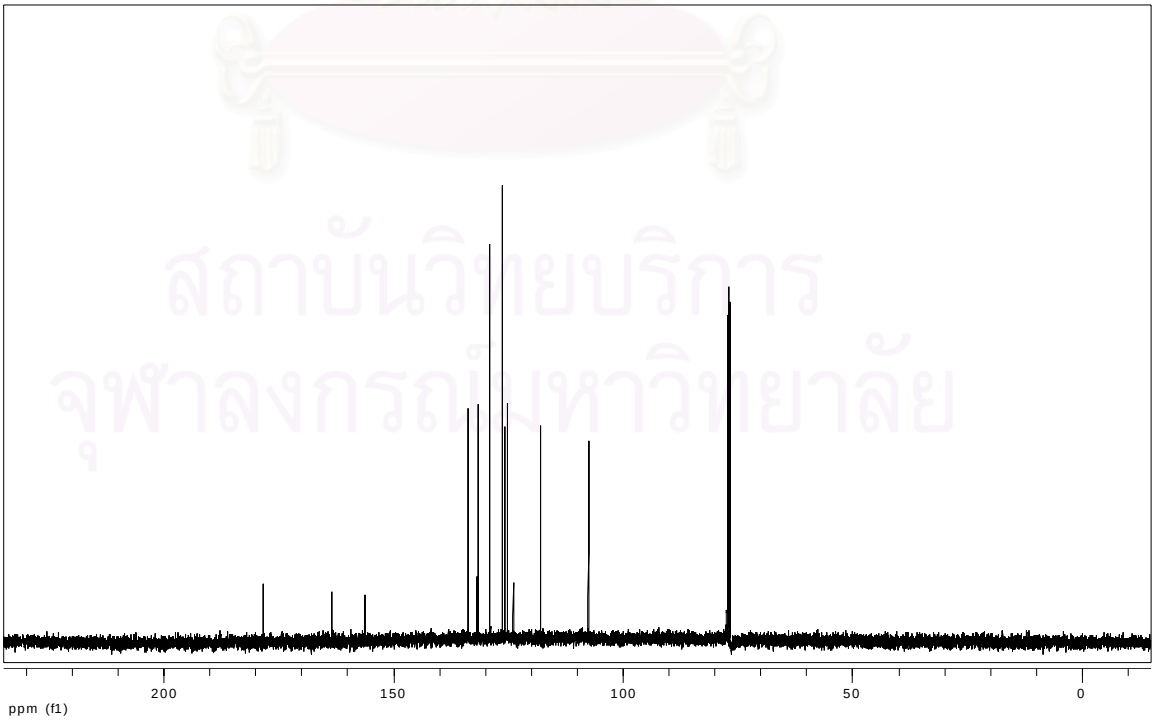

Figure C-2 The ^{13}C-NMR spectrum of compound **3**

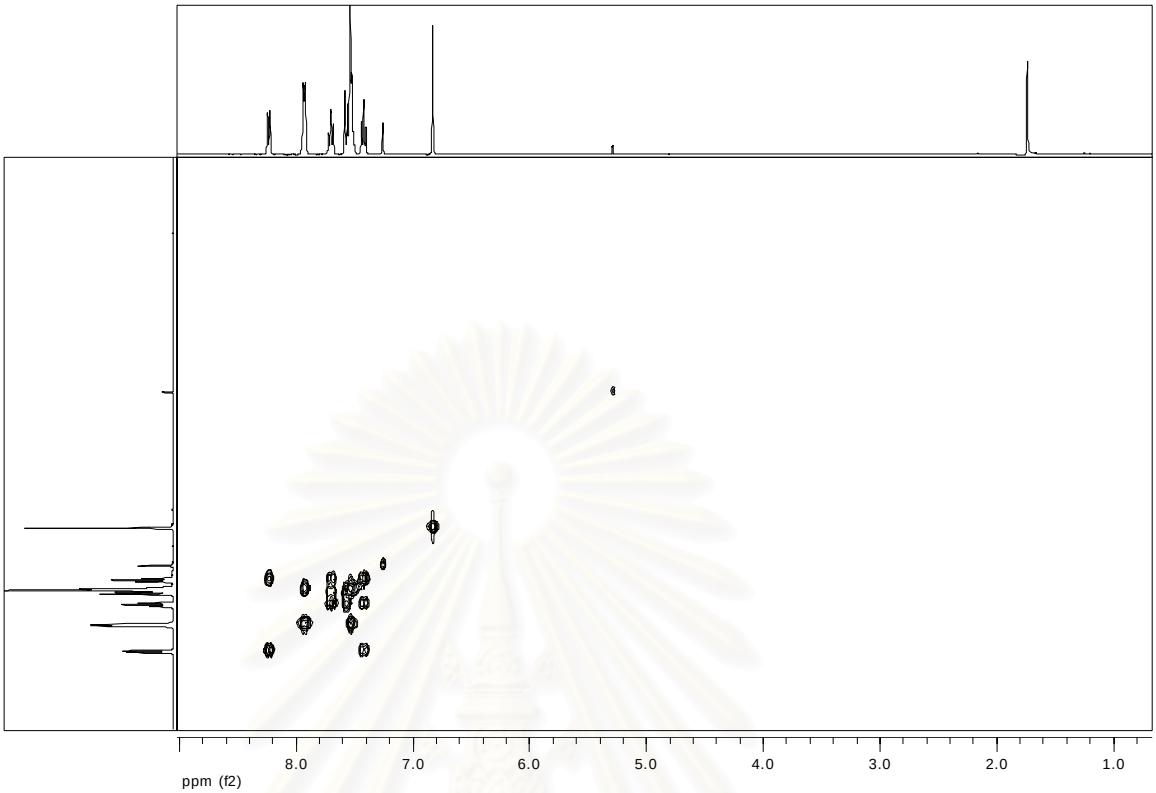

Figure C-3 COSY spectrum of of compound **3**

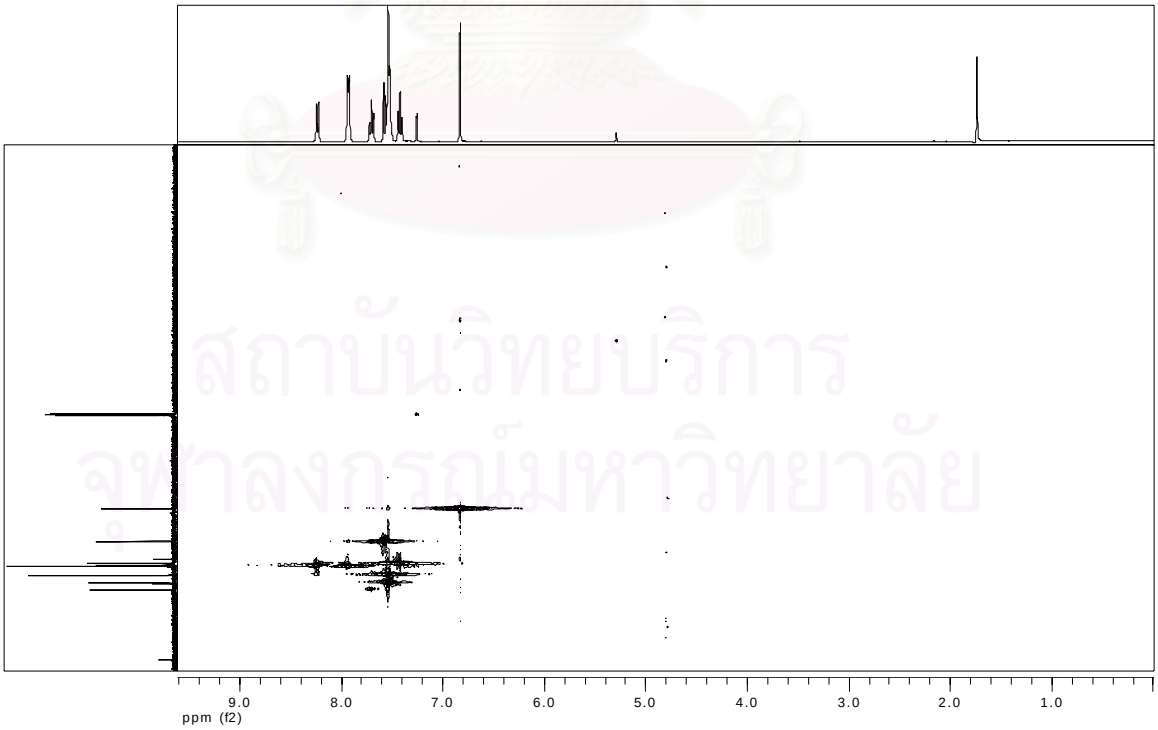

Figure C-4 HMQC spectrum of compound **3**

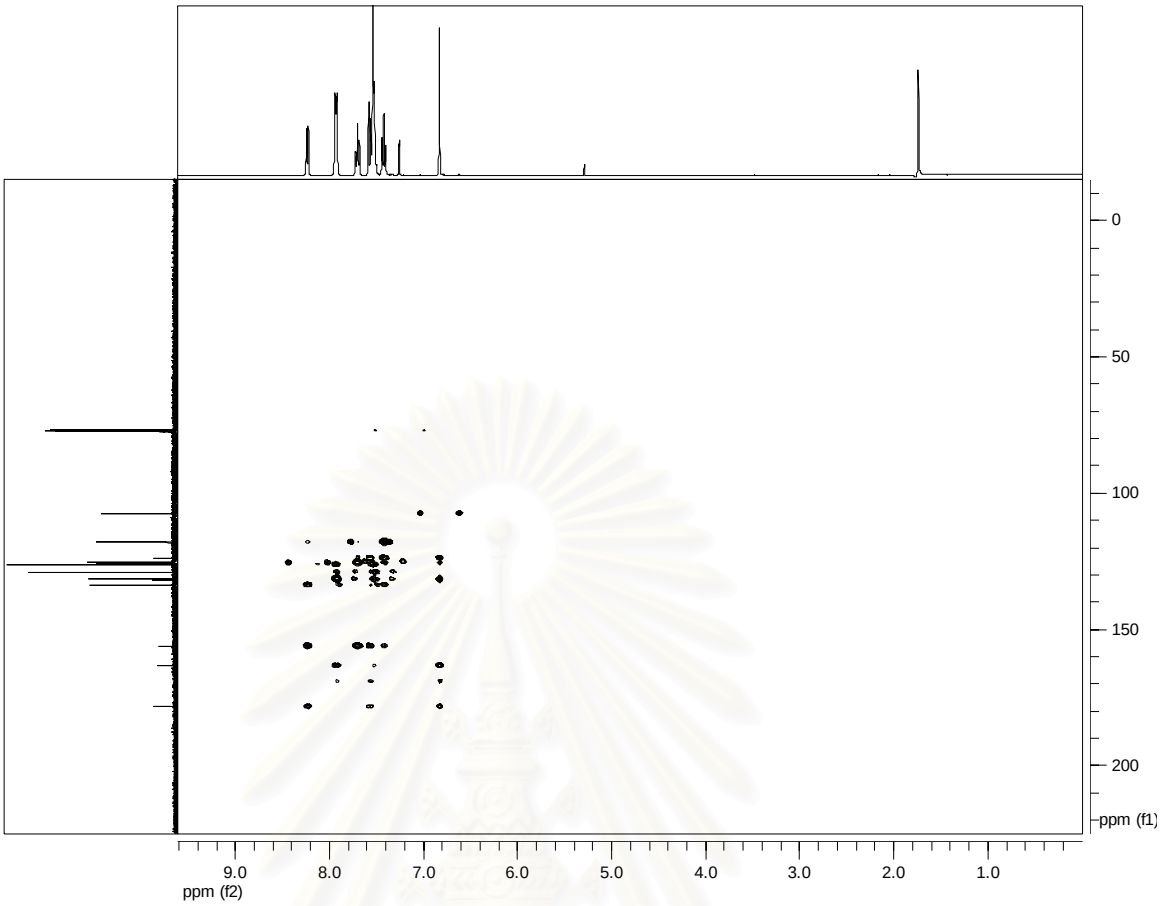

Figure C-5 HMBC spectrum of compound **3**

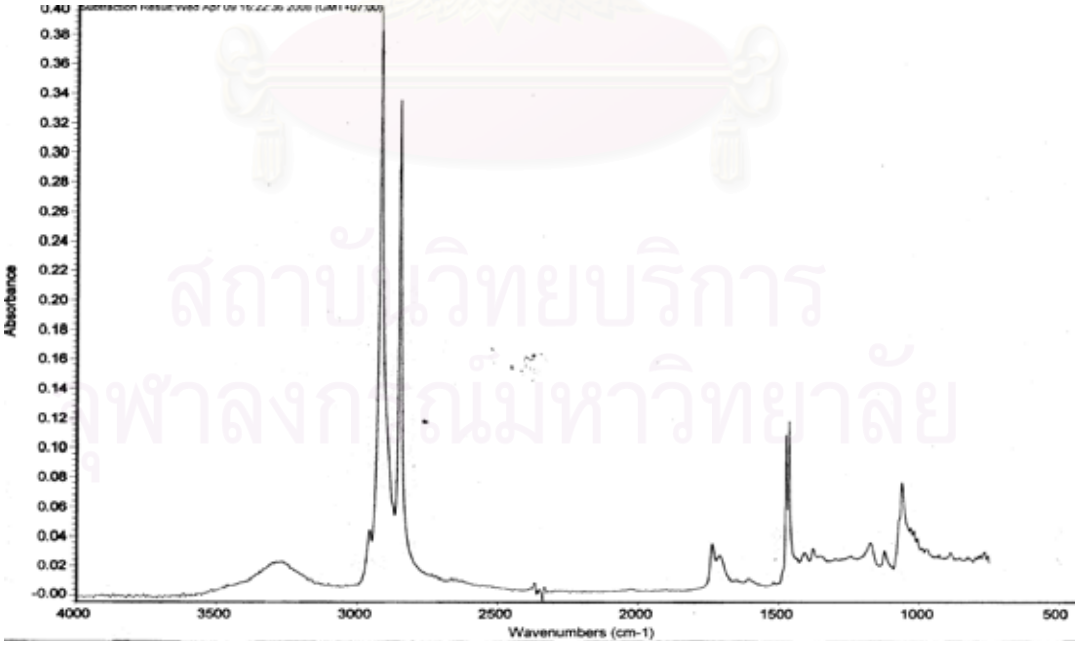

Figure D-1 The IR spectrum of substance **2**

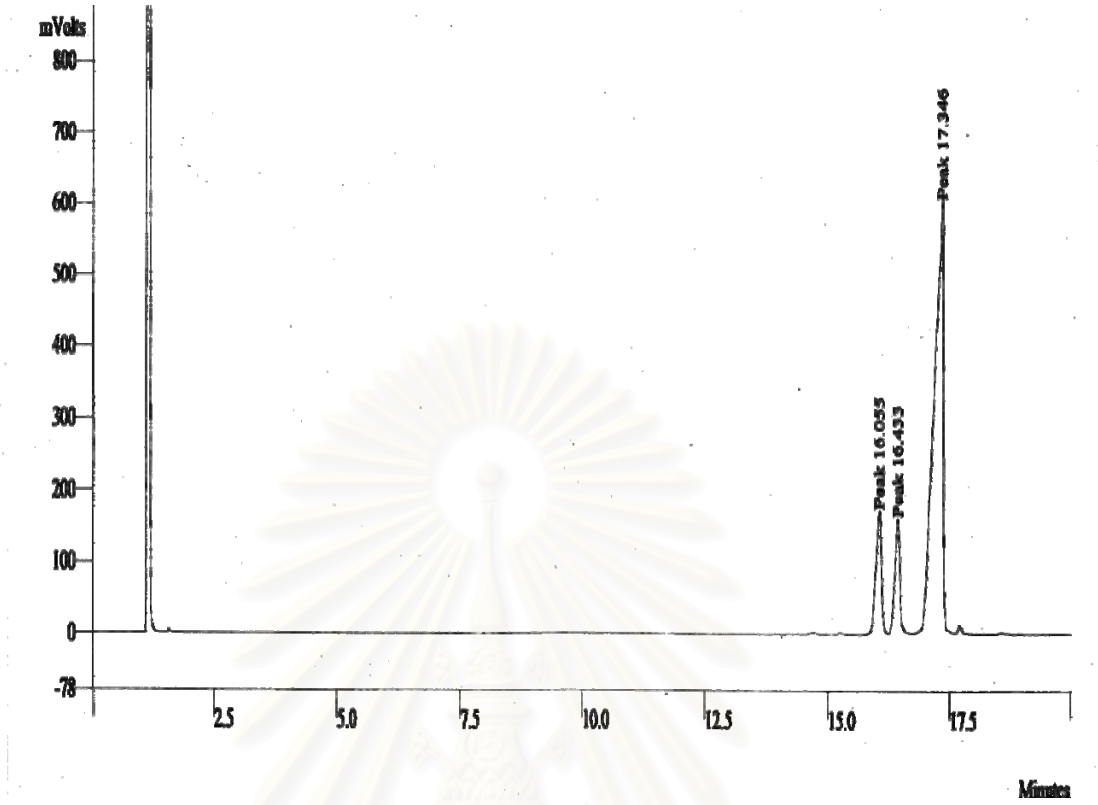

Figure E-1 The GC chromatogram of **F2411**

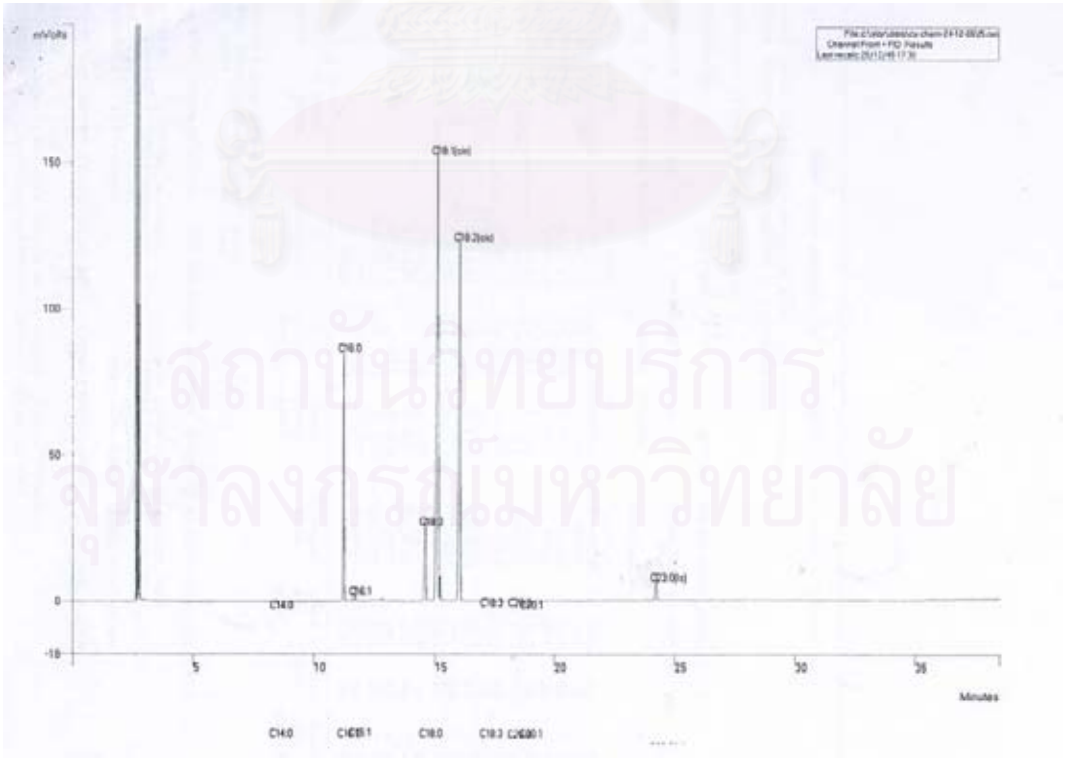

Figure F-1 The GC chromatogram of kernel oil

ANOVA-The extract of the leaves

Oneway 12-5-49 (1321-The extract of the leaves)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	38.224	3	12.741	15.075	.000
Within Groups	158.896	188	.845		
Total	197.120	191			

Post Hoc Tests

Homogeneous Subsets

clearzone

Duncan

treatment	N	Subset for alpha = .01	
		1	2
Me	48	1.0729	
DM	48		1.8854
Ex-DM	48		2.0938
Ex- Me	48		2.2188
Sig.		1.000	.095

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 48.000.

initial inoculum = 8.3175×10^{12} cfu

ANOVA -the extract of the brance

Oneway 31-3-49 (1321-the extract of the brance)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	140.368	3	46.789	119.926	.000
Within Groups	73.349	188	.390		
Total	213.717	191			

Post Hoc Tests

Homogeneous Subsets

clearzone

Duncan

treatment	N	Subset for alpha = .01		
		1	2	3
Me	48	.5000		
DM	48	.5000		
Ex-DM	48		2.0208	
Ex- Me	48			2.3646
Sig.		1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 48.000.

initial inoculum = 4.2×10^9 cfu

Oneway 2-5-49 (1321-The extract of the latex)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	681.639	3	227.213	240.717	.000
Within Groups	177.453	188	.944		
Total	859.092	191			

Post Hoc Tests

Homogeneous Subsets

clearzone

Duncan

treatment	N	Subset for alpha = .01			
		1	2	3	4
Me	48	.0000			
Ex-Me	48		.7396		
DM	48			2.4792	
Ex-DM	48				4.8958
Sig.		1.000	1.000	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 48.000.

initial inoculum = 7.04×10^{12} cfu

Oneway 27-5-49 (1321-the hexane extract of kernel oil)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	129.503	1	129.503	87.111	.000
Within Groups	139.745	94	1.487		
Total	269.247	95			

Post Hoc Tests

Post hoc tests are not performed for CLEARZON because there are fewer than three groups**.**

initial inoculum = 2.11×10^{12} cfu

Oneway 15-3-49 (1466-The extract of the leaves)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	30.484	3	10.161	59.991	.000
Within Groups	31.844	188	.169		
Total	62.328	191			

Post Hoc Tests

Homogeneous Subsets

clearzone

Duncan

treatment		Subset for alpha = .01		
	N	1	2	3
Ex-DM	48	1.1667		
DM	48		1.4063	
Me	48			1.9792
Ex- Me	48			2.1354
Sig.		1.000	1.000	.064

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 48.000.

initial inoculum = 8.725×10^{12} cfu

Oneway 5-4-49 (1466-The extract of the brance)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	242.057	3	80.686	99.946	.000
Within Groups	151.771	188	.807		
Total	393.828	191			

Post Hoc Tests

Homogeneous Subsets

clearzone

Duncan

treatment		Subset for alpha = .01		
t	N	1	2	3
Me	48	.5000		
DM	48		1.7292	
Ex-Me	48		1.8125	
Ex- DM	48			3.6458
Sig.		1.000	.650	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 48.000.

initial inoculum = 4.357×10^{11} cfu

Oneway 2-5-49 (1466-The extract of the latex)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	570.130	3	190.043	80.539	.000
Within Groups	443.615	188	2.360		
Total	1013.745	191			

Post Hoc Tests

Homogeneous Subsets

clearzone

Duncan

treatment		Subset for alpha = .01		
t	N	1	2	3
Me	48	.0000		
DM	48	.5000		
Ex- DM	48		1.5729	
Ex-Me	48			4.4479
Sig.		.112	1.000	1.000

Means for groups in homogeneous subsets are displayed.

a Uses Harmonic Mean Sample Size = 48.000.

initial inoculum = 2.54×10^{12} cfu

Oneway 2-7-49 (1466-The hexane extract of kernel oil)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	211.523	1	211.523	113.487	.000
Within Groups	175.203	94	1.864		
Total	386.727	95			

Post Hoc Tests

Post hoc tests are not performed for CLEARZON because there are fewer than three groups**.**

Initial inoculum = 2.75×10^{12} cfu

Oneway 2-7-49 (931- The hexane extract of the kernel oil)

ANOVA

clearzone

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	96.000	1	96.000	64.457	.000
Within Groups	140.000	94	1.489		
Total	236.000	95			

Post Hoc Tests

Post hoc tests are not performed for CLEARZON because there are fewer than three groups**.**

initial inoculum = 1.707×10^{12} cfu

VITAE

Miss Sutthiduean Chunhakant was born on November 22, 1979 in Pare province, Thailand. She graduated a Bachelor Degree of Science in Chemistry, from the Department of Chemistry, Phranakorn Rajabhat University , Bangkok, Thailand in 2002. She graduated in Master of Science in Biotechnology in 2007 from the program of Biotechnology, faculty of Science, Chulalongkorn University, Bangkok, Thailand. During the course of study, she obtained financial support from Graduate School Chulalongkorn University.