ISOLATION AND IDENTIFICATION OF BIOACTIVE PHYTOCONSTITUENTS AND THEIR BIOLOGICAL ACTIVITIES OF *LIMONIA ACIDISSIMA* L. (THEE)

PhD DISSERTATION

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MYANMAR

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ISOLATION AND IDENTIFICATION OF BIOACTIVE PHYTOCONSTITUENTS AND THEIR BIOLOGICAL ACTIVITIES OF *LIMONIA ACIDISSIMA* L. (THEE)

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THIS DISSERTATION IS SUBMITTED TO THE BOARD OF EXAMINERS IN CHEMISTRY, UNIVERSITY OF YANGON FOR THE DEGREE OF DOCTOR OF PHILOSOPHY.

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TO MY PARENTS

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ABSTRACT

Limonia acidissima L. (Thee) has invaluable effect on skin and enormous range of pharmacological activities. Although the efficacy of this bark extract on skin has been studied, no study has been done the skin whitening effect with the sunscreen lotions from the various extracts of this plant. Present study is designed to get the scientific proof of the sunscreen lotions from the herbal extract.

Sample collection and identification, preliminarily phytochemical investigation and elemental analysis by AAS method have been carried out. Some biological activities such as antimicrobial activity, acute toxicity and antioxidant activity of bark of *L. acidissima* (Thee) have been studied.

Nutritional values such as moisture content, ash content, protein content, fibre content, fat content, carbohydrate content and energy value of *L. acidissima* (Thee) were determined. The antimicrobial activity of PE, $CHCl_3$, MeOH, CH_3COCH_3, EtOAc, EtOH and watery extracts from bark of *L. acidissima* and MeOH, EtOAc and EtOH extracts from fruit pulp have been preliminarily screened on six microorganisms such as *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacilus pulmalis, Candida albicans* and *Escherichia coli.*

Acute toxicity of Thee bark was also studied by Litchfield and Wilcoxon method using mice models. From acute toxicity, it has been found that the 95 % ethanol extract of bark was free from toxic effect. Antioxidant activity test assessed by DPPH radical scavenging activity assay revealed ethanol extract of Thee bark possesses more radical scavenging activity than other extracts (PE and EtOAc) when compared to standard antioxidant, ascorbic acid (IC_{50} = 28.4 μg / mL).

Three isolated compounds were isolated from ethyl acetate extract of bark of *L. acidissima* (Thee) by thin layer and silica gel column chromatographic methods. The isolated compound I (xanthotoxin, 0.025% yeild), compound II (isopimpinellin, 0.025%) and compound III (marmesin, 0.005%) were identified by physicochemical determination and modern spectroscopic techniques such as UV, FT IR, ^{1}H NMR, ^{13}C NMR, HSQC, HMBC and HR ESI Mass spectrometry as well as by comparing with the reported data.

In addition, the isolated compound IV (auraptene) was also isolated from dichloromethane extract of Thee bark and purified by vacuum liquid column chromatography, flash column chromatography and high performance liquid chromatography. The isolated compound IV was identified by modern spectroscopic techniques such as ^{1}H NMR, ^{13}C NMR, HMBC, ESI mass spectrometry as well as comparing with the reported data.

The ethanol extract of bark and fruit pulp of *L. acidissima* (Thee) were formulated as sunscreen lotions. The skin irritation test was performed by using albino rats. The efficacy of sunscreen lotions was determined by UV visible spectrophotometer. Moreover, the skin whitening effect of sunscreen lotions (thee bark and fruit pulp) was studied by adopting the method reported by Yusutami *et al.*, 2004 with some modification.

Moreover, the effectiveness of the sunscreen lotions was evaluated using SPF. From the result, sunscreen lotions were proved to be nonirritant, possess SPF for normal skin.

Keywords : *Limonia acidissima*, xanthotoxin, isopimpinellin, marmesin, auraptene, antimicrobial, antitumor, antioxidant, irritation, sunscreen lotion, SPF

ABBREVIATIONS

i.e,	:	idest., that is
etc	:	et cetera, and other rings
viz	:	videlicet, namely
et al.	:	et alli, and others
°C	:	Degree Celsius
g	:	gram
mg	:	milligram
µg	:	microgram
mL	:	milliliter
cm	:	centimeter
cm^3	:	cubic centimeter
%	:	percent
hr	:	hour
m	:	meter
min	:	minute
UV	:	Ultraviolet
FT IR	:	Fourier Transform infrared spectroscopy
^1H NMR	:	Proton Nuclear Magnetic Resonance
^{13}C NMR	:	Carbon Nuclear Magnetic Resonance
^1H ^1H COSY	:	Proton-Proton Correlation Spectroscopy
HSQC	:	Heteronuclear Single Quantum Coherence
HMBC	:	Hetronuclear Multiple Bond Coherence

ESI MS	:	Electron Spray Ionizing Mass Spectrometry
$CDCl_3$	:	Deuterated chloroform
d	:	doublet
t	:	triplet
dd	:	doublet of doublet
s	:	singlet
m	:	multiplet
ppm	:	part per million
Hz	:	Hertz
MHz	:	Mega Hertz
ppt.	:	precipitate
b.wt.	:	body weight
v/v	:	volume by volume
ND	:	Not Detected
h	:	hour
min	:	minute
R_f	:	Rate of flow/ Retention factor
M	:	Molarity
DPPH	:	1,1-diphenyl-2-picryl hydrazyl
RSA	:	Radical Scavenging Activity
IC_{50}	:	50% Inhibitory Concentration
AA	:	Ascorbic Acid
g	:	gram
mg	:	milligram

kg	:	kilogram
µg	:	microgram
v	:	volume
w	:	weight
m.pt.	:	melting point
DW	:	Distilled Water
gp	:	group
CC	:	Column Chromatography
TLC	:	Thin Layer Chromatography
VLCC	:	Vacuum Liquid Column Chromatography
FCC	:	Flash Column Chromatography
HPLC	:	High Performance Liquid Chromatography
F	:	Fraction
λ_{max}	:	wavelength of maximum absorption
ν	:	stretching vibration
δ	:	in plane bending, chemical shift

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CHAPTER I

1. INTRODUCTION

1.1 Selected Medicinal Plants

Natural products are important in health care. They can be used as starting materials for semisynthetic drugs. The main examples are plant steroids, which led to the manufacture of oral contraceptives and other steroidal hormones. Today, almost every pharmacological class of drugs contains a natural product or natural product analog (Eba, 2005). Nearly 80% of the world's population relies on traditional medicines for primary health care, most of which involve the use of plant extracts (Akindele and Adeyemi, 2007).

The blind dependence on synthetics is over and people are returning to the naturals with hope of safety and security. Also the development of adverse effect and high microbial resistance to the chemically synthesized drugs, has forced men into ethnopharmacognosy. More so, in our local situation, degree of ignorance and illiteracy has forced many to abandon or neglect pharmaceutically formulated drugs in favour of locally prepared herbal remedies coupled with the fact pharmaceutical products are increasingly being faked. Thus, the herbal products today symbolize safety in contrast to the synthetics that are regarded as unsafe to human and environment (Bukke, 2011).

Herbs are staging a comeback, herbal "renaissance" is happening all over the globe and people returning to the naturals with hope of safety and security. The use of herbal medicines continues to expand rapidly across the world. By and large, the public is gradually drifting towards acceptance and usage of herbal preparations (Adotey *et al.,* 2012).

Throughout the ages, humans have relied on nature for their basic needs for the production of food-stuffs, shelters, clothing, means of transportation, fertilizers, flavours and fragrances, and, not the least, medicines. Plants have formed the basis of sophisticated traditional medicine systems that have been in existence for thousands of years and continue to provide mankind with new remedies. The vast majority of

people on this planet still rely on their traditional material medica (medicinal plants and other materials) for their everyday health care needs (Qureshi *et al.,* 2010).

It is also a fact that one quarter of all medical prescriptions are formulations based on substances derived from plants or plant-derived synthetic analogs, and according to the WHO, 80% of the world's population-primarily those of developing countries rely on plant-derived medicines for their healthcare. It is likely that the profound knowledge of herbal remedies in traditional cultures developed through trial and error over many centuries, and that the most important cures were carefully passed on verbally from one generation to another. People who use traditional remedies may not understand the scientific rationale behind their medicines, but they know from personal experience that some medicinal plants can be highly effective if used at therapeutic doses. Since we have a better understanding today of how the body functions, we are thus in a better position to understand the healing powers of plants and their potential as multi-functional chemical entities for treating complicated health conditions. Medicinal plants typically contain mixtures of different chemical compounds that may act individually, additively or in synergy to improve health (Qureshi *et al.,* 2010).

Biological organisms particularly plants produce two distinctly different types of chemical products. The first type, primary metabolites, which consists of compounds such as sugars and proteins that are common to most organisms and are essential for functional metabolism. Secondary metabolites, on the other hand, are chemicals unique to a single species or related group of organisms. These chemicals can function as communications tools, defense mechanisms or sensory devices (Swerdlow, 2000).

The biological activity of these chemicals is beneficial to the organism that produces them, but it is often harmful to other species, including humans. This toxicity can adversely affect the functions of the entire human body or only a species biological process, such as growth of cancer cells. In this way, certain foreign, naturally produced chemicals can acts as power. Also, many beneficial biological activity such as anticancer, antimicrobial, antioxidant, antidiarrheal, analgesic and wound healing activity of plants have been reported. In this way, certain foreign,

naturally produced chemicals can act as powerful drugs when administered at the proper concentration. Natural products have been used by native cultures as a source of remedies for thousands of years, dating back to ancient empires in Egypt, China, Greece and Rome (Adotey *et al.*, 2012).

It is a fact that traditional systems of medicine have become a topic of global importance. Although modern medicine may be available in many developed countries, people are still turning to alternative or complementary therapies including medicinal herbs. Yet, few plants species that provide medicinal herbs have been scientifically evaluated for their possible medicinal applications. Similarly, the herbal drugs contain many chemical compounds naturally. In many cases, traditional healers claim the good benefit of certain natural or herbal products. But, it is only a few herbs, their extracts and active ingredients and also, the preparation containing them that their safety and efficacy data are available (Adotey *et al.*, 2012).

No dought, plants extracts either as pure compounds or as standardized extracts, provide unlimited apportunities for new drug discoveries because of the unmatched availability of chemical diversity (Cosa *et al.*, 2006).

As such, it is therefore essential to separate out those compounds which are responsible for therapeutic effect and characterize them. They are called active constituents or principles. Phytochemical screening is very important in identifying new sources of therapeutically and industrially important compounds such as alkaloids, flavonoids, phenolic compounds, saponins, steroids, tannins, terpenoids etc (Akindele and Adeyemi, 2007).

Also, isolation is a part of natural product chemistry, through which it is possible to separate different components and biologically active ones which can be incorporated as ingredients in the modern system of medicine. Modern medicine has largely confined itself to the isolation or synthesis of single active ingredient for the treatment of specific disease (Shoge, 2010). Chromatographic techniques are widely used for the separation, isolation and purification of chemical constituents from natural drugs (Devi *et al.*, 2012).

Many plants are chemically very variable depending on the locality where they are found with some of the constituents occurring only at certain seasons of the year (Adelani, 2007).

In the present works, the medicinal plant *Limonia acidissima* L. (Thee) belonging to the family of *Rutaceae* was chosen to study some of their chemical constituents and pharmacological activities.

Medicinal plants have played a vital role in maintaining and improving human health from past thousands of years. History of human civilization and discovery of herbal medicines are running parallel from ancient time till date. Among medicinal plants, *L. acidissima* (Thee) is used in several systems of medicine for a variety of ailments and has a significant role in promoting health and alleviate illness. *L. acidissima* (Thee) belonging to family *Rutaceae* synonomically *Feronia limonia swingle* L. and commonly also called as wood apple.

L. acidissima (Thee) has invaluable effect on skin and enormous range of pharmacological activities like antidiabetic, antimicrobial, antiviral, antifungal, antitumor, CNS depressant activities and wound healing etc.

1.2 *Limonia acidissima* L. (Thee)

Family : *Rutaceae*

Genus : *Limonia*

Species : *L. acidissima*

Botanical Name : *Limonia acidissima* L.

Myanmar Name : Thee

1.2.1 Botanical description and distribution of *L. acidissima* (Thee)

Thee is an aromatic, slow growing deciduous tree 9 m tall grows all over India in dry and warm areas up to 450 m elevation. Often polygamonoecious tree with rough, spiny bark. The spines are axillary, short, straight, 2-5 cm long on some of the zigzag twigs. The leaves are deciduous, alternate, dark-green, leathery, 3-5 inch long. Small fragnant light red flowers 1-2 inch wide are borne in small, loose, terminal or lateral panicles. The fruit is berry round to oval, globose, large, 2-5 inch wide, with a hard, wood rind, which is grayish-white, scurfy rind about 6 mm thick. The pulp is sticky brown, aromatic odorous, resinous, astringent, acid or sweetish, white seeds scattered through it. The wood- apple is native and common in the wild in the dry plains of India and Ceylon and cultivated along roads and edges of fields and occasionally in orchards. It is also frequently grown throughout Southeast Asia, in northern Malaysia and on Penang Island. In India, the fruit was traditionally a "poor man's food" until processing techniques were developed in the mid- 1950's. Naturally grow of *L. acidissima* (Thee) is at the upper portion of Myanmar, we can call at dry zone. Shwebo, Mandalay, Magway, Pakokku and Monywa areas are the famous (Kumawat *et al.*, 2012).

The tree grow up to elevation of 1,500 ft (450 m) in the western Himalayas. It is said to require a monsoon climate with a distinct dry season. Throughout its range there is a diversity of soil types, but it is best adapted to light soils. The wood-apple is generally grown from seeds, seedlings will not bear fruit until at least 15 years old. Multiplication may also be root cuttings, air-layers, or by budding onto self-seedlings to include dwarfing and precociousness. In Malaysia, the leaves are shed in January, flowering occurs in February and March, and fruit matures in October and November. In Indian, the fruit ripens from early October through March. The fruit is tested for maturity by dropping onto a hard surface from a height of 1ft (30 cm). Immature fruits bounce, while mature fruit do not. After harvest, the fruit is kept in the sun for 2 weeks to fully ripen (Morton, 1987) (Figure 1.1).

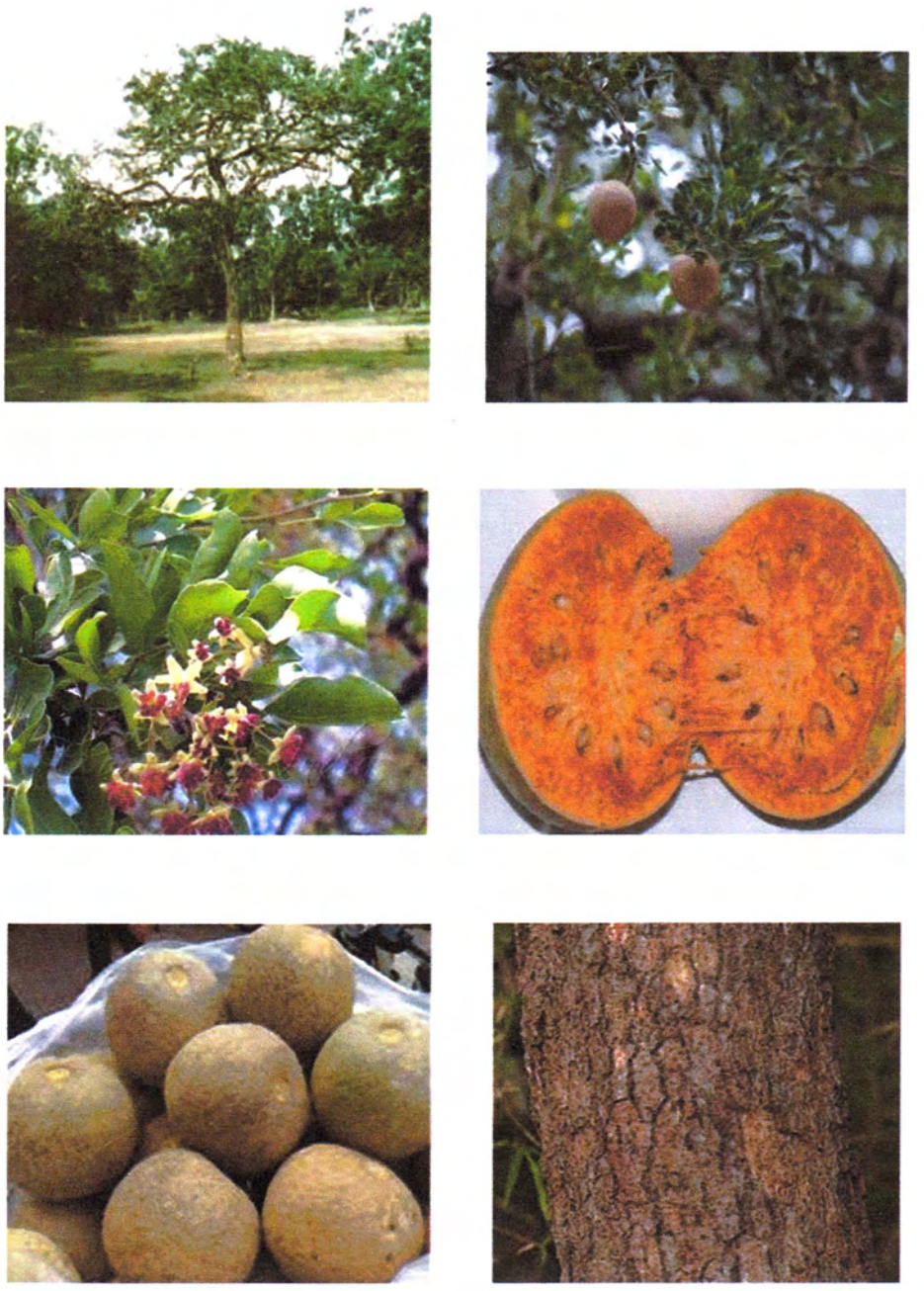


Figure 1.1 Photograph of *L. acidissima* (Thee)

1.2.2 Uses

The rind of the fruit is so thick and hard it can be carved and used as a utensil such as a bowl or ashtray. The bark also produces an edible gum. The tree has hard wood which can be used for woodworking.

Fruit pulp has a soap-like action that made it a household cleaner for hundreds of years. The sticky layer around the unripe seeds is household glue that also finds use in jewelry- making. The glue, mixed with lime, waterproofs wells and cement walls. The glue also protects painting when added as a coat on the canvas.

The fruit is eaten plain, blended into an assortment of drinks and sweets, or well-preserved as jam. The scooped-out pulp from its fruit is uncooked with or without sugar, or is combined with coconut milk and palm-sugar syrup and drunk as a beverage, or frozen as an ice cream. It is also used in chutneys and for making fruit preserves jelly and jam. Indonesians eat the pulp of the ripe fruit with palm sugar and eat the mixture at breakfast. The sugared pulp is a foundation of sherbet in the subcontinent. Jam, pickle, marmalade, syrup, jelly, squash and toffee are some of the foods of multipurpose fruit. Young leaves are a salad green in Thailand. Indians eat the pulp of ripe fruit with sugar or jiggery. The ripe pulp is also used to make chutney. The raw pulp is varied with the yoghurt and make into rata. The raw pulp is bitter in taste, while the ripe pulp would be having a smell and taste that's a mixture of sourness and sweet.

Ground *L. acidissima* bark is also used as a cosmetic called Tha-nat-khar in Southeast Asia. The fruit rind yields oil that is popular as a fragrance for hair, it also produces a dye used to color silks and calico.

Traditionally, the constituents (in paste form) from the stem bark of *L. acidissima* is mixed with water and applied mainly to the face. It may be used to remove small spots or lesions which appear on the skin. Myanmar people frequently use the bark of *L. acidissima* (in paste form) as a substitute for Tha-nat-khar (a famous cosmetic bark). It is recognized to be great help to bear the heat of sun and thus an ideal cosmetic for those who have to work under direct sunshine. Women who work in paddy fields always wear thick layers of Tha-nat-khar to help themselves tolerate the intense heat of the sun. "Thee" paste also has the same properties as Tha-

nat-khar. It is believed that the regular application on the skin helps to keep skin cool, smooth, fair and well-textured complexion. It also cures pimples and acne. It is also known to be protecting against skin cancer by blocking UV rays. The term "cosmeceutical" is a category of cosmetic products claimed to have biologically active ingredients with medicinal or drug like benefits. Moreover, they satisfy the needs of beauty and health. Many substances, either chemically synthesized or extracted from plants or animals, can be used as functional ingredients (Kilgman, 2000).

Cosmeceuticals are intended to carry out their functions as protection, whitening, tanning, anti-wrinkling, deodorants, anti-aging and nail and hair care. Cosmeceutical may, however, cause the unwanted problems. The common ones are irritability to the skin, contact dermatitis, photosensitivity, comedoge nicety, hair and nail damage, hyper or hypo pigmentation infectivity, carcinogenicity and even systemic adverse effects. Popularity of herbal cosmetics in society and technological advances in manufacturing process has resulted in flooding of market with herbal formulations. Natural substances extracted from plants have recently been considered as potential sunscreen resources owing to high ultraviolet ray absorption and anti-oxidant activity. The decrease in the intensity of UV radiation reaching the skin through sunscreen may reduce the risk of sun induced skin cancer. Now herbal cosmetics have gained much recognition and become popular among people. These products claimed to have efficacy and intrinsic acceptability due to routine use in daily life and devoid of side effects commonly seen with synthetic products.

All parts of the *L. acidissima* tree are medicinally useful. Literature in Indian traditional medical systems like Ayureda, Siddha and Unani were prescribed this as an Indian folk medicine which has much potential information its therapeutic uses. In Ayureda, *L. acidissima* is used as a folk medicine for renitent fever, puerperal fever, lightening of skin, diarrhea, ulcer, inflammation, skin irritation, dyspepsia, diabetes and many other diseases (Merinal *et al.*, 2012).

L. acidissima has traditionally been used in many herbal remedies such as digestive, stimulant, astringent, carminative and as an anti-diarrheal. All the parts of the plants are prescribed in indigenous system of medicine for the treatment of

various ailments. The unripe fruits are sour, aromatic, astringent, constipating, alexipharmic and are useful in diarrhea, pruritus and pharyngodynia. The unripe fruit is alexipharmic, astringent to the bowels and removes itching of the body. Useful in whooping cough.

The ripe fruits are considered as sour, sweet, acrid, with flavor and taste; difficult to digest; refrigerant, aphrodisiac, alexipharmic; cures cough, dysentery, heart diseases, vomiting; removes biliousness, and blood impurities, fatigue, thirst, hiccough; good for throat, asthma, consumption, tumours, opthalmia, leucorrhoea, the juice put in the ear cures earache. The fruits are cardio tonic, tonic to the liver and the lungs, astringent and binding, diuretic, strengthening the gums; the juice is good for stomatitis, and sore throat; useful in biliousness; topically it relieves pain due to stings of wasps and other insects. They are beneficial in scurvy and sore throat. Fruit pulp is sour, sweet, edible stomachic, stimulant and astringent. The pulp is applied externally as a remedy for the bites of venous insects. Pulp with honey and pipil is given for hiccup and difficulty of breathing. Pulp is used for affections of gums and throat and to tone the breast. The leaves are aromatic and carminative, and are prescribed in the indigestions and slight bowel affections of children. The juice of leaves given to the children suffering from stomach troubles (Qureshi *et al.*, 2010).

Leaves, bark, roots and fruit pulp are used against snakebite. The bark is chewed with that of *Barringtonia* and cardiac tonic, in diarrhea and dysentery, in effective treatment for hiccough, in sore throat and diseases of the gums. The pulp is poultice onto bites and strings of venomous insects. Mixture of young leaves juice, milk and candy is given as a remedy for biliousness and intestinal troubles of children. Fruits, leaves and stem bark of *L. acidissima* have been studied for anti-tumour, larvicidal and antimicrobial activity. Fruit pulp showed anti-inflammatory, antipyretic and analgesic activity and leaves of *L. acidissima* showed anthelminitic activity (Intekhab *et al.*, 2009).

1.2.3 Chemical constituents of *L. acidissima* (Thee)

The different parts of the plant have been investigated by several workers and found to contain coumarins, furocoumarins, lignans, alkaloids, steroids and flavonoids. The unripe fruits contain stigmasterol. Root bark yielded osthol, geranyl umbelliferone, marmin, marmesin, aurapten, bergapten, isopimpinellin and feroil. The heart wood contains ursolic acid and a flavanone glycoside 7-methyl porial-β-d-xylopyranosyl-d-glucopyranoside. The stem bark of *L. acidissima* yielded flavonone, alkaloids, coumarins, lignin, sterols and triterpene. Psoralen, bergapten, orientin, vitexin and saponarin have been isolated from leaves were reported by Intekhab *et al.*, 2009.

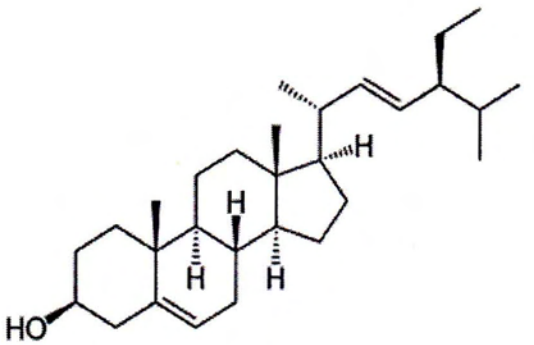
The fruit contains fruit acids, vitamins and mineral. The dried pulp contains 15 % of citric acid, and a small quantity of deliquescent ash consisting of potassium, calcium and iron salt. Seeds and fruits contained oil and protein; oil composed of palmitic, oleic, linoleic and linolenic acids besides traces of palmitoleic and stearic acids; β-sitosterol, β-amyrin, lupeol and stigmasterol from unsaponifiable matter of seed oil. An acidic heteropolyssacharide has been isolated from the ripe fruit which shows antitumour activity against ascites carcinoma cell growth. Three volatile flavor components are obtained from fresh wood apple fruit; they are namely methyl hexanoate, ethyl 3-hydro hexanoate, and butanoic acid. The acid- insoluble fraction of the ethereal extract of the dry whole unripe fruit gives stigmasterol (Ghosh *et al.*, 1994).

The leaves and stems contain the coumarins, luvangetin, xanthotoxin and marmesin; the triterpenoids, lupeol and limonin; and the steroids, sitosterols-O-β-D-glucoside, anisic acid isolated from leaf essential oil as well as methylchavicol, trans-anethole, thymol and p-cymen-7-ol. Estragole, trans-anethole and cis-anethole also determined in leaf oil. The leaves after hydrodistillation yielded an essential oil (0.4%). The essential oil from leaves has been found rich in methyl chavicol, linalool, caryophyllene, cis-amethole, p-methoxy phenyl-2-propanone, elemicine, 3, 4-dimethoxy benzaldehyde and alcohol were reported (Gupta *et al.*, 2009).

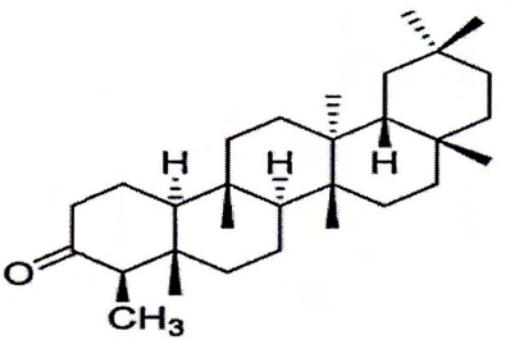
The stem bark yielded 5,3-dihydroxy-4-methoxy-6,6-dimethyl chromeno-flavone along with several known compounds including an alkaloid, five coumarins, a

flavonone, a lignin, three sterols and triterpene. The root and root bark were found to contain amino acids, phenolic compounds, sterol and tannins in addition to the alkaloid (Talapatra, 1973). Among the amino acids, phenyl alanine, tyrosine and cysteine are present. Maltose and inositol are the sugars present. Phenolic compounds like p-cresol and stigma sterols were also detected. Aurapten, bergapten, isopimpinellin, 6-methoxy-7-geranyloxycoumarin and marmesin isolated from root bark. Roots yielded geranylumbelliferone, bergapten, osthol, isopimpinellin, xanthotoxin, marmesin and marmin were also reported (Qureshi *et al.*, 2010).

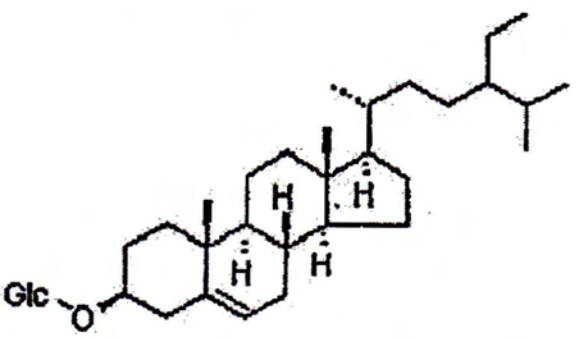
The bark mainly contains marmesin, bergapten, psoralen, acidissima, (-)-5′, 3′-dihydroxy -4′-methoxy-6′, 6′-dimethoxy chromeno-(7,8,2′,3′)-flavonone, alkaloid, coumarins (luvangetin, xanthotoxin and marmesin), lignin, steroids (sitosterol and sitosterol-o-beta-d-glucoside) and triterpenoid (lupeol and limonin) were also reported by Kumawat *et al.*, 2012. The chemical constituents of *L. acidissima* (Thee) were shown in Figure 1.2.



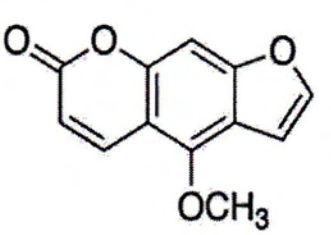
Stigmasterol



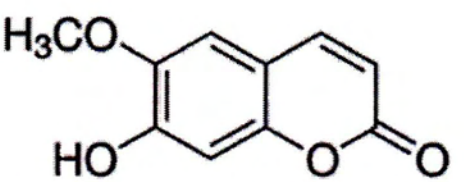
Friedelin



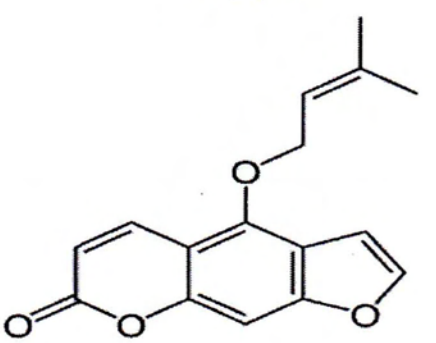
β-sitosterol-3-O-β-D-glucopyranoside



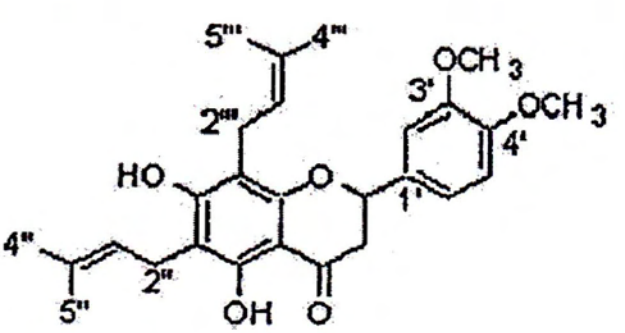
Bergapten



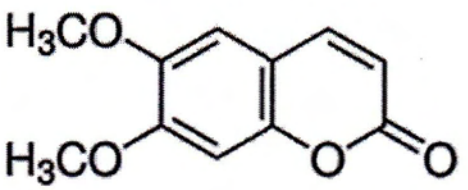
Scopoletin



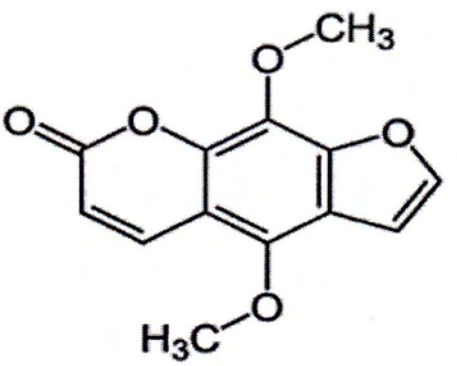
Isoimperatorin



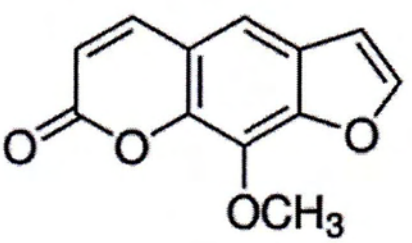
5,7-dihydroxy-3′,4′-dimethoxy-6,8-di(3-methylbut-2-enyl) flavone



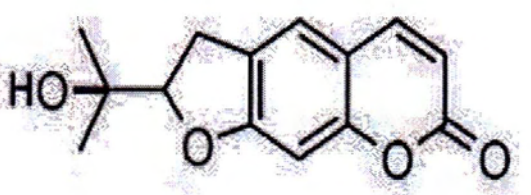
6,7 - Dimethoxycoumarin



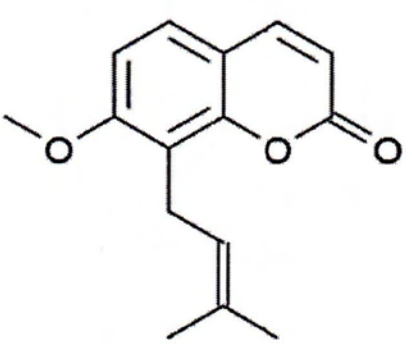
Isopimpinellin



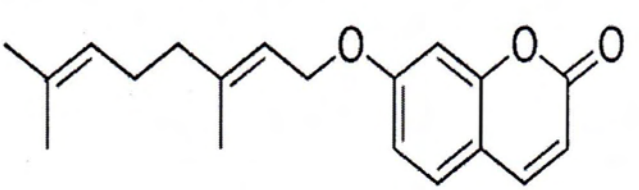
Xanthotoxin



Marmesin



Osthol



Auraptene

*** Syamasunda *et al.*, 2012**

Figure 1.2 The chemical constituents of *L. acidissima* (Thee)

1.3 Antimicrobial Activity

Antimicrobial activity is that a substance or an extract produced by a living thing inhibits the growth and activity of various pathogenic microbes. Although the chief sources come from moulds, anti-nomycetes and bacteria, higher plants often show antimicrobial activities.

1.3.1 Bacteria

Bacteria are prokaryotic cells whose single chromosome is not contained within a nuclear membrane. The bacterial cytoplasm is surrounded by a plasma membrane. The cytoplasm contained RNA and chromosome contains DNA. No mitochondria are present.

Some species of bacteria which after gram's strain retain the violet iodine combination and appear violet are called gram-positive, while those which yield it to the alcohol and turned pink or red are called gram-negative (Monica, 1984).

1.3.2 Fungi or moulds

Fungi or Moulds are multicellular microorganisms. Some moulds can produce antibiotics such as penicillin group of moulds. Many species of moulds are capable of producing toxins, referred to mycotoxins, during spoilage or growth in food. Mycotoxins can cause liver, kidney and central nervous disorders in humans and other animals (Frazier, 1967).

1.3.3 Classification of bacteria

Bacteria are classified according to their shape and structure. Spherical or oval bacteria are called cocci; rod shape or cylindrical – bacilli; comma shaped – vibrios; chains – strepto; clusters – staphylo, non-flexuous, spiral – spirllia; flexuous, spiral – spirochetes and filamentous and branching – Actinomycetas (Monica, 1984).

1.3.3.1 *Bacillus* Species

Genus *Bacillus* with the various species is widely distributed in nature. *Bacillus* genus comprises a large group of gram positive, aerobic, spore-forming rods that are abundant in soil and are commonly found as laboratory contaminants. They are gram positive and are divided into two classes based on oxygen requirement. *Bacillus* species are catalase positive, most are motile, some produce capsule, and some are thermophilic. *Bacillus subtilis* is one of the commonest of non-pathogenic aerobic spore formers. It is found in dusty places everywhere and especially in hay. *Bacillus pumilus* is most resistant to gamma radiation. They are found in plants. It is common contaminant of culture media (Finegold and Martin, 1982).

1.3.3.2 *Staphylococcus aureus*

Staphylococci are gram-positive spherical bacteria that occur in irregular cluster, the individual cells being approximately 1 μ in diameter. They can be isolated from air, dust, water, soil, human and animals sources. *Staphyloccus aureus* can grow at a temperature range of 15 to 45°C.

1.3.3.3 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa are gram-negative bacteria and non-sporing *bacillus*, measuring 1.5 μ , actively motile by virtue of polar flagella which rarely numbers more than three, many strains are monotrichoirs, non-capsulate and some strains are fimbirate. Essentially aerobic but a few strain grow, although poorly, under an aerobic conditions, the temperature range from 5° - 43° C, while the optimum 37°C. *Pseudomonas aeruginosa* occur widely in nature in soil, water, plants and animals. They grow on ordinary media, producing a musty odour like trimethylamine (Cruickshank, 1975).

1.3.3.4 *Candida albicans*

Candida albican is a genus of yeast that can cause fungal infections in human and other animals. It grows in the medical laboratory as large, round, white or cream colonies on agar plates, which reproduce by budding. *Candida albicans* is the only important pathogenic species in the genus *Candida. Candida albicans* organisms grows partly as spherical or oval yeast cells, 2.5 – 4.0 μ in diameter which reproduces by budding. It is a common cause of acute and sub acute infection in man, animals and birds. In man, *candida albicans* produce superficial infections of the skin and mucous membranes. It is found on the bedding of patients and in the air, and it may be spread by contact or by air borne infected dust (Soltys, 1963).

1.3.3.5 *Escherichia coli*

Escherichia coli are a gram-negative, rod-shaped bacterium that is commonly found in the lower intestine of warm-blooded organisms. *Escherichia coli* are a short plump rod about 2 to 3 μ long and about 0.6 μ in breadth. Coccoid and long filamentous forms are occasionally seen. Some strains produce a definite capsule. Most of the strains are motile, although in some cases motility may be extremely difficult to detect. The organisms are gram-negative and strain uniformly with the usual aniline (Soltys, 1963). Table 1.1 showed classification of microorganisms and their related diseases.

Table 1.1 Classification of Microorganisms and their Related Diseases *

No.	Microorganisms	Types	Effects
1	*Bacillus subtilis*	Bacteria	Food poisoning
2	*Staphylococcus aureus*	Bacteria	Skin infections, respiratory tract infections, food poison pneumonia, urinary infection meningitis
3	*Pseudomonas aeruginosa*	Bacteria	Respiratory infections, eye infection, skin infections, bone and joint infections
4	*Bacillus pumilus*	Bacteria	Eye infection, food poisoning
5	*Escherichia coli* (E-coli)	Bacteria	Dysentery, urinary tract infection, body diarrhoea intestinal disease
6	*Candida albican*	Fungus	Skin rashes, can infect both oral and vaginal cavaties, causes itching and discomfort

* (Cruickshank, 1975)

1.4 Acute Toxicity Study

Toxicology is the science of harmful effects of chemicals or drugs on living organism or is the science dealing with poison. To know the harmful effects of a new chemical or drug, toxicity test must be done the potential toxicity of new chemicals or drugs must be evaluated first on the laboratory animals.

The principle purpose for conducting toxicity test on animals is to evaluate the nature and the degree of harmful effects or deaths. Animal toxicity test also prevent distinctly harmful agents from becoming readily available to man.

There are three basic types of toxicity test on animals for the purpose of detecting toxic effect from the drugs. They differ primarily in their duration. They are acute test (single dose, 24 – hours test and survivors followed for 7 days), subacute or prolonged test (daily dose for 3 months), and chronic test (daily dose for 1 – 2 years).

Acute toxicity test is a single test to determine the symptoms consequent to administration of the test agent and to determine the LD_{50} of that agent. The route of administration selected on animal would be intended route for its administration to human.

Acute toxicity describes the adverse effects of a substance that result either from a single exposure or from multiple exposures in a short space of time (usually less than 24 hours). To be described as acute toxicity, the adverse effects should occur within 14 days of the administration of the substance. Acute toxicity is distinguished from chronic toxicity, which describes the adverse health effects from repeated exposures, often at lower levels, to a substance over a longer time period (months or years). It is widely considered unethical to use humans as test subjects for acute (or chronic) toxicity research. However, some information can be gained from investigating accidental human exposures (e.g., factory accidents). Otherwise, most acute toxicity data comes from animal testing or, more recently, *in vitro* testing methods and inference from data on similar substances (Walum, 1998).

1.5 Antioxidants

Antioxidants are substances that retard oxidation by atmospheric oxygen at moderate temperatures (auto oxidation). They are sometimes referred to simple as inhibitors. An important characteristic of antioxidants is their great effectiveness in very low concentrations and they significantly inhibit or delay oxidative processes. Free radicals damage the cells in our bodies and an imbalance of free radicals causes oxidative stress which can cause grave disturbances in cell metabolism.

Although there are several enzyme systems within the body that scavenge free radicals, the principle micronutrient antioxidants are vitamin C and vitamin E. Antioxidant reduce the effect of dangerous oxidants by binding together with these harmful molecules, decreasing their destructive power.

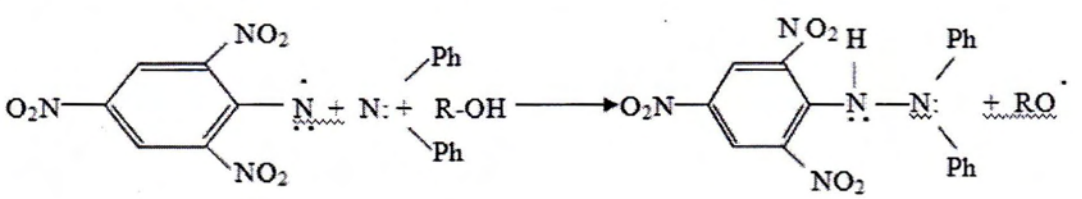
Antioxidant can also help to repair damage already sustained by cells. Our bodies gain antioxidants from two sources;

(i) From body production

(ii) From consumption of plants (fruits, vegetables, nuts and grains)

As we grow older our body's defense system loses its effectiveness and we hold fewer antioxidants. So, to reduce the damage to our bodies causes by free radicals. Antioxidant compounds like flavonoids, organic acid, ascorbic acid and some polyphenols. The delay or inhibit cellular damage mainly through their free radicals scavenger property (Soares, 1997).

The mechanism of free radical scavenging of antioxidant is as follow:



DPPH (purple) Antioxidant DPPH-H (yellow) Radical

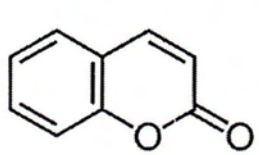
1.6 Coumarin

Coumarins have been identified from a number of natural sources, especially green plants. Coumarins are found free or as heterosides in many dicotyledons families, including the Asteraceae, Fabiaceae, Rosaceae, Rubiaceae and Rutaceae. The important major constituents that contributed to these samples are aurapten, bergapten, isopimpinellin and other coumarins.

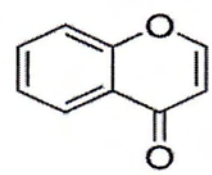
Coumarin is a fragrant organic chemical compound in the benzopyrone chemical class, which is a colorless crystalline substance in this standard state. It is a natural substance found in many plants. Most coumarins occur in higher plants, with the richest sources being the Rutaceae and Umbelliferae. Although distributed throughout all parts of the plants, the coumarins occur at the highest levels in the fruits, followed by the roots, stems and leaves. Environmental conditions and seasonal changes can influence the occurrence in diverse parts of the plant (Keating *et al.*, 1997).

The benzopyrones are a group of compounds whose members include coumarins and flavonoids. Dietary exposure to benzopyrones is quite significant, as these compounds are found in vegetables, fruits, seeds, nuts, coffee, tea and wine. It is estimated that the average western diet contains approximately 1 g/ day of mixed benzopyrones. It is, therefore, not difficult to see why extensive research into their pharmacological and therapeutic properties is underway over many years. Coumarin is a natural substance that shown anti-tumour activity *in vivo*, with the effect believed to be due to its metabolites (eg. 7-hydrocoumarin).

Coumarin is classified as a member of benzopyrone family compounds all of which consist of a benzene ring joined to a pyrone ring the benzopyrone can be subdivided into the benzo-α-pyrone to which the coumarins belong and the benzo-γ-pyrone, of which the flavonoids are principal members (Lacy *et al.,* 2004)



α-benzopyrone



γ-benzopyrone

Coumarins are a group of plant-derived poly phenolic compounds. They belong to the benzopyrone family and possess a wide range of pharmaceutical applications including anti-inflammatory, anti-proliferative, anti-coagulant, anti-carcinogenic, cytoprotective, heptaprotective and modulatory functions etc, which may be translated into therapeutic potential for multiple diseases. Several natural and synthetic coumarins and derivatives, such as coumarin glycosides, possess potent biological activities. Coumarin, a 1,2-benzopyrone is the simplest compound of a large class of naturally occurring phenolic substances made of fused benzene and a pyrone ring, have recently drawn much attention due to their broad pharmacological activities. The recognition of important structural features within coumarin family was crucial for the design and development of new analogues with much improved activity (Karayil *et al.*, 2014).

Coumarin has clinical medical value by itself, as an edema modifier. The coumarins have long been recognized to possess anti-inflammatory, antioxidant, antiallergic, hepatoprotective, antithrombotic, antiviral and anticarcinogenic and analgesic activities. Other than the medicinal properties, coumarin has a history of importance in perfumery. Coumarin has been used as an aroma enhancer in pipe tobaccos and certain alcoholic drinks, although in general it is barned as a flavorant food additive, due to concern regarding its hepatotoxicity in animal models (Sudha *et al.*, 2014).

Coumarins have been roughly categorized as follows: simple coumarins, furanocoumarins, pyranocoumarins, biscoumarins and triscoumarins, and coumarinolignans (Borges *et al.*, 2005).

1.6.1 Furanocoumarins

The furanocoumarins or furocoumarins, are a class of organic compounds produced by a variety of plants. The furocoumarins are a therapeutically important subtype as they have various clinical applications. They are biosynthesized partly through the phenylpropanoid pathway and mevalonate pathway, which is biosynthesized by a coupling of dimethyl allyl pyrophosphate (DMAPP) and 7-hydroxycoumarin (umbelliferone).

The chemical structure of furocoumarins consists of a five-membered furan ring attached to the coumarin nucleus. The furan may be fused in different ways producing several isomers. The compound that form the core structure of the two most common isomers are psoralen and angelicin. Derivatives of these two core structures are referred to respectively as linear and angular furanocoumarins.

Many furanocoumarins are produced by plants as a defense mechanism against various types of predators ranging from insects to mammals. This class of phytochemical is responsible for the phytophotodermatitis seen in exposure to the juices of the wild parsnip and Gaint Hogweed. Furanocoumarins have other biological effects as well. For example, in humans, bergamottin and 6′, 7′-dihydroxybergamottin are responsible for the "grape fruit effect", in which these furanocoumarins affect the metabolism of certain drugs (Berenbaum, 2010).

Two categories of furanocoumarins are produced; the linear furanocoumarins have the furan ring in line with the benz-2-pyrone nucleus, while the angular furanocoumarins have the furan ring oriented at an angle to the nucleus. Two of the most important and well known furanocoumarins are psoralen (linear) and angelicin (angular). The terms linear and angular refer to the orientation to the furan ring with respect to the coumarin nucleus.

Furanocoumarins are toxic compounds found primarily in species of the *Apiaceae* and *Rutaceae.* They come in variety of flavours and have adverse effects on wide variety organisms, ranging from bacteria to mammals. Some of the furanocoumarins are photoactive- their toxicity is enhanced in the presence of ultraviolet radiation. Furanocoumarins are chemical substances that sensitize the skin to the effects of the sun, thus leading to irregular pigmentation and increasing the risk of sunburn and phototoxicity.

Psoralens (linear furanocoumarins) are naturally occurring plant biosynthetic metabolites that have been used since ancient times in photochemotherapy to treat a number of skin disorders including mycosis fungoides, psoriasis and vitilligo. Psoralens have recently found application in the regulation of human cervical carcinoma cell proliferation in conjunction with anti-sense technology. Oligonucleotides and their analogs have been used to inhibit protein biosynthesis by

suppressing the gene expression in a sequence specific manner. The method is called antisense strategy and has been applied to gene therapy for incurable diseases such as cancers and viral infections (Diawara *et al.*, 1997).

Upon UVA irradiation psoralen derivatives have the ability to crosslink covalently with pyrimidine bases (eg. thymine and uracil). As psoralen derivatives can inactive gene expression *via* cross-linking, they have been conjugated with oligonucleotides to reinforce antisense effects. During *in vitro* expreiments psoralen-conjugated S-oligos have shown resistance to nucleases and, therefore, have exhibited significant inhibitory effects upon UVA irradiation. Psoralen-conjugated S-oligos (Ps-S-oligo) were prepared and used to inhibit the proliferation of human cervical carcinoma cells (Murakami *et al.*, 2001).

Linear furocoumarins such as 8-methoxypsoralen (8-MOP) and 5-methoxypsoralen (5-MOP) are phototoxic their toxicity is enhanced in the presence of ultraviolet A radiation and they cause acute skin reactions. These reactions are manifested as itching, pigmentation and erythema. Long term PUVA treatment with 8-MOP can cause persistent pigmentation and other skin changes.

Furocoumarins constitute a family of natural chemicals present in different plant extracts. These plant extracts are widely used as ingredients in fragrances. Due to the phototoxic, photomutagenic and photocarcinogenic properties reported for certain furocoumarins, they are not permitted for use in cosmetic products as such, except for the normal content in natural essences, if the total concentration of furocoumarin-like substances in the finished cosmetic product do not exceed 1ppm.

Isopimpinellin is a natural chemical belonging to the group furocoumarins. It is a natural product synthesized by Umbelliferae (or Apiaceae), also known as the carrot or parsley family. It can be found in celery, garden angelica, parsnip, fruits and in the rind and pulp of limes. There have been several studies looking in to the effects of isopimpinellin and other so called naturally occurring coumarins (such as bergmottin and imperatorin) as anticancinogens. These studies have shown possible inhibition of 7, 12-Dimethylbenz-anthracene, which are initiators of skin tumours. Evidence has also been reported that links these compounds to the inhibition of breast cancers (Kleiner *et al.*, 2002).

1.7 Harmful Effects of Ultraviolet Radiation

The harmful effects from exposure to ultraviolet radiation can be classified as acute or chronic. The effects of UV-A and UV-B exposure are both short-lived and reversible. These effects include mainly sunburn (or erythema) and tanning (or pigment darkening). The chronic effects of UV exposure can be much more serious, even life threatening, and include premature aging of the skin, suppression of the immune system, damage to the eyes, and skin cancer (Kane *et al.*, 1999).

1.7.1 Sunburn (Erythema)

Sunburn (or erythema) is redness of the skin, which is due to increased blood flow in the skin caused by dilatation of the superficial blood vessels in the dermis as a result of exposure to UV radiation. High UV doses may also results in edema, pain, blistering and peeling of the skin a few days following exposure. UV-B radiation is believed to be mainly responsible for sunburn as it is more erythmogenic by a factor of 1000, however since there is more UV-A radiation reaching the earth's surface, UV-A contributes 15-20 % to the sunburn reaction in the summer months. Risk factors for sunburn include fair skin, red or blond hair, blue eyes and freckles. For people with fair skin, it takes only 15-30 minutes in midday sun to include erythema. In terms of areas of the body that are more susceptible to sunburn, the face, neck and trunk are two to four sensitive than the limbs. In addition, children and the elderly are believed to be more sensitive to UV radiation and may burn more easily. A sunburn reaches its maximum redness 8-12 hours after exposure and fades one to two days (Kane *et al.*, 1999).

1.7.2 Tanning

Tanning refers to delayed pigmentation of the skin, or melanin pigmentation. It usually becomes noticeable one to two days after exposure to the sun and gradually increases for several days persisting for weeks or months. Tanning results from an increase in the number of functions melanocytes (pigment cells) resulting in increased activity of the enzyme tyrosinase. This lead to the formation of new melanin and an increase in the number of melanin granules throughout the epidermis. Tanned skin

need not only be considered a harmful effect as it does confer some protection for subsequent exposure to the sun, but the degree of protection is through to be moderate and not sufficient as a sunscreen for Caucasian skin. However, there is another mechanism that may provide more protection for subsequent exposures. In addition to tanning and sunburn, thickening (or hyperplasic) of the epidermis also occurs and through to be moderate a significant component of a mild sunburn reaction. A single moderate exposure to UV-B radiation is sufficient to include 3-fold thickening in the stratum cornea that last one to two months. This thickening is likely to be more important than tanning for providing endogenous photoprotection for those with Caucasian skin (Kane *et al.*, 1999).

1.7.3 Premature aging of the skin

One of the chronic effects resulting from repeated exposure to UV radiation is premature aging of the skin, which encompasses a number of clinical signs that reflect structural changes in the dermis. These clinical signs include dryness, wrinkles, accentuated skin furrows, sagging, loss of elasticity and mottled pigmentation, and are the result of degenerative changes in elastin and collagen. The degenerative changes accumulate over time and are largely irreversible. It is believed that as much as 80 % of the premature aging of the skin may occur within the first 20 years of life. UV-A radiation has been found to be an important contributor to premature aging of the skin. Whereas UV-B is 1000 to 10,000 times more efficient than UV-A in terms of induction of sunburn and non-melanoma skin cancer, respectively, with premature aging of the skin UV-B radiation is only 20-50 times more efficient than UV-A (Kane *et al.*, 1999)

1.7.4 Skin cancer

Skin cancers are the most commonly occurring cancers in terms of incidence in the world. There are different types of skin cancer including the non-melanoma skin cancers, basal cell carcinoma (BCC) and squamous cell carcinoma (SCC), and melanoma. Exposure to UV radiation as it induces DNA damage, however the types of exposure necessary to cause the different types of skin cancer may vary. For the

non-melanoma ski cancers, cumulative sun exposure is believed to be important, whereas for melanoma the intermittent exposure hypothesis has been postulated. This hypothesis purposes that infrequent intense exposure of unacclimated skin to sunlight is related to the increasing incidence of melanoma and is more important than chronic sun exposure. This incidence of all types of skin cancer is increasing (Kane *et al.*, 1999).

1.8 Sunscreen Lotions

Skin is composed of very specific cells and tissues. The subcutaneous layer of skin contains blood vessels which branch infinitely into the dermis to supply the sweat glands, hair follicles, sebaceous glands and erector muscles with blood. Dermis contains capillaries, which provides the essential nutrients to the cells in the dermis, and also helps the skin to perform cooling function. The epidermis is not supplied directly by blood, but is fed by the dermis. Dermis contains several different nerve endings; heat-sensitive, cold-sensitive, pressure-sensitive, itch-sensitive and pain-sensitive. Epidermis consists of one inner layer, malpighian layer, which creates the dead cells and is in direct contact with the dermis, which feeds and supports it only it is affected during sun tanning.

The malpighian layer has a basal layer, a spinous layer, a granular layer, and an outer stratum corneum. The cells in stratum corneum are filled with a protein called keratin. Keratin is a very interesting protein because it is tough-horns, hair, hoofs, finger nails and feathers all gain their strength from keratin. In the basal cells malpighian layer consists cells called melanocytes, which produces melanin, which is a pigment that is the source of tanning. The appearance of the skin is partly due to the reddish pigment in the blood of the superficial vessels and it is determined by melanin, a pigment manufactured by the dendritic cells called melanocytes, found among the basal cells of the epidermis. Colour differences are due to solely to the amount of melanin produced and the nature of the pigment granules. When the skin becomes tanned on the exposure to sunlight, the melanocytes do not increase in number, they only became more active. Not only do melanocytes produce a tan, they are also responsible for the form of cancer called melanoma. Melanoma is caused by

UV radiation damage to melanocytes. Repeated exposure to UV can cause cancerous mutations (Singh *et al.*, 2010).

The rapid growth of commercially available products containing sunscreens indicates that even through a suntan is still desired, people are conscious of the possible dangers of photoaging and skin cancer, occurring as a result of sun exposure. Every year, about one million people are diagnosed with skin cancer and about 10,000 die from malignant melanoma. Most skin cancer occurs on the areas of the body that are most frequently exposed to the sun, such as the face, neck, head and back of the hands (Sax, 2000).

The harmful effects of solar radiation are caused predominantly by the ultraviolet (UV) region of the electromagnetic spectrum, which can be divided into three regions: UVA, from 320 to 400 nm; UVB, from 290 to 320 nm and UVC, from 200 to 290 nm. UVC radiation is filtered by the atmosphere before reaching earth. UVB radiation is not completely filtered out by the ozone layer and is responsible for the damage due to sunburn. UVA radiation reaches the deeper layers of the epidermis and provokes the premature aging of the skin. Ultraviolet radiations have been implicated as a causative factor of skin cancer (Dutra, 2004).

On exposure to sunlight human body experiences both beneficial and harmful effects, depending on the length and frequency of exposure, sunlight intensity and the sensitivity of the individual concerned.

On exposure to sunrays the first effect produced is erythema of skin, followed by the formation of tan, which actually is a protective reaction of human body to minimize the adverse and harmful effects of solar radiation. The intensity of erythema (reddening) produced on exposure to sunlight depends on the amount of UV energy absorbed by skin. It usually develops after a latent time period of 2-3 hour and reaches its maximum level within 10-24 hours. Due to these facts, sunscreen substances are now incorporated into everyday products such as moisturizers, creams, lotions, shampoos, mousses, and other hair and skin preparations (Wilkinson, 1982).

Sunscreens are the agents which are used to prevent the skin from the harmful ultraviolet radiation of sun. These agents act as a barrier for the sun rays. Sunscreen can be physical, chemical or natural sunscreen. The regular use of these products may

help to reduce the chance of the harmful effects of ultraviolet radiation. However, it is necessary that a very efficient sunscreen substance is used in the cosmetic formulation. The effectiveness of sunscreen agents can be measured in terms of Sun Protection Factor (SPF) (Dutra, 2004).

1.8.1 Sun protection factor

The SPF is a quantitative measurement of the effectiveness of a sunscreen formulation. To be effective in preventing sunburn and other skin damage, a sunscreen product should have a wide range of absorbance between 290 nm and 400 nm. Evaluation of the efficiency of a sunscreen formulation has for a long time been assessed through *in vivo* test, which is performed with human volunteers. *In vivo* test is time –consuming, is normally subject to certain degree of variability, not mention the ethical problems of testing with human. *In vitro* SPF is useful for screening test during product development.

1.9 Aim and Objectives of the Present Research

The aim of the present work is to isolate and identify some bioacitve phytoconstituents from bark and fruit pulp of *L. acidissima* (Thee) and to investigate some of their biological activities.

Objectives of the work can be summarized as follow:

- To collect *Limonia acidissima* L. (Thee) bark and fruit pulp
- To perform the botanical identification of the selected samples
- To investigate the phytochemical constituents from the samples
- To analyze the elemental contents by AAS method
- To prepare the crude extracts with various solvents
- To determine the nutritional values of the samples
- To evaluate the antimicrobial activity of some crude extracts by agar well diffusion method
- To investigate the acute toxicity of ethanol extract from bark sample
- To study the antioxidant activity of *L. acidissima* bark
- To isolate the organic constituents from bioactive extracts

- To elucidate the isolated compounds by modern spectroscopic methods such as UV, FT IR, ^{1}H NMR, ^{13}C NMR, H SQC, HMBC and HR ESI MS
- To formulate the sunscreen lotions from the bark and fruit pulp of *L. acidissima* (Thee)
- To evaluate the sunscreen lotions of the bark and fruit pulp of *L. acidissima* (Thee)
- To study the skin whitening effect of the sunscreen lotions by using the albino rat models
- To study the skin whitening effect of the sunscreen lotions by using sun burning human skins
- To determine the SPF value of the sunscreen lotions

CHAPTER II

2. MATERIALS AND METHODS

2.1 Collection and Preparation of Plant Samples

Myanmar people apply Tha-nat-khar on the face as a sun protection back. Some places in Myanmar, especially Upper Myanmar, is very hot and dry region. So, people at this region is exposed UV radiation from the sun-ray. Long term exposure to UV radiation raised the risk of skin diseases such as edema, erythema, hyperpigmentation, photo aging, inflammation, mutations and skin cancer. Most of the people feel the sunburn, tanning and other skin diseases concerning with the harmful effect of UV radiation. *L. acidissima* (Thee) plant has invaluable effect on skin and enormous range of pharmacological activities like antidiabetic, antimicrobial, antiviral, antiviral, antifungal, antitumor, CNS depressant activities and wound healing etc. Most of the people apply Thee bark as a cosmetic back. Thee bark is recognized to be great help to bear the heat of sun and thus an ideal cosmetic for those who have to work under direct sunshine. Women who work in paddy fields always wear thick layers of Tha-nat-khar to help themselves tolerate the intense heat of the sun. Thee paste also has the same properties as Tha-nat-khar making the skin cool and smooth, having a refreshing and cool fragrance, beautifying the users. It also cures pimples and acne. Thee is a rare and endangered tree species and also a valued plants for its edible fruits and immense medicinal properties. Therefore, *L. acidissima* was also chosen to be studied.

L. acidissima (Thee) was collected from Yenangyaung Township, Magway Region, in February 2012. After collection, the scientific name of this plant was verified by authorized botanist at Botany Department, University of Yangon.

The bark sample was cleaned by washing with water and air dried at room temperature. The dried samples were cut into small pieces and then ground into powder by means of a grinding mill. The fruit pulps were shade dried and pulverized. The dried powdered samples were separately stored in the air-tight containers.

2.2 Preliminary Phytochemical Investigation of Plant Samples

In order to find out the types of organic constituents present in the samples, preliminary phytochemical investigation was carried out according to reported methods.

(i) Materials

Air-dried powder of bark and fruit pulp of *L. acidissima* (Thee).

(ii) Chemical and reagents

The reagents used for phytochemical screening were Mayer's reagent, modified Dragendroff's reagent, Wagner's reagent, 10% α- naphthol, Benedict's solution, acetic anhydride, H_2SO_4 solution, NaOH solution, 5% $FeCl_3$, 1% $FeCl_3$, I_2, NH_3 vapour, Ninhydrin reagent, 1% $AlCl_3$, 10% lead acetate and gelatin.

2.2.1 Test for alkaloids

The powdered sample (10 g) was boiled with 1% hydrochloric acid for about 10 min and allowed to cool and it was filtered. The filtrate was divided into four portions and tested with Mayer's reagent, sodium picrate, modified Dragendroff's reagent, Wagner's reagent respectively. Observation was made to see if treatment with alkaloid reagents finished alkaloidal precipitate (Trease and Evans, 1980).

2.2.2 Test for α-amino acids

The powdered sample (10 g) was boiled with distilled water (50 mL) and filtered. A few drops of filtrate was spotted on a filter paper allowed to dry and sprayed with ninhydrin reagent. The filter paper was then dried at room temperature and kept in the oven at 100 °C for a few minutes. Appearance of a pink colour indicated the presence of α-amino acid (Marini-Bettolo *et al.*, 1981).

2.2.3 Test for carbohydrates

The powdered sample (10 g) was boiled with distilled water for about 20 min and then filtered. The filtrate was collected in a test tube and a few drops of 10% α-

naphthol was added into the test tube and shaken. This test tube was inclined at an angle of 45° and about 1 mL of concentrated sulphuric acid was slowly added to enter along the inner side of the test tube to see if red ring was formed at the interface of two liquids (Vogel, 1956).

2.2.4 Test for flavonoids

10 g of powdered sample were extracted with 50 mL ethanol and filtered. A few drops of concentrated hydrochloric acid were added to the filtrate. A few pieces of magnesium ribbon were added to the above mixture. Appearance of reddish-pink colour indicated the presence of flavonoids (M-Tin Wa, 1972).

2.2.5 Test for glycosides

10 g of powdered sample were boiled with 50 mL of water for about 10 min and filtered after cooling at room temperature. The filtrate was treated with 10% lead acetate solution. Observation was made to see if the solution turned to white precipitates which indicated the presence of glycosides (Marini-Bettolo *et al.*, 1981).

2.2.6 Test for phenolic compounds

10 g of powdered sample were boiled with 50 mL of ethanol for about 10 min and then filtered. The filtrate was treated with a few drops of 5% $FeCl_3$ and $K_3Fe(CN)_6$ solutions. Appearance of a deep blue colour indicated the presence of phenolic compounds (Marini-Bettolo *et al.*, 1981).

2.2.7 Test for reducing sugar

About 3 g of dried powdered sample was boiled with dilute sulphuric acid (25 mL) for about 10 minutes and filtered. The filtrate was then neutralized with dilute sodium hydroxide solution. The resulting solution was mixed with two drops of Benedict's solution and boiled for about 2 minutes. Then the solution was allowed to cool. Brick red precipitate indicates the presence of reducing sugar (M-Tin Wa, 1972).

2.2.8 Test for starch

Dried powdered sample (3 g) was boiled with distilled water (25 mL) for 30 minutes and filtered. Two drops of 1 % (w/v) iodine solution were added to the filtrate and an observation was made to see if a deep blue precipitate was formed, indicating the presence of starch (M-Tin Wa, 1972).

2.2.9 Test for steroids

10 g of the powdered sample were refluxed with Pet-ether and the solvent was removed by distillation under reduced pressure. 3 drops of acetic anhydride were added and the mixture was manually shaken for 1 minute. Then a few drops of concentrated H_2SO_4 acid were carefully added and shaken. Observation was made to see if the solution turned to green colour which indicated the presence of steroids (M-Tin Wa, 1972).

2.2.10 Test for tannins

3 or 4 drops of 10% NaCl solution were added to the ethanolic extract of plant material, followed by filtration. About 3 mL of the filtrate were transferred to the test tube and added 2-3 drops to 1% gelatin solution. Observation was made to see if white precipitate were formed (Trease and Evans, 1980).

2.3 Quantitative Elemental Analysis of Plant Samples by Atomic Absorption Spectroscopy (AAS)

In order to determine the heavy toxic metals and macronutrient elements in plant samples, quantitative elemental analysis was performed by AAS method at the Universities' Research Centre, University of Yangon.

Samples

L. acidisima (Thee)

Chemicals

20% HCl, 20% HNO_3

Apparatus

Porcelain crucible, hot plate, filter paper, vortex mixer, volumetric flask (50 mL)

Procedure

The sample was weighed and then pre-ashing was carried out on a hot plate until all the combustible materials were burnt. Pre-ash sample was placed inside the electric muffle furnace and heated gradually raising temperature until 450°C. The process of heating, cooling and weighing were repeated, until constant weight of ash sample was obtained.

0.5 g of dried sample was placed into a porcelain crucible. 5 mL of HNO_3: HCl (1:4) concentrated acid mixture was also added it. The solution was evaporated to dryness overnight on a hot plate. Leach the residue on a sample was mixed with 10 mL of HNO_3 weak acid mixture at a temperature of about 70°C for 30 minutes. The solution was stirred by using vortex mixer. The solution were filtered and aspirated on an atomic absorption spectrophotometer (Varian Tectron Model AAS) at Applied Geology Department.

2.4 Determination of Nutritional Values

In the present study, some nutritional values such as moisture, ash, protein, fiber, fat, carbohydrate and energy values of *L. acidissima* (Thee) were determined by AOAC methods (A.O.A.C, 2000).

2.4.1 Determination of moisture content

The moisture content of the selected samples was determined by oven drying method (A.O.A.C, 2000).

Sample

Stem bark of *L. acidissima* (Thee)

Apparatus

Porcelain crucibles, air-tight desiccators, oven and electric balance

Procedure

A clean dry crucible with lid was weighed. Sample (2 g) was placed in this crucible and weighed again. The difference was the weight of the sample taken. The sample was dried in an oven at 100 °C for 6 hours. After that the crucible containing dried sample was cooled to room temperature in a desiccator and weighed. Heating, cooling and weighing were repeated until a constant weight was obtained. The loss in weight is reckoned as weight of moisture. The percent content of moisture in the sample could be calculated by the equation shown in APPENDIX II.

Moisture content in the sample was determined by above procedure for three more times and the average moisture content is shown in Table 3.3.

2.4.2 Determination of ash content

The ash content of the sample was determined by the method given in "The Chemical Analysis of Foods" (Joslyn, 1970).

Sample

Stem bark of *L. acidissima* (Thee)

Apparatus

Porcelain crucible with lid, hot plate, electric oven, desiccator

Procedure

Carefully weighed dried sample (5 g) was placed in a preheated, cooled and weighed porcelain crucible. The crucible was heated carefully on a hot plate until the organic matter burned off without the flame. The partially decomposed sample was then incinerated in a muffle furnace at (600 °C, 873 K) until the resultant ash was uniform in colour (i.e., white or gray). Crucible containing ash was then cooled to room temperature in a desiccator and weighed. Heating, cooling and weighing were repeated until a constant weight was obtained.

The percent of ash in the sample was calculated by the equation shown in APPENDIX II.

Ash content in the sample was determined by this method for three more times and the average ash content is shown in Table 3.3.

2.4.3 Determination of crude fibre content

Crude fibre content in the sample was determined by acid-base digestion method (Raghuramulu, 1983).

Sample

Stem bark of *L. acidissima* (Thee)

Chemicals requirement

Sulphuric acid and sodium hydroxide

Apparatus

Round-bottomed flask, glass rod, Buchner funnel, water suction pump, condenser, filter paper, beaker, oven, measuring cylinder and conical flask

Procedure

The dried sample (2 g) was placed into the 500 mL round-bottomed flask, the hot sulphuric acid solution (1.25 % v/v) was added to the mark and the mixture was refluxed for about half an hour. The level constant was maintained by adding water periodically to prevent loss of solvent by evaporation. The solution was filtered through a fine piece muslin cloth into Buchner funnel. The residue was washed with distilled water until free from acid. The residue was transferred into the flask and 200 mL of hot sodium hydroxide solution (1.25 % w/v) was added. The mixture was again refluxed for about half an hour and made the level constant by adding water at intervals. Then it was filtered through a Buchner funnel. The residue was washed with distilled water till the washing was neutral. It was finally washed with ethanol (5 mL) and petroleum ether 10 mL. The fiber was transferred to a clean and dry crucible, dried at 100 °C to get constant weight. The difference in weight of the fiber before and after incineration gives the weight of fiber and the fiber content was calculated by using the equation shown in APPENDIX II.

The fibre content was determined three times and average fibre content was shown in Table 3.3.

2.4.4 Determination of fat content

Fat content was determined by the soxhlet extraction method (Pearson, 1970) using petroleum ether.

Sample

Stem bark of *L. acidissima* (Thee)

Apparatus

Soxhlet extractor, 500 mL round-bottomed flask, condenser, water bath, hot plate, beaker, cloth bag

Chemical

Petroleum ether (bp 60-80 °C)

Procedure

The dried sample (30 g) was weighed and placed in a cloth bag and the bag was then placed in a soxhlet extractor. Petroleum ether (300 mL) was poured into the extractor until some of it over flowed into the flask and some pieces of pumice stones were added into a flask. The extraction was allowed to continue for 8 hours at the boiling point of petroleum ether. After the completion of extraction, most of the petroleum ether in the extract was distilled off. Then the content in the flask were carefully combined and transferred to a weighed specimen tube. The remaining ether in the specimen tube was vaporized at (100 °C, 373 K) until a constant weight was obtained. The difference in weights of before and after refluxing with petroleum ether was the fat extracted from the leaves sample. The fat content can be calculated using the equation shown in APPENDIX II.

The experiment was repeated three times. The average fat content of dried samples is shown in Table 3.3.

2.4.5 Determination of protein content

Protein content in the sample was determined by macro-Kjeldahl method (Steyermart, 1961).

Sample

Stem bark of *L. acidissima* (Thee)

Apparatus

Kjeldahl's digestion flask (30 mL), macro-Kjeldahl's distillation apparatus, digestion flask, conical flask, round-bottomed flask and burette

Chemicals

Potassium sulphate, anhydrous copper (II) sulphate, concentrated sulphuric acid, 40 % sodium hydroxide, 2 % boric acid, 0.1 M hydrochloric acid, distilled water and methyl red indicator

Preparation of required reagents

(a) Standard 0.1 M hydrochloric acid solution

Hydrochloric acid 8.9 mL was dissolved in distilled water and the volume was made up to 100 mL to obtain a 1 M HCl solution.

This 1 M HCl solution 100 mL was diluted with water and the volume was made up to 1 L in a volumetric flask to obtain 0.1 M HCl solution. This solution was standardized by standard 0.05 M carbonate solution.

(b) 2 % (w/v) Boric acid solution

Boric acid (2 g) was dissolved in 10 mL of distilled water and the volume was made up to 100 mL with distilled water.

(c) 40 % (w/v) Sodium hydroxide solution

Sodium hydroxide pellets (40 g) was dissolved in some distilled water and make up to 100 mL with distilled water.

(d) Methyl red indicator

Methyl red (0.1 g) was dissolved in 50 mL of ethanol and diluted with distilled water and made up to 100 mL of solution

Procedure

The dried sample (0.5 g), potassium sulphate, anhydrous copper (II) sulphate (0.05 g) and concentrated sulphuric acid (10 mL) were added into a Kjedahl's flask. The flask was partially closed with a glass funnel and the content was digested by heating the flask in an inclined position in a digester. The mixture was heated for about 30 minutes until the solution became clear. It was allowed to cool and transferred into the 500 mL of the round-bottomed flask. In the receiving flask, 50 mL of 2 % boric acid and 2 drops of methyl red indicator were added to the flask rotated to mix the contents thoroughly. The distillation was then set up, some pieces of pumice stone were placed in the distillation flask containing the digested liquid to prevent bumping and 50 mL of 40 % sodium hydroxide was added to the solution by the use of a dropping funnel. The flask and its contents were heated when ammonia diffused into the acid solution. The color of boric acid solution gradually changed from pink to yellow. The distillation was continued until 200 mL of distillate was collected in the receiving flask. The stopper of the flask was opened and the condenser and delivery tube were washed down with distilled water. The distillate in the flask was titrated with standardized 0.1 M hydrochloric acid solution. A blank determination was carried out in the same manner but without including the sample. The protein content in the sample was calculated by using the equation shown in APPENDIX II. The results are shown in Table 3.3.

2.4.6 Determination of carbohydrate content

The total carbohydrate content of a food can be obtained as the difference between 100 and the sum of percentages of moisture, protein, fat, ash and fibre. Although the individual carbohydrates can, if necessary be estimated separately by chemical methods the "total" carbohydrate content of a food obtained by calculation

as described above is sufficiently accurate practical nutrition work. The resultant carbohydrate content (percent) is shown in Table 3.3.

2.4.7 Determination of energy value

The energy value was calculated using the sum of 4 kilocalories per gram of carbohydrate and of protein and 9 kilocalories per gram per fat. The energy values are expressed to the nearest kilocalories per 100 gram. The amount of energy value was calculated.

Energy value (kcal/100g) = (4 x protein) + (4 x carbohydrate) + (9 x fat)

2.5 Preparation of Various Crude Extracts from the Bark of *L. acidissima* (Thee)

The dried powdered sample (500 g) of the bark of *L. acidissima* (Thee) was extracted with ethanol (1.5 L) in an air-tight bottle for about two weeks at room temperature and then filtered and concentrated by using a rotatory evaporator at 40 °C under reduced pressure. This similar procedure was made for three times. The crude ethanol extract (11 g) was obtained. The marc remained was similarly extracted with pet-ether (100 mL × 3) for 3 days. The above similar procedure is used to obtain pet-ether crude extract. The pet-ether extract (4 g) was obtained. Finally, ethyl acetate extract (2 g) was prepared from the marc of the pet-ether by maceration with ethyl acetate (100 mL x 3) for 3 days. The flow diagram of extraction method of the bark of *L. acidissima* (Thee) is shown in Figure 2.1.

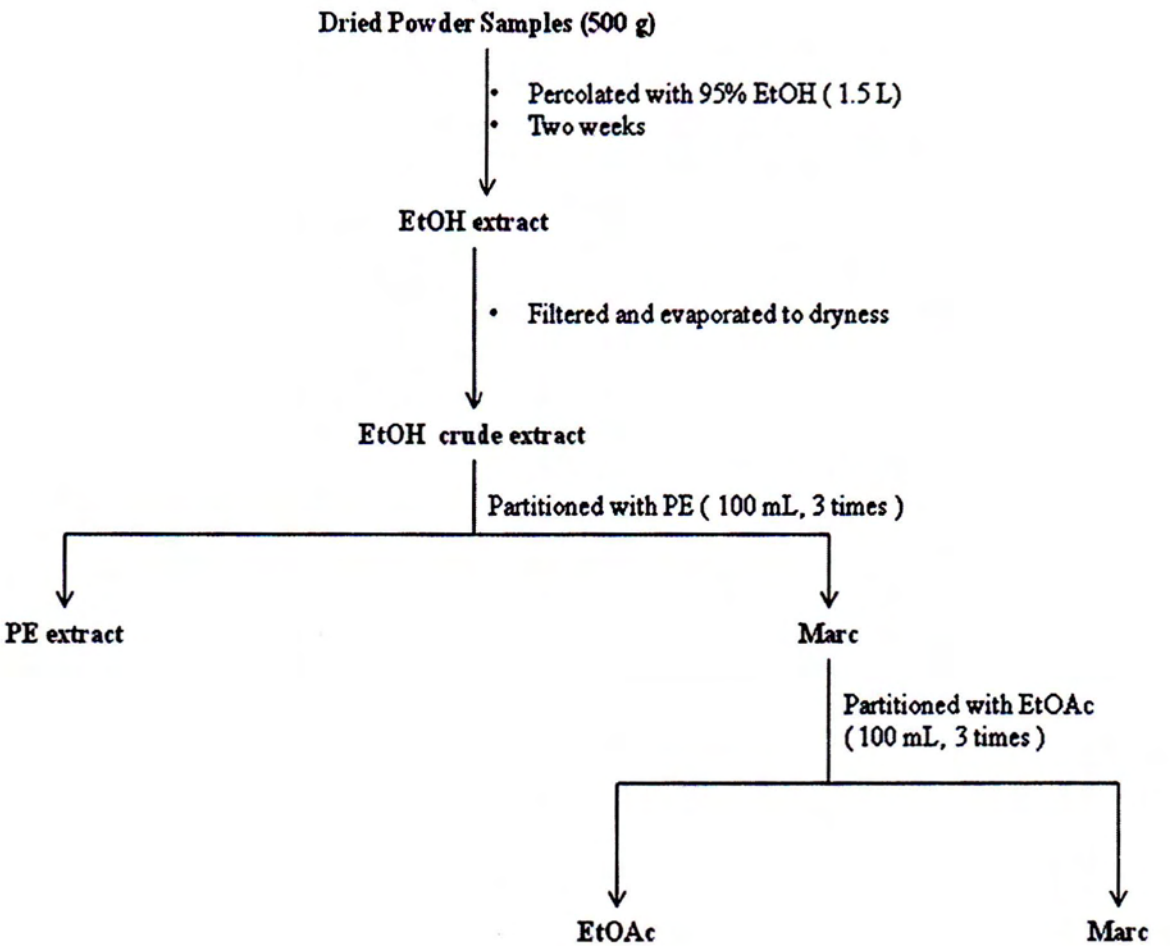


Figure 2.1 **Flow diagram of preparation of plant extracts for the *L. acidissima* (Thee)**

2.6 Isolation of some Phytoconstituents

2.6.1 Isolation of some phytoconstituents from ethyl acetate extract of the bark of *L. acidissima* (Thee)

Before separation of chemical constituents present in ethyl acetate extract, selection of solvent system for column chromatography was carried out by using pre-coated silica gel GF_{254} with Pet-ether : EtOAc (20:1, 19:1, 9:1, 5:1, 3:1, 2:1, 1:1, 1:2, 1:3, 1:5, 1:7), ethyl acetate only and MeOH only increasing polarity. The ethyl acetate extract (11 g) of *L. acidissima* (Thee) was subjected to column chromatography over silica gel eluted with Pet-ether (60–80 °C) and EtOAc of increasing polarity and finally with MeOH which yielded a total of thirteen fractions. Based on Thin Layer Chromatography (TLC) analysis, fractions III subjected to the solvent system ratio of Pet-ether: EtOAc (9:1). Pet-ether and EtOAc in a ratio of 9:1 to yield compound I and II mixture (colorless needled crystal) and in a ratio 1:1 to yield compound III (white needle crystal) were obtained. TLC chromatograms were visualized under UV-lamp (254 nm) as well as by spraying with 5% H_2SO_4 and heated 110°C with orange for compound I and II mixture and with dark blue for compound III. Compound III on TLC was visualized under (254-365 nm) as well as spraying with 10% $FeCl_3$ gave greenish blue coloured. Compound I and II mixture and compound III gave yellow colour while spraying with I_2. Flow diagram for the isolation of phytoconstituent from the EtOAc extract from bark of *L. acidissima* (Thee) is given in Figure 2.2.

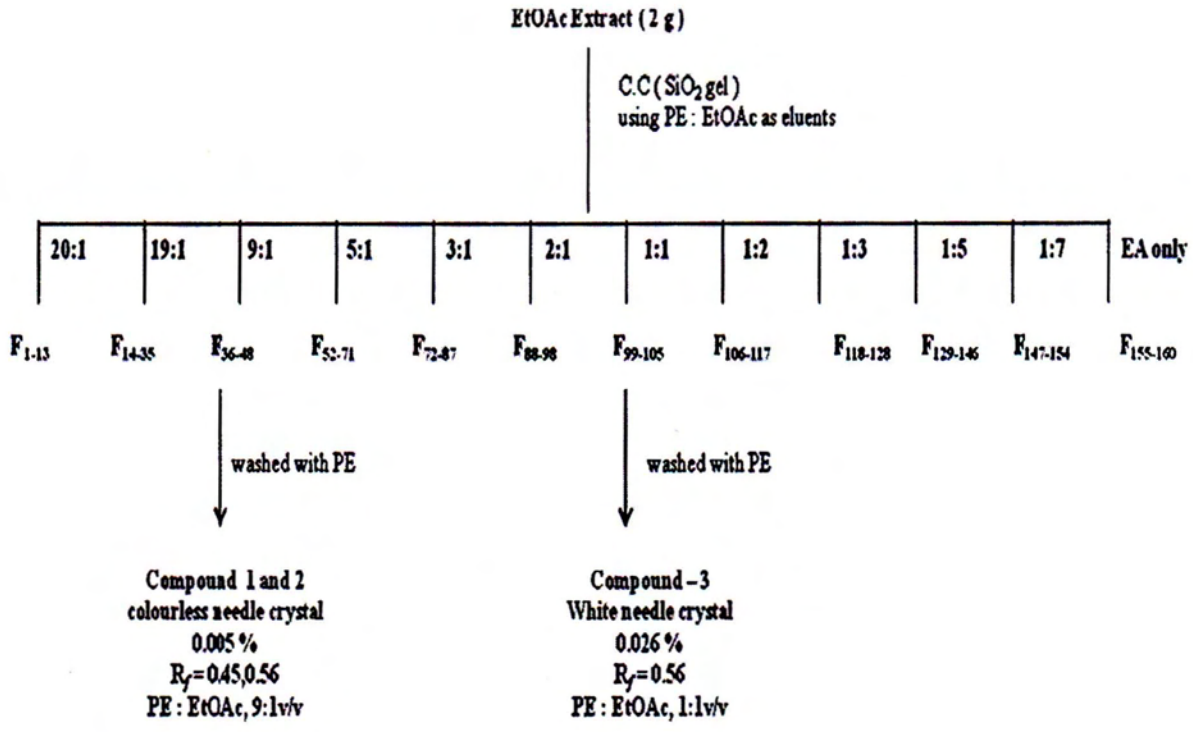


Figure 2.2 Procedure for extraction and isolation of compound I, compound II and compound III

2.6.2 Isolation of some phytoconstituents from dichloromethane extract of the bark of *L. acidissima* (Thee)

Samples

Dried bark powdered samples of *L. acidissima*

Chemicals

MeOH, hexane, distilled water, dichloromethane

Apparatus

Beakers, round bottomed flask, funnel, sonicator, separation funnel, filter paper

Procedure

The dried powdered sample was soaked in methanol for three days. On the last day, before filtering it out into a big bottle, sonicator was used to extract out solvent soluble components. Sonication process was done for five minutes. Then, filtration was done and the solvents were removed using rotatory evaporator. This procedure is repeated for three times. And then, MeOH crude extract was obtained.

MeOH crude extract was dissolved in 500 mL of MeOH: H_2O (9:1). It was then transferred into a separating funnel and 500 mL of hexane were added. The stopper was placed on top of the separatory funnel and the two layers were mixed by rocking the funnel back and forth. The stopcock was opened to vent the gas with the funnel invented. The stopper was opened and the layers were allowed to settle. The aqueous layer will be at the bottom and was allowed to drain into a clean round bottomed flask. This step was done three times. This aqueous layer dilutes to make the concentration to MeOH: H_2O (7:3). It was then extracted with 200 mL of dichloromethane. The extraction with dichloromethane was done three times and dichloromethane layer was collected.

2.6.2.1 Separation by thin layer chromatography

Sample

Dichloromethane extract, MeOH crude extract, aqueous MeOH extract, hexane extract

Chemicals

Hexane, acetone, methanol, dichloromethane

Apparatus

TLC plate, TLC tanks, capillary tubes

Procedure

TLC was used to determine the best solvent system that is useful for separating organic compounds. Hexane: acetone (7:3) and dichloromethane: methanol (9:1) were prepared using measuring cylinders and then transferred to their respective labelled TLC. A pencil was used to draw a straight line across the TLC plate approximately 1 cm from the bottomed and top. Four dots were drawn on the base line on each TLC plates and were labelled according to their sample names. A capillary tube was used to spot each sample on their respective dots. After spotting, these plates were then put into the TLC tanks filled with the solvent reach the solvent front, the plates were removed from the tank and were allowed to evaporate. UV box was used to observe the spots. It will be used for separation in column chromatography analyzing of compounds after separation and combining fractions of the same type. TLC method was used to spot all the fractions collected after gravity column chromatography to determine which ones are the same before combining them together. Also, this is a primary method that was used to roughly know how many compounds are there.

2.6.2.2 Separation by vacuum liquid column chromatography

Sample

Dichloromethane extract

Chemicals

Dichloromethane, methanol, silica gel

Apparatus

Conical flask, column, vacuum pump, funnel, droppers

Procedure

The dichloromethane extract was chosen to go through vacuum liquid column chromatography as it have more materials. The column was packed using dry packing method. Silica gel was added and air bubbles were removed by applying vacuum for about 10 minutes. In the meantime, the extract was prepared using pre-absorption method. The solvent systems of CH_2Cl_2 (100 %), CH_2Cl_2: MeOH (9:1), CH_2Cl_2: MeOH (4:1), CH_2Cl_2: MeOH (7:3), CH_2Cl_2: MeOH (1:1) and MeOH (100 %) were prepared. After 10 minutes, the vacuum was turned off and the extract was loaded into the column. The vacuum was then turned on for another 5 minutes before applying the gradient solvent system. After 5 minutes, vacuum was turned off and 100 % dichloromethane was poured into the column. The vacuum was turned on and fraction A was collected. The same steps were repeated for other solvent systems and then collected for each fraction. Rotary evaporator was used to remove the solvents in each fraction.

2.6.2.3 Separation by flash column chromatography

Sample

Fraction CH_2Cl_2: MeOH (7:3)

Chemicals

MeOH, dichloro methane, silica gel

Apparatus

Column, test tubes, droppers

Procedure

Fraction CH_2Cl_2: MeOH (7:3) was chosen to go through FCC. The column was packed using wet method and crude extract of fraction D was prepared using pre-absorption method and loaded into the column. The isocratic solvent system was then applied and fractions were collected using test tubes. The solvent system of hexane: acetone (7:3) (2 litres) was used. The column was then washed with CH_2Cl_2: MeOH (7:3) (1 litre) followed by about 1 litre of MeOH only. This was done to ensure that all samples were eluted out. Rotary evaporator was used to remove the solvents.

2.6.2.4 Separation by high performance liquid chromatography

(a) Preparative high performance liquid chromatography

Sample

CH_2Cl_2: MeOH (7:3) extract

Chemicals

Methanol, distilled water, formic acid

Apparatus

Vials, injector, Waters 2695 HPLC (alliance)

Procedure

Preparative HPLC was used for separation used to get pure compound. All four machines together with computer were turned on. LCMS solution program followed by HPLC icon were clicked to open up the program. Everything was ensured that it showed the ready sign. The instrument icon was clicked to turn it on. The column was washed using methanol followed by water and lastly 0.1% formic acid in distilled water. The proper method set was then selected. Line A (0.1 % formic acid in acetonitrile) was prime first followed by line B (0.1 % formic acid in distilled water). After priming, the column was then equilibrated with the starting concentration. The flow for analytical and preparative column is 10 mL/min and 20 mL/min, respectively. The sample to be injected was then prepared. The single start icon was then clicked. The sample injector port was turned to the load position and the arm was ensured that it moves to right position before injecting the sample. Once the arm was at the right position, the sample was injected and the sample injector port was turned to the inject position. The drain icon was then clicked to collect the fractions.

(b) Analytical HPLC

Sample

Samples from each fraction

Chemicals

Formic acid, distilled water

Apparatus

HPLC machine

Procedure

The degassing button was turned on and line D (0.1 % formic acid in distilled water) was primed. Then, the column was equilibrated with the initial concentration for 15 minutes. While equilibrating, the sample set were created and saved. Once the pressure is stable and samples were placed on the sample tray, the run button was clicked. Flow diagram for the isolation of compound IV from CH_2Cl_2 extract from bark of *L. acidissima* (Thee) is given in Figure 2.3.

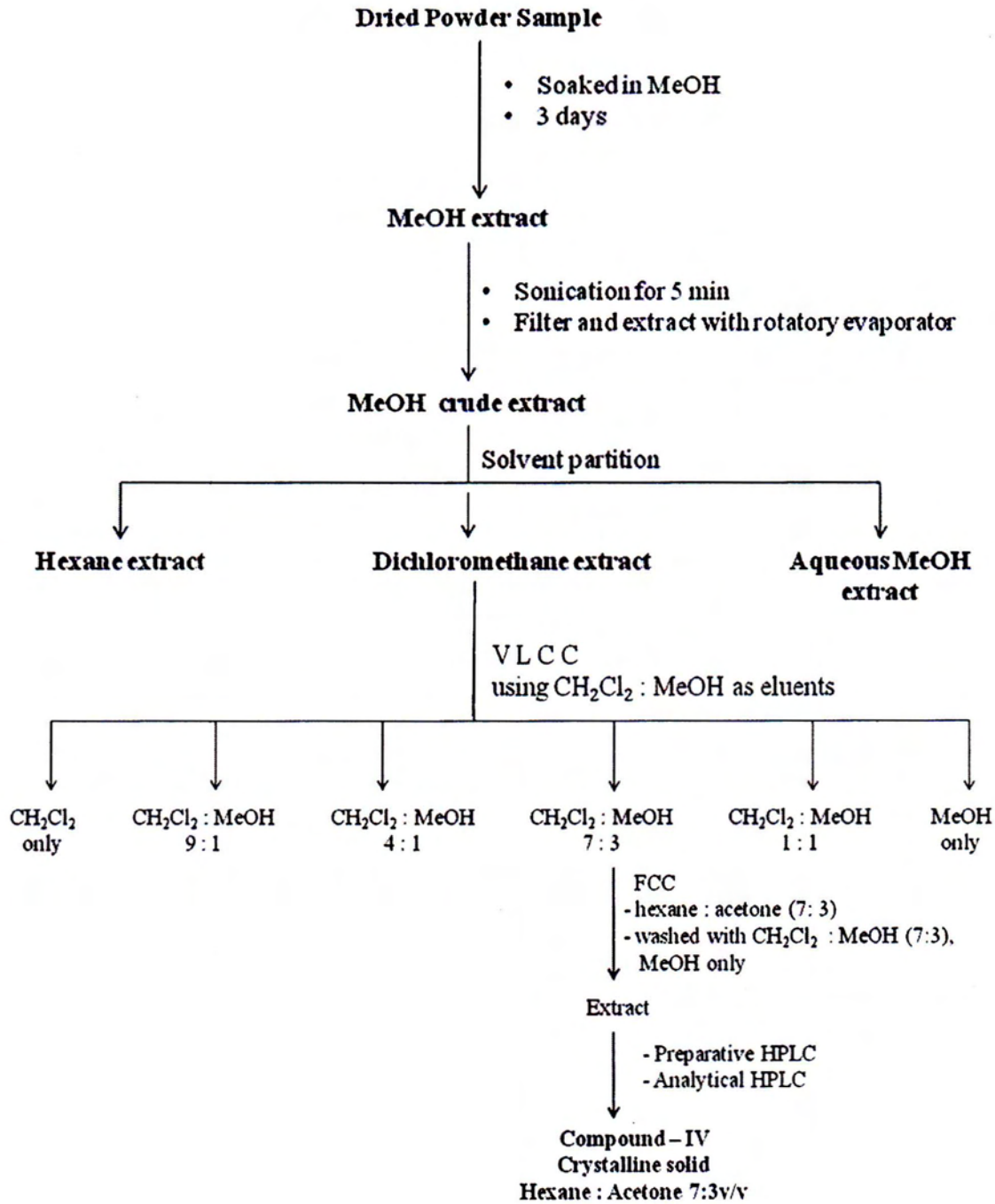


Figure 2.3 Flow diagram for the isolation of compound IV from CH_2Cl_2 extract from bark of *L. acidissima* (Thee)

2.7 Identification of Isolated Compounds

Isolated compounds were identified by their TLC behaviors and by their spectral data, namely, UV, FT IR, ^{1}H NMR, ^{13}C NMR, 2D NMR (COSY, HSQC, HMBC) and EI MS.

2.7.1 Determination of R_f value

Each isolated compound was developed on a silica gel GF_{254} TLC plates with a suitable solvent system. The compounds on the plate were located under UV lamp (254 nm and 365 nm wavelength) for visualization. The compounds on the plate were also localized by spraying with respective reagents followed by heating at 110 °C if necessary. The R_f values of the respective spots were determined.

2.7.2 UV/ Vis - spectroscopy

The UV spectra of isolated compounds in methanol were recorded with a Shimadzu UV-240, UV-visible spectrophotometer (Japan) at the Universities' Research Centre, University of Yangon and a Shimadzu UV-1800, UV-visible spectrophotometer (Japan) at the AMTT Company.

2.7.3 Determination of FT IR spectroscopy

The FT IR spectra of all isolated compounds were taken with KBr pellets and recorded on Shimadzu FT IR 8400 Fourier Transform Infrared Spectrometer at the Department of Chemistry, University of Yangon.

2.7.4 ^{1}H NMR spectroscopy

The ^{1}H NMR spectra of all isolated compounds were determined in $CDCl_3$, CD_3OD and acetone using TMS as internal standard and recorded on INOVA-600 (600 MHz for proton) Spectrophotometer at the Department of Organic and Biomolecular Chemistry, University of Goettingen, Germany and the Department of Organic Chemistry, Korea University, South Korea.

2.7.5 ^{13}C NMR spectroscopy

The ^{13}C NMR spectra of isolated compounds were determined in $CDCl_3$ using TMS as internal standard and recorded on INOVA 500 (125 MHz for carbon) Spectrophotometer at the Department of Organic and Bio molecular Chemistry, University of Goettingen, Germany.

2.7.6 COSY, HSQC and HMBC spectroscopy

COSY and HSQC spectra of isolated compounds were determined in $CDCl_3$ and CD_3OD recorded on INOVA 600 (600 MHz for proton and 125 MHz for carbon) at the Department of Organic and Biomolecular Chemistry, University of Goettingen, Germany.

2.7.7 EI MS spectroscopy

For the identification of isolated compounds, the mass spectra were recorded to examine the molecular weight and fragmentation patterns. EI MS spectra of isolated compounds were determined at the Department of Organic and Biomolecular Chemistry, Georg-August University, Göettingen, Germany.

2.8 Screening of Bioactivities

In this section, screening of bioactivities of the selected plants the bark and fruit pulp of *L. acidissima* (Thee) were performed including antimicrobial activity, acute toxicity test, and *in vitro* antioxidant activity.

2.8.1 Screening of antimicrobial activity of *L. acidissima* (Thee) by agar well diffusion method

For the examination of *in vitro* antimicrobial activity successive solvent extracts such as 95% EtOH, PE, $CHCl_3$, MeOH, CH_3OCH_3, EtOAc and watery extracts, compound I and II mixture, compound III from the bark of *L. acidissima* (Thee) and 95% EtOH, MeOH and EtOAc extracts from the fruit pulp of *L. acidissima* (Thee) were investigated by using agar well diffusion method.

Samples

95% EtOH, PE, $CHCl_3$, MeOH, CH_3OCH_3, EtOAc and watery extracts, compound I and II mixture, compound III from the bark of *L. acidissima* (Thee) and 95% EtOH, MeOH and EtOAc extracts from the fruit pulp of *L. acidissima* (Thee)

Chemicals

Agar 0.5%, 100 mL of distilled water, nutrient agar 2.8%

Apparatus

100 mL conical flask, petridishes, 500 mL beaker, measuring cylinder, micropipettes, test tube, centrifuge bottle, flask bottle

Instruments

Autoclave, water-bath, clean bench, hot-air sterilizer, incubator, centrifuges, loops

Microorganisms

Bacillus subtilis, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Bacillus pumilus, Candida albicans* and *Escherichia coli.* These were obtained from the Central Research and Development Center (CRDC), Yangon.

Procedure

Nutrient agar was prepared according to method described by Cruickshank, 1975. Nutrient agar was boiled and 20–25 mL of the medium were poured into the test tube and plugged with cotton wool and autoclaved at 121 °C for 15 minutes in an autoclave. After this, the tubes were cooled down to 30–35 °C and poured into sterilized petridished and 0.02 mL of spore suspension was also added into the dishes. The agar was allowed to set for 2 hours after which 10 mm plate agar disc was made with the help of sterilized cork borner. After that, about 0.1 mL of sample was introduced into the agar –disc and incubated at 37 °C for 24 hours. The inhibition zone (clear zone) which appeared around the agar-disc indicated the presence of antimicrobial activity.

2.8.2 Acute toxicity test

Preparation of plant extracts

Dried powdered sample of (1 kg) of bark of *L. acidissima* (Thee) was extracted three times with 95% ethanol (2 L) at room temperature and filtered. The filtrates were combined and concentrated under vacuum rotatory evaporator to get the crude extract. 95% ethanolic extract of *L. acidissima* (Thee) was made to give the serial dilution of 2, 4, 8, 12 and 16 g/kg in volume of 10 mL for the acute toxicity test.

Theory

The purposes of acute toxicity testing are to obtain information on the biological activity of a chemical and gain insight into its mechanism of action. Long term studies usually start with a dose-finding exercise under acute conditions. Furthermore, the information on acute systemic toxicity generated by the test is used in hazard identification and risk management in the context of production, handling and use of chemicals. The LD_{50} value (precise or approximate) is currently the basis for toxicological classification of chemicals and is thus required by government authorities in different situations. The dosed animals are closely observed during the first 24 h and then day by day for as long as 2 weeks and changes in appearance and behaviors are noted. A large number of clinical signs can be used to characterize acute systemic toxicity and describe its progression (Walum, 1998).

Procedure

The acute toxicity test was done by the method of Litchfield and Wilcoxon. In this study a total of 60 adult albino mice of both sex weighing (25-30 kg) were selected for this experiment. The selected animals were housed in acrylic cages at standard environmental condition at 25 ± 2 °C, in a well- ventilated room maintained at 12: 12h light: dark cycle. The mice were fasted for 18 hours and they were divided into 5 groups, each group containing of 10 mice. The mice were individually marked on the part of body with picric acid staining for each group in different sites (e.g., Head, neck, back, tail, head back, head neck, head tail, neck tail, right leg, left leg) and administered orally with various concentrations of extracts.

Group I, II, III, IV and V mice were treated with 95% ethanolic extract from the bark of *L. acidissima* (Thee). The groups were treated with different doses by oral route using intragastration of the extract. Another group mice was treated with 10 mL/kg of distilled water and served as control.

After administration of the drug, the mice were separately housed in standard acrylic cages and allowed to food and water.

The mice were observed continuously for the first six hours for mortality and behavioral changes if any and then every 24 h for 14 days. During this period, the observation for the number of death of mice was noted. Each study was done separately. The mice were also closely examined for signs of intoxication, lethargy, behavioral modification and co-morbid state and the results obtained will be discussed in Section 3.6.2. The LD_{50} of 95% ethanolic extract was determined by using the simplified statistical methods of Litchfield and Wilcoxon for evaluation dose effect experiments. Then the LD_{50} was calculated by data obtained from varying dose of plant extract (Litchfield and Wilcoxon, 1949).

2.8.3 Screening of antioxidant activity of *L. acidissima* (Thee) by using DPPH free radical scavenging assay

DPPH (1, 1-diphenyl- 2-picryl-hydrazyl) radical scavenging assay was chosen to assess the antioxidant activity of plant materials. This assay has been widely used to evaluate the free radical scavenging effectiveness of various flavonoids and polyphenol in food systems.

In this experiment, the antioxidant activity was studied on watery extract from selected plant samples by DPPH free radical scavenging assay.

Sample

95% ethanolic extracts of from the bark of *L. acidissima* (Thee)

Chemicals

95% EtOH, 1, 1-diphenyl- 2-picryl-hydrazyl (DPPH) and Ascorbic acid.

Apparatus

Cell (5 cm^3), vortex mixer, UV-visible spectrophotometer, electric balance and micro pipette (10 mL and 5 mL).

Preparation of solutions

Preparation of 60 μ M DPPH solution

DPPH (2.364 mg) was thoroughly dissolved in 95% ethanol (100 mL). This solution was freshly prepared in the brown colour flask. Then it must be stored in the fridge for no longer than 24 hours.

Preparation of test sample solution

Each 2 mg of test sample and 10 mL of 95% EtOH were thoroughly mixed by vortex mixer. The mixture solution was filtered and the solution was obtained. Desired concentration 12.5 μg/mL, 25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL and 400 μg/mL of solution were prepared from this stock solution of dilution with appropriate amount of 95% ethanol.

Preparation of blank solution

Blank solution was prepared by mixing the test sample solution (1.5 mL) with 95% ethanol (1.5 mL).

Procedure

DPPH radical scavenging activity was determined by UV spectrophotometric method. The control solution was prepared by mixing 1.5 mL of 60 μM DPPH solution and 1.5 mL of 95% ethanol using vortex mixer. The sample solution was also prepared by mixing thoroughly 1.5 mL of 60 μM DPPH solution and 1.5 mL of test sample solution. The solutions were allowed to stand at room temperature for 30 min. After 30 min, the absorbance of these solutions was measured at 517 nm by using UV spectrophotometer. Absorbance measurements were done in triplicate for each

solution and then mean values so obtained were used to calculate percent inhibition of oxidation by the following equation.

$$\% \text{ Inhibition} = \frac{A - (B - C)}{A} \times 100$$

where,

% Inhibition = % inhibition of test sample

A = absorbance of control solution

B = absorbance of sample solution

C = absorbance of blank solution

$$\text{Average, } X = \frac{x_1 + x_2 + \ldots + x_n}{n}$$

where, X = average % inhibition

$x_1 + x_2 + \ldots x_n$ = % inhibition of test sample solution

n = number of times

$$\text{Standard Deviation (SD)} = \sqrt{\frac{(\bar{x} - x_1)^2 + (\bar{x} - x_2)^2 + \ldots + (\bar{x} - x_n)^2}{n - 1}}$$

Then, % inhibition were plotted against respective concentrations used and from the graph the IC50 was calculated using ascorbic acid, as a positive control. Three replicates of each sample were used for statistical analysis and the values are reported as mean ± SD. Then, IC_{50} (50% inhibitory concentration) values were also calculated by linear regressive excel program.

2.9 Preparation of Sunscreen Lotions from the Bark and Fruit Pulp of *L. acidissima* (Thee) and Screening of their Whitening Effect

Preparation of plant extracts

Each of the dried powdered of *L. aciissima* (Thee) (500 g) was macerated for three days with 95 % ethanol and filtered. The filtrates were concentrated under vacuum rotary evaporator under reduced pressure. Each extract was weighed and dissolved in ethanol.

Procedure

Accurate quantities of cetyl alcohol, zinc oxide, stearic acid, glycerin and hydroxyl propyl cellulose (HPC) were weighed (Table 2.1). Accurate quantity of water was measured and taken in 400 mL beaker. 1.0 g of tri-ethanolamine was added to water and stirred. The water solution was heated up to a temperature of 80-85 °C. After the solution has reached the required temperature, melted cetyl alcohol, zinc oxide, stearic oxide, glycerine, HPC mixture and propyl paraben were slowly poured into the solution a little at a time, stirring constantly. Stirring was continued until a smooth and uniform paste was obtained. The prepared sunscreen lotions were set aside to cool. Then weighed quantity of extracts and vitamin E were added and stirred well until all the ingredients mixed uniformly. Finally rose oil was added as a flavoring agent (Arun *et al.,* 2012).

Table 2.1 Composition of Various Sunscreen Formulations

Ingredients	F1 (%)	F2 (%)
Ethanol bark extract	10.0	-
Ethanol fruit extract	-	10.0
Olive oil	3.0	3.0
Rose oil	1.0	1.0
Rose water	3.0	3.0
Cetyl alcohol	2.0	2.0
Zinc oxide	12.0	12.0
Stearic acid	4.0	4.0
Glycerine	2.0	2.0
Vitamin E	1.0	1.0
Triethanolamine	1.0	1.0
Hydroxy propyl cellulose	10.0	10.0
Propyl paraben	0.50	0.50
Distilled water	50.50	50.50

2.9.1 Study on the skin irritation test *in vivo* with the wistar albino rats

The skin irritation test was performed on albino rats of both sexes weighing about 150-200 g. Fifteen albino rats, originated from the animal house, Department of Medical Research (Lower Myanmar, Yangon) breeding colony was used. The animals were maintained on standard animal feed and free access to water and kept in a mild light room. All rats had hair removed from the back (2 cm x 2 cm) to facilitate contact with the sunscreen lotions. A total of five experimental groups with three animals per group were formed as follows: G1 the group served as control, G2 the group tested with Thee bark sunscreen lotion (F 1), G3 the group tested with the Thee fruit pulp sunscreen lotion (F 2), G4 the group tested with the marketed sunscreen lotion SPF 22 and G5 the group tested with the marketed sunscreen SPF 50. 5 mL of lotions was applied twice aday for three days and the site as observed for any sensitivity, edema and erythema.

2.9.2 Evaluation of the skin whitening effect of sunscreen lotions on albino rats

Skin whitening effect was studied by adopting the method reported by (Yusutami *et al.*, 2004) with some modification.

Test animals

Diseases free six female albino rats with average weight (500 g) were obtained from DMR (Department of Medical Research).

Procedure

Sunscreen lotion F1 was formulated from the bark of extract and F2 was formulated from the fruit pulp extract of *L. acidissima* (Thee). F1 and F2 were selected to test the skin whitening activity. The 2 albino rats were selected for each group. Group 1 as a control, group 2 was for treatment with F1 and group 3 was for F2. The back of skin of albino rats was washed and about (2 cm x 2 cm) area was cleanly shaved. The shaven skin was exposed to sunlight daily (10-11 AM and 2-3 PM, 2h/day) for one week. After two weeks, the hyperpigmentation (tanning) on skin was clearly noticed by naked eye.

After the sunlight induced on hyperpigmented skin of the albino rats, 3 mL of sunscreen lotions F1 and F2 were applied twice aday for two successive weeks. Finally, the whitening effect of the sunscreen lotions on hyperpigmented skin was evaluated by viewing with naked eye while comparing with control group.

2.9.3 Evaluation of the skin whitening effect of sunscreen lotions on human skin

Skin whitening activity of sunscreen lotions F1 and F2 were evaluated by using sun burning skin of human.

Procedure

Sunscreen lotions F1 and F2 were selected to test skin whitening activity. The back of the hand and foot of sun burning human skin was cleaned. (2 cm x 2 cm) area was supposed for application area. And then, 5 mL of sunscreen lotion F1was applied on back of the hand and 5 mL of sunscreen lotion F2 was applied on back of the foot. Sunscreen lotions were applied thrice a-day for 3 days successively.

Finally, the whitening effect of sunscreen lotions on tested area was evaluated by viewing with naked eye while comparing with the control area.

2.9.4 Determination of sun protection factor (SPF) of sunscreen lotions by ultraviolet spectrophotometry

Preparation of sunscreen lotions

0.10 % solution (w/v) of the sunscreen lotions in n-propyl alcohol was prepared by dissolving 0.05g of the sunscreen lotions in 50.0 mL of n-propyl alcohol. 0.10 % solution of the two selected commercial sunscreen lotions (SPF 22 and 50) in n-propyl alcohol was also prepared.

Procedure

The sunscreen lotions of the *L. acidissima* (Thee) were analysed for the *in vitro* by using UV spectrophotometer. The method *in vitro* are in general of two types. Methods which involve the measurement of absorption or the transmission of UV

radiation through sunscreen product films in quartz plates or biomembranes, and methods in which the absorption characteristics of the sunscreen agents are determined based on the spectrophotometric analysis of dilute solutions. Scanning spectra of the sunscreen lotion sample in solution was obtained by running from 290 nm to 320 nm with 5 nm intervals. The SPF was calculated by using the equation derived by Mansaur (Mansaur 1986; Santo., 1999). Mansur *et al.*(1986), developed a very simple mathematical equation which substitutes the *in vitro* method proposed by Sayre *et al.*, (1979), utilizing UV spectrophotometry and the following equation:

$$SPF = CF \times \Sigma_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

Where EE (λ) – erythemal effect spectrum; I (λ) - solar intensity spectrum; Abs (λ) – absorbance of sunscreen product; CF – correction factor (= 10). The values of EE x I are constants. They were determined by Sayre *et al.*, (1979), and are shown in Appendix I.

CHAPTER III

3. RESULTS AND DISCUSSION

3.1 Collection of Plant Sample

The stem bark of *Limonia acidissima* L. (Thee) was collected from Yenangyaung Township, Magway Region in February, 2012. Authentication of plant was done by the authorized botanist at Botany Department, University of Yangon. After cleaning the collected sample, it was air-dried at room temperature to prevent some reactions of phytochemical constituents with sunlight. Then it was ground into powder by a motor and stored in air-tight container to prevent moisture and other contaminations and kept to use for isolation of organic compounds and bioactivity tests.

3.2 Phytochemical Investigation of *L. acidissima* (Thee)

In order to classify the types of phytochemical constituents present in selected sample of *L. acidissima* (Thee), preliminary phytochemical investigation was carried out according to the procedures as mentioned in Section 2.2.

According to these results, α-amino acids, alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, starch, steroids and tannins were found to be present in the sample. These results are summarized in Table 3.1.

Table 3.1 Results of Preliminary Phytochemical Investigation on the Stem Bark and Fruit Pulp of *L. acidissima* (Thee)

Sr. No	Tests	Extracts	Test Reagents	Observations	Remarks	
					Stem bark	Fruit pulp
1.	Alkaloids	1%HCl	Mayer's reagent	White ppt	+	+
			Dragendroff's reagent	Orange ppt	+	+
			Wagner's reagent	Brown ppt	+	+
2.	α-amino acids	H_2O	Ninhydrin reagent	Pink spot	+	++
3.	Carbohydrates	H_2O	10% α-naphthol & conc. H_2SO_4	Red ring	+	+
4.	Flavonoids	EtOH	Mg ribbon & conc. HCl	Reddish	+++	+++
5.	Glycosides	H_2O	10% lead acetate	White ppt	++	+++
6.	Phenolic compounds	EtOH	5% $FeCl_3$ solution $K_3Fe(CN)_6$	Deep blue	++	+++
7.	Reducing sugars	dil H_2SO_4	Dil NaOH solution & benedict's solution	Brick red ppt	+	+
8.	Starch	H_2O	Iodine	Blue color	+	+
9.	Steroids	PE	Acetic anhydride & H_2SO_4 (conc.)	Green	+++	+++
10.	Tannins	H_2O	Gelatin and 1% $FeCl_3$	White ppt	++	+

(+) = present

(–) = absent

(++), (+++) = present in large amount

3.3 Qualitative Elemental Analysis of Plant Samples by Atomic Absorption Spectroscopy (AAS) Method

In this work, heavy toxic metals and macronutrient mineral elements present in the dried powdered samples of bark and fruit pulp of *L. acidissima* (Thee) were determined by atomic absorption spectrophotometer. The experimental details have been described in Section 2.3. The data of the samples are shown in Table 3.2. It can be found that Na (0.742 ppm), K (14.90 ppm), Zn (0.134 ppm) and Mn (0.211 ppm) are present in the bark of *L. acidissima* (Thee). It can be also found that Na (1.129 ppm), K (14.80 ppm), Zn (0.2015 ppm) and Mn (0.163 ppm) are present in the fruit pulp of the *L. acidissima* (Thee).

It can be seen that potassium is the most predominant element in these two samples. The high content of potassium can protect the body against paralysis, muscles weakness, kidney damage and regulate the heartbeat.

3.4 Determination of Nutritional Values of Bark of *L. acidissima* (Thee)

Some nutritional values, such as moisture, ash, fibre, fat, protein, carbohydrates and energy value of the bark of *L. acidissima* (Thee) were determined. The experimental details have been shown in Section 2.4. The results for these contents are summarized in Table 3.3.

Table 3.2 Elemental Content in the Bark and Fruit Pulp of *L. acidissima* (Thee) by AAS Method

Element	Bark (ppm)	Fruit Pulp (ppm)
Na	0.742	1.129
K	14.90	14.80
Zn	0.134	0.2015
Mn	0.211	0.163

Table 3.3 Nutritional Values of Bark of *L. acidissima* (Thee)

No.	Nutritional Values	AOAC Methods	Content (%)
1	Moisture	Oven drying method	9.25
2	Ash	Muffle furnace method	6.72
3	Fibre	Acid- base treatment	55.20
4	Fat	Soxhlet extraction method	0.48
5	Protein	Kjeldahl digestion method	5.01
6	Carbohydrate	100 - (protein+ fat + moisture + fibre+ ash)	23.07
7	Energy value (kcal/100g)	[Protein (g) x 4 + Fat (g) x 9 + Carbohydrate (g)x 4]	121

3.5 Identification of some Isolated Compounds from Bark of *L. acidissima* (Thee)

3.5.1 Identification of isolated compound I and II

Compounds I and II were isolated as a mixture. It appeared as a colorless needled crystals (0.005 %). From EtOH soluble fraction of EtOAc extract of bark of *L. acidissima* (Thee) via column chromatographic separation. Its R_f value on TLC with PE: EA (9:1 v/v) solvent system was found to be 0.45 and 0.56.

3.5.1.1 Study on UV spectrum of isolated compounds I and II

The UV spectra of compounds I and II are described in Figure 3.1. The wavelength of maximum absorption of compounds I and II were found to be at 248 nm, 266 nm and 307 nm (see Table 3.4).

3.5.1.2 Study on FT IR spectrum of isolated compounds I and II

The functional groups present in compounds I and II were studied by FT IR spectrum peak (see Figure 3.2). Its assignment is described in Table 3.5. Peaks in weak intensity at 3025 cm^{-1} was attributed at the C-H stretching vibration of coumarin. In addition, aliphatic C-H stretching vibration appeared at 2886 cm^{-1}. A strong band at 1715cm^{-1} that represented C=O stretching vibration for α, β unsaturated ketone. The weak peaks at 1580 cm^{-1} and 1473 cm^{-1} indicating the C=C stretching vibration for aromatic ring. The bending vibration of aliphatic C-H groups appeared at 1353 cm^{-1}. Band at 1124 cm^{-1} could be interpreted as C-O stretching vibration for methoxy group. In addition, C=H bending vibration of aromatic system was found at 824 cm^{-1}.

Muller *et al.* (2004) reported characteristic absorption peaks of xanthotoxin at $\bar{\nu}$ 3121 cm^{-1}, 3080 cm^{-1}, 3040 cm^{-1}, 1705 cm^{-1}, 1626 cm^{-1}, 1580 cm^{-1}, 1540 cm^{-1} and 875 cm^{-1} and those of isopimpinellin at 1720 cm^{-1}, 1597 cm^{-1}, 1477 cm^{-1}, 1356 cm^{-1}, 1142 cm^{-1}, 1069 cm^{-1}, 818 cm^{-1} and 748 cm^{-1}.

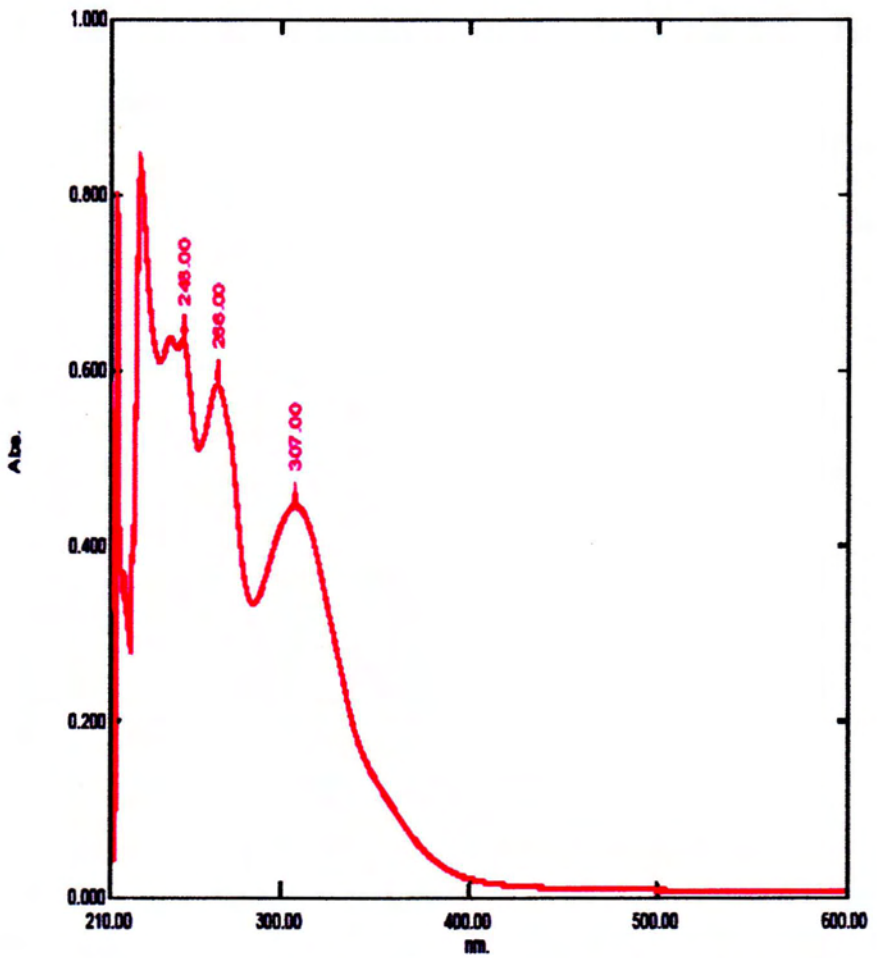


Figure 3.1 UV spectrum of isolated compounds I and II (in MeOH)

Table 3.4 UV Spectral Data Assignment of Isolated Compounds I and II

Reagent	Observed λ_{max} (nm)	Reported λ_{max} (nm)	
		Compound I*	Compound II**
MeOH	248	249	248
	266	269	263
	307	313	303

* Wu *et al.*, 2003 ** Mu *et al.*, 2004

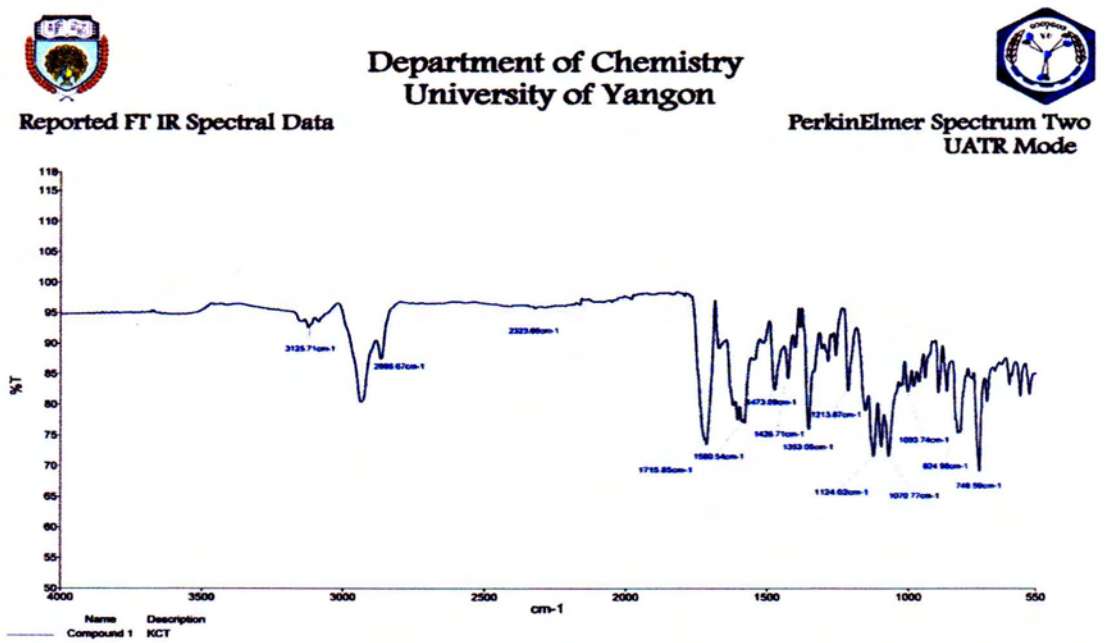


Figure 3.2 FT IR spectrum of isolated compounds I and II

Table 3.5 FT IR Spectral Data Assignment of Isolated Compounds I and II

Wave number (cm^{-1})	Band Assignment
3025	= CH stretching for aromatic ring
2886	C – H stretching (asymmetric and symmetric) for CH_3
1715	C = O stretching vibration for α, β unsaturated ketone
1580	C = C stretching for aromatic ring
1473	C – H bending for CH_2 and CH_3
1353	C – H bending for CH_3
1124	C – O stretching for methoxy group
824	C – H bending for aromatic ring

3.5.1.3 Study on ^{1}H NMR spectra of isolated compounds I and II

^{1}H NMR spectra of isolated compounds I and II ($CDCl_3$, 300 MHz) are shown in Figures 3.3 and 3.4. Band assignments are presented in Table 3.6. Examination of ^{1}H NMR clearly revealed compounds I and II as a mixture of two coumarins. Both compounds exhibited the typical linear furocoumarin skeletons. In addition, the presence of aromatic methoxy group suggested that these compounds would be methoxylated furanocoumarins. It revealed the presence of 8 protons including 3 protons of one methoxy group for compound I at C-9 that was found at δ 4.26 ppm as one singlet. It also showed signals due to the pyrone ring at δ 7.75 (1H, *d*, J = 2.4 Hz, H-5) and 6.35 (1H, *d*, J = 9.6 Hz, H-6).The aromatic methoxyl group at C-9 which was found at δ 4.26 (3H, s, 9-OCH_3) as a singlet. The protons on the furan ring appeared as two doublets at δ 6.82 (1H, *d*, J = 2.4 Hz, H-3) and 7.65 (1H, *d*, J = 9.6 Hz, H-2), both protons were mutually coupled. These couplings were confirmed by using ^{1}H-^{1}H COSY spectrum. Furthermore, the benzene ring proton at δ 7.38 (1H, s) occurred at H-4 position.

The ^{1}H NMR spectral data of isolated compound II were similar to those of I except for the appearance of methoxy groups at δ 4.15 ppm (H-4OCH_3) and (H-9OCH_3) and the disappearance of one of the aromatic proton (H-4).

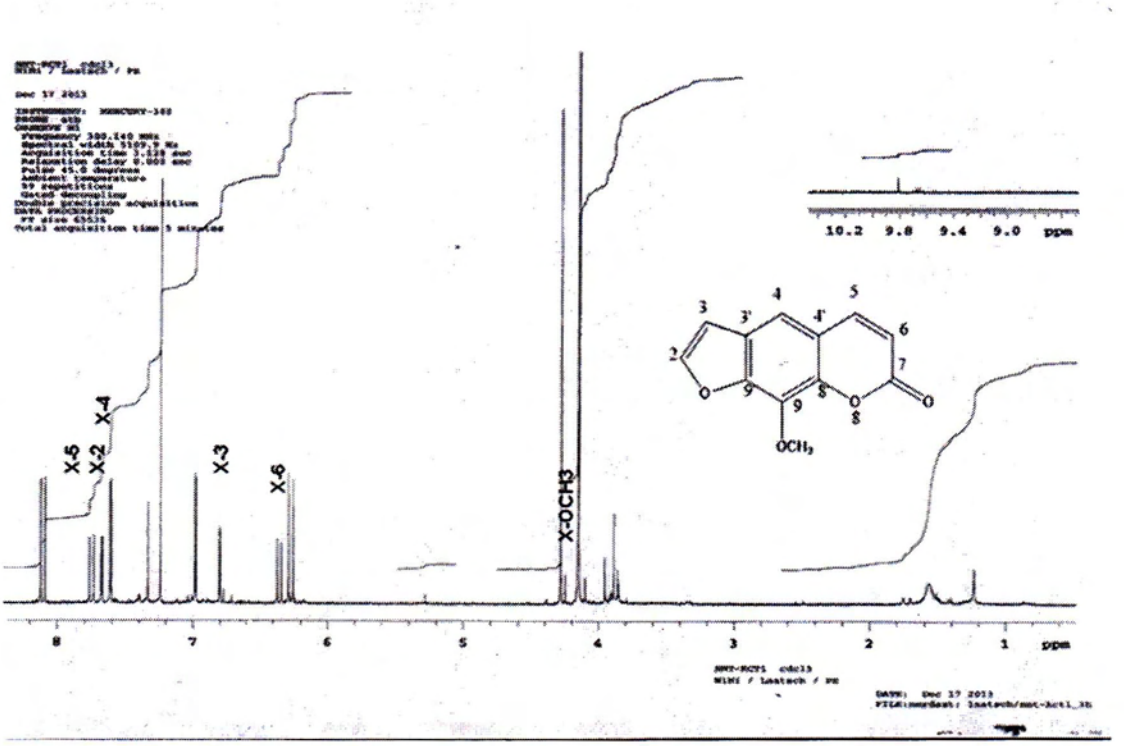


Figure 3.3 ^{1}H NMR (300MHz, $CDCl_3$) spectrum of isolated compound I

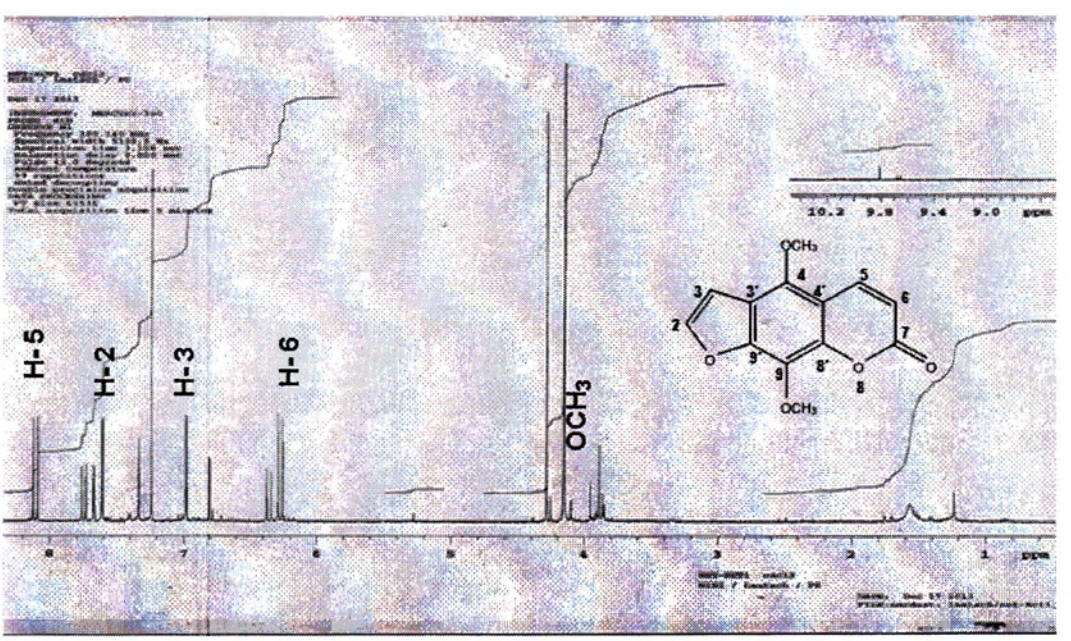


Figure 3.4 ^{1}H NMR (300MHz, $CDCl_3$) spectrum of isolated compound II

Table 3.6 **^{1}H NMR Spectral Data of Isolated Compounds I and II and Reported Xanthotoxin and Isopimpinellin**

Position	δ_H (ppm), *J* (Hz)			
	Compound I	**Xanthotoxin ***	**Compound II**	**Isopimpinellin ****
H-2	7.65 *(1H,d)*	7.67	7.6 *(1H, d, 2.3Hz)*	7.56
H-3	6.82 *(1H,d)*	6.82	6.98 (1H, *d*, 2.3Hz)	6.93
H-4	7.38 *(1H,s)*	7.34	-	-
H-5	7.75 *(1H,d)*	7.76	8.10 *(1H, d, 9.8Hz)*	8.06
H-6	6.35 *(1H,d)*	6.36	6.28 *(1H, d, 9.8Hz)*	6.22
4OCH$_3$	-	-	4.15 *(3H, s)*	4.10
9OCH$_3$	4.26 *(3H,s)*	4.27	4.15 *(3H, s)*	4.11

* **Wu *et al.*, 2003**

** **Mu *et al.*, 2004**

3.5.1.4 Study on ^{13}C NMR spectra of isolated compounds I and II

The ^{13}C NMR spectra of isolated compounds I and II ($CDCl_3$, 75 MHz) are shown in Figures 3.5 and 3.6. The ^{13}C NMR spectrum revealed 12 carbon atoms for compound I that consistent with the molecular structure explored from ^{1}H NMR spectrum. These carbon peaks were classified as one ester carbonyl carbon, one methoxyl carbon, four quaternary aromatic carbons, five aromatic methine carbons and one oxygenated aromatic carbon. Peak assignment is shown in Table 3.7. The peak appeared at δ 160.4 could be assigned as C=O group of (C-7). One methoxyl carbon peak at δ 61.3 (9-OCH_3). Four aromatic quaternary carbon peaks observed at δ 126.1, 116.5, 143.0 and 147.7 were due to C-3′, C-4′, C-8′ and C-9′. Five aromatic methine carbon peaks observed at δ 146.5 (C-2), 106.7 (C-3), 112.4 (C-4), 144.3 (C-5) and 114.5 (C-6). In addition, the remaining peak found at δ 132.8 could be designated as oxygenated aromatic carbon (C-9).

^{13}C NMR spectrum of compound II ($CDCl_3$, 75 MHz) exhibited 13 carbons present in the molecule. The spectrum showed two methoxyl groups at δ 61.7 (C-4OCH_3) and 60.8 (C-9OCH_3). The most downfield signals of the carbon spectrum at 107.6 (C-4′), 114.8 (C-3′), 143.0 (C-9), 145.1 (C-2′) and 150.0 (C-9′) were characteristic of quaternary carbons bearing oxygen functions. The ^{1}H NMR and ^{13}C NMR assignments were confirmed by using HSQC and HMBC heteronuclear coupling experiments.

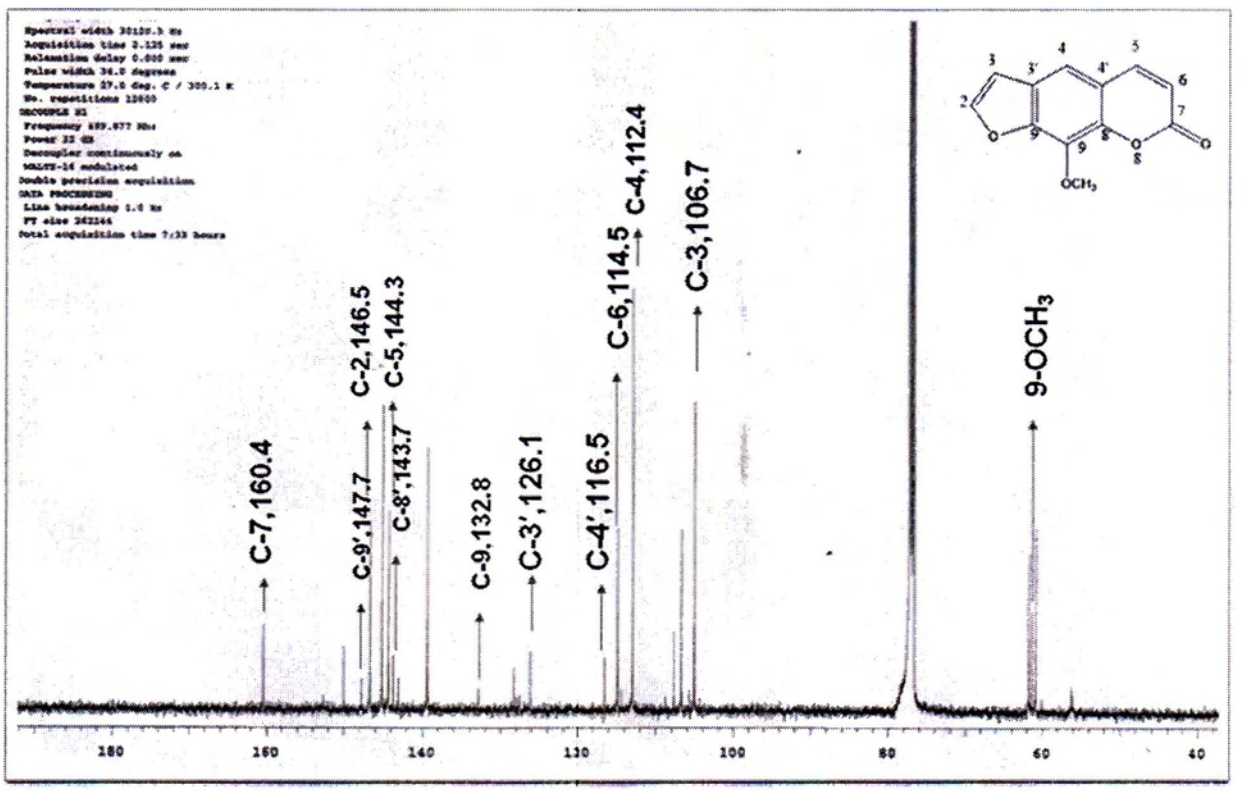


Figure 3.5 ^{13}C NMR (75 MHz, $CDCl_3$) spectrum of isolated compound I

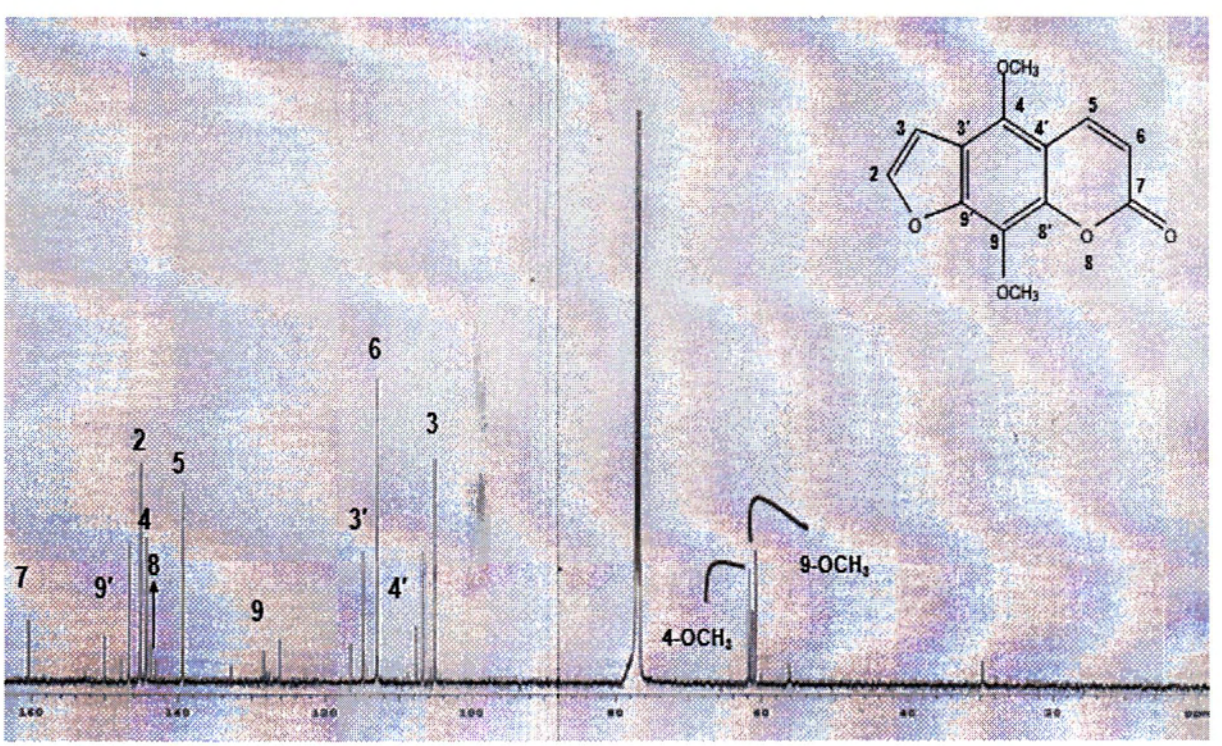


Figure 3.6 ^{13}C NMR (75 MHz, $CDCl_3$) spectrum of isolated compound II

Table 3.7 **^{13}C NMR Spectral Data of Isolated Compounds I and II and Reported Xanthotoxin and Isopimpinellin**

Position	δ_C (ppm)			
	Compound I	Xanthotoxin *	Compound II	Isopimpinellin **
C-2	146.5	146.6	145.1	145.1
C-3	106.7	106.7	105.0	105.0
C-3′	126.1	126.3	114.8	114.8
C-4	112.4	112.9	144.3	144.0
C-4′	116.5	116.5	107.6	107.6
4OCH_3	-	-	61.7	61.7
C-5	144.3	144.4	139.3	139.3
C-6	114.5	114.8	112.9	112.9
C-7	160.4	160.7	160.5	160.5
C-8′	143.0	143.9	143.0	143.0
9OCH_3	61.3	61.2	60.8	60.8
C-9	132.8	132.6	128.2	128.2
C-9′	147.7	147.7	150.0	150.0

* Wu *et al.*, 2003

** Muller *et al.*, 2004

3.5.1.5 Study on the 2D NMR spectra of isolated compounds I and II

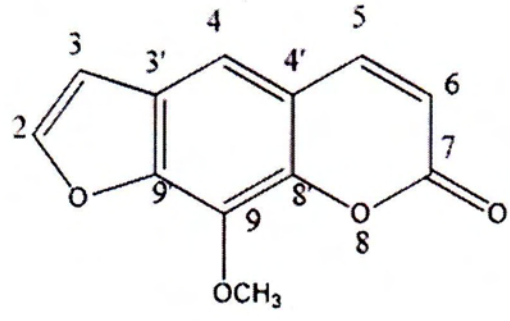
The ^{1}H-^{13}C Heteronuclear Single Quantum Coherence (HSQC) spectrum revealed all ^{1}H-^{13}C direct couplings and helped in the unambiguous assignment of ^{1}H-^{13}C NMR signals for all methine and methoxyl groups. The spectrum of HSQC for compound I revealed that the resonances for H-2 proton at δ 7.65 correlated with carbon signal for C-2 (146.5). The signals of aromatic methine protons of H-3, H-4, H-5 and H-6 at (δ 6.82, 7.38, 7.75 and 6.35) showed correlation with carbon signals at δ 106.7 (C-3), 112.4 (C-4), 144.3 (C-5) and 114.5 (C-6). In addition, the signal of methoxy proton of H-9OCH_3at (δ 4.26) directly attached with the respective carbon signal C-9OCH_3 (61.3).

For compound II, the aromatic proton signals at δ 7.6, 6.98, 8.10 and 6.28 were correlated with the carbons at δ 145.1, 105.0, 139.3 and 112.4. Therefore, the compounds I and II are very similar compounds, and the only difference is compound I has one methoxyl group, but compound II has two methoxyl groups.The methoxy protons at δ 61.7 (H-4OCH_3) and 60.8 (H-9OCH_3) were joined with the carbons at δ 4.15 pointing out the presence of two methoxy groups. The HSQC spectra for compound I and II are shown in Figures (3.7 and 3.8). The HSQC spectral data is summarized in Table 3.8.

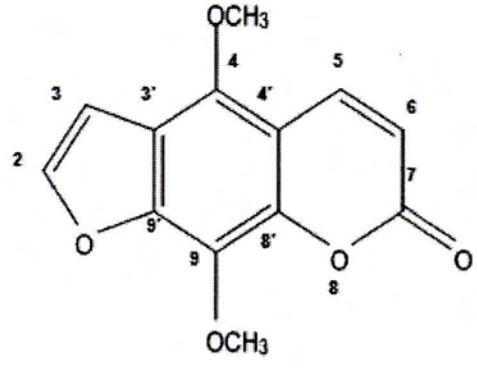
Some of the long range C-H correlation of the compound I are displayed by HMBC spectrum (Figure 3.9). According to the HMBC spectrum, the attachment of methoxy and carbonyl groups could be confirmed. The position of the methoxy group was confirmed by correlation of methoxy proton signal at δ 4.26 (H-9OCH_3) with carbon signal at 61.3 (C-9). The presence of carbonyl group was also confirmed by correlation of carbon peak at δ 112.4 (C-4), 160.4 (C-7) and 143.0 (C-8′) with proton signal at δ 7.75 (H-5). In the HMBC spectrum (Figure 3.10), the carbonyl carbon (C-7) can be correlated with H-5. The position of furan ring was confirmed by correlation of proton peak at 7.6 (H-2) with carbon signals at 145.1 (C-3), 112.4 (C-3′) and 61.7 (C-9′). Moreover, two methoxy protons H-4 and H-9 coupled with carbon peaks at 61.7 (C-4OCH_3) and 60.8 (C-9OCH_3). The detail data for HMBC spectra for compound I and compound II are shown in Figure 3.11 and 3.12.

Proton couplings (H-2 and H-3; H-5 and H-6) were observed in the ^{1}H-^{1}HCOSY (600 MHz, $CDCl_3$) spectrum. The H-H COSY spectra for compound I and compound II are shown in Figure 3.13 and 3.14.

The molecular formula and molecular mass of compound I and II could be seen in the ESI MS spectra (see Figures 3.15 and 3.16). From this spectrum, the molecular ion peak for compound I was observed at *m/z* 216 indicating the compound I has molecular weight 216 and which was consistent with the molecular formula $C_{12}H_8O_4$. And then, the molecular ion peak for compound II was observed at *m/z* 246 indicating the compound II has molecular weight 246 and which was with the molecular formula $C_{13}H_{10}O_5$.



Isolated compound I (Xanthotoxin)



Isolated compound II (Isopimpinellin)

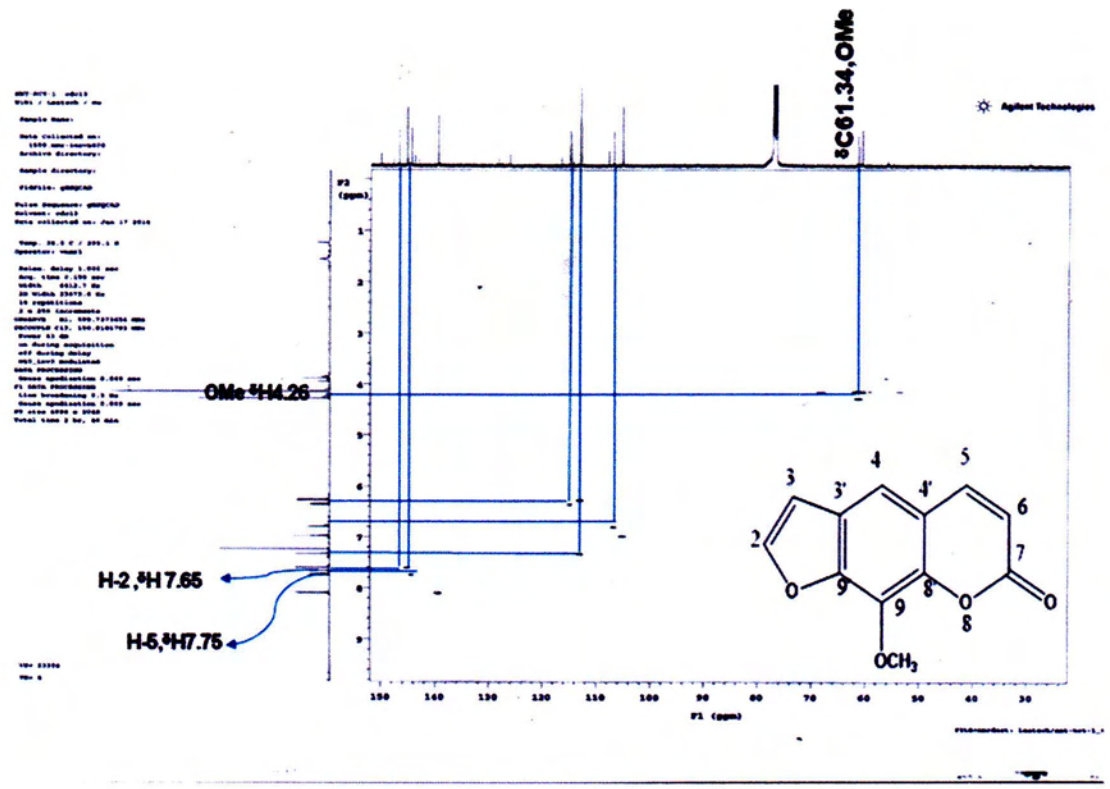


Figure 3.7 HSQC ($CDCl_3$, 600 MHz) spectrum of isolated compound I

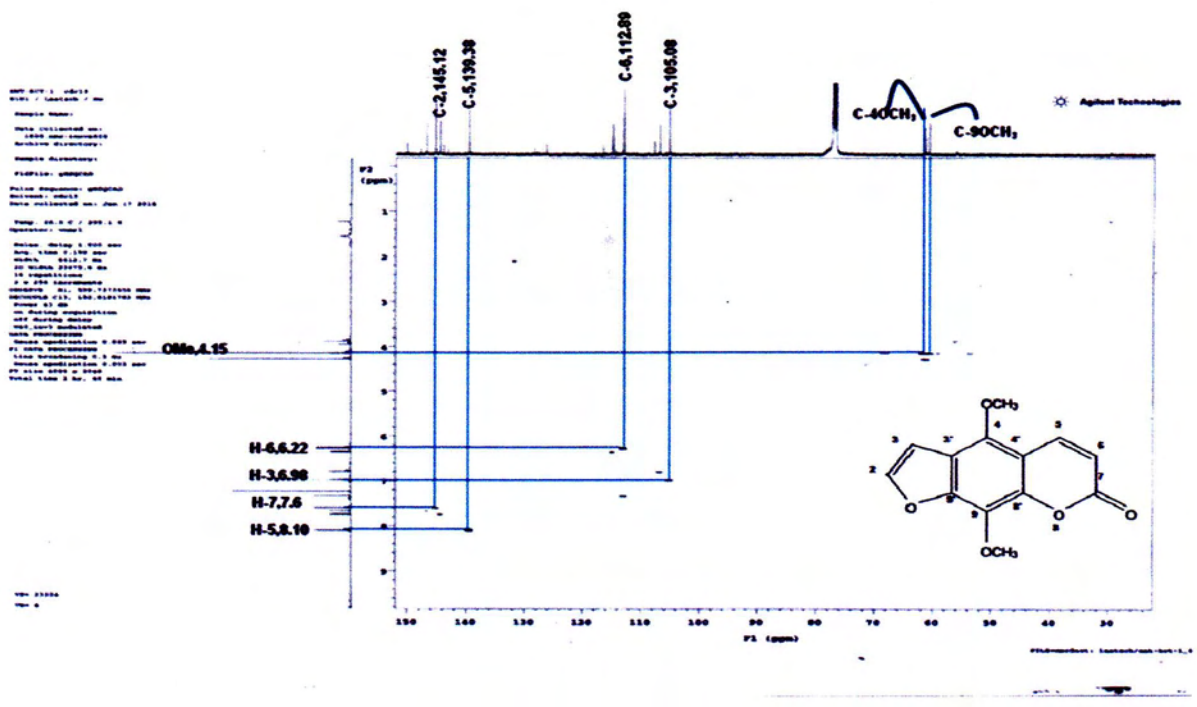


Figure 3.8 HSQC ($CDCl_3$, 600 MHz) spectrum of isolated compound II

Table 3.8 HSQC Spectral Data of Isolated Compounds I and II

Position	δ_H (ppm), *J* (Hz) ($CDCl_3$, 300MHz)		δ_C (ppm) ($CDCl_3$, 75 MHz)	
	Compound I	**Compound II**	**Compound I**	**Compound II**
C-2	7.65 (1H, *d*, *J* = 9.6 Hz)	7.6 (1H , *d* , 2.3 Hz)	146.5	145.1
C-3	6.82 (1H, *d*, *J* = 2.4 Hz)	6.98 (1H , *d* , 2.3 Hz)	106.7	105.0
C-4	7.38 (1H, *s*)	-	112.4	-
C-5	7.75 (1H, *d*, *J* = 2.4 Hz)	8.10 (1H , *d* , 9.8 Hz)	144.3	139.3
C-6	6.35 (1H, *d*, *J* = 9.6 Hz)	6.28 (1H, *d* , 2.3 Hz)	114.5	112.4
4-OCH_3	-	4.15 (3H, *s*)	-	61.7
9-OCH_3	4.26 (3H, *s*)	4.15 (3H , *s*)	61.3	60.8

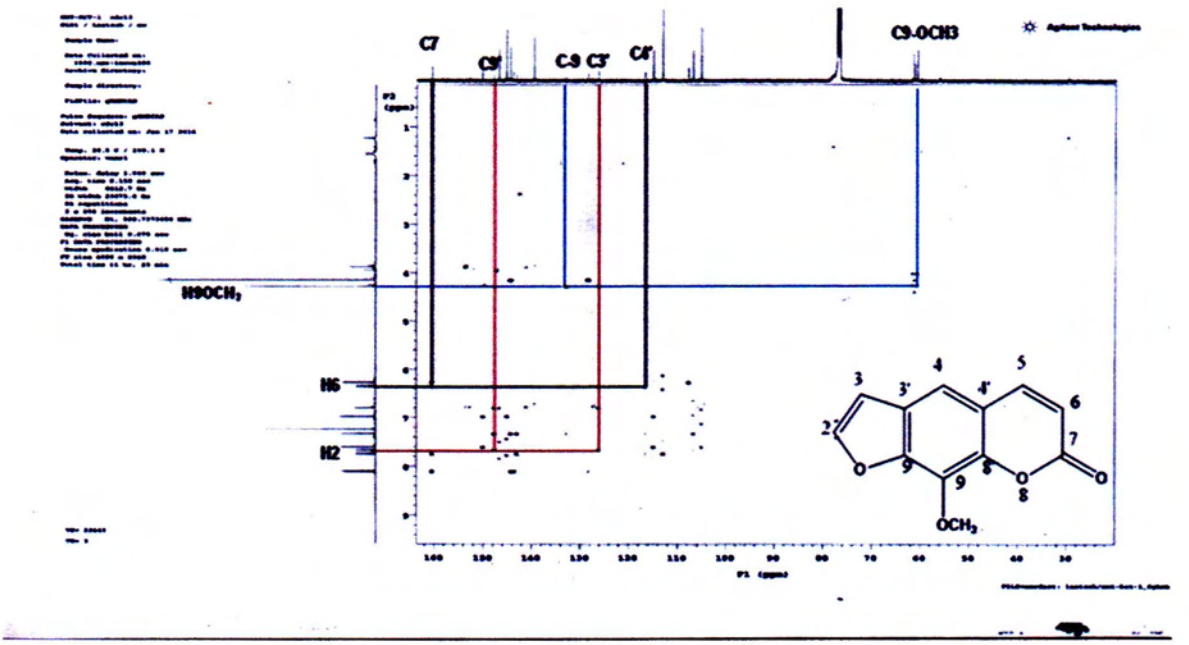


Figure 3.9 HMBC ($CDCl_3$, 600 MHz) spectrum of isolated compound I

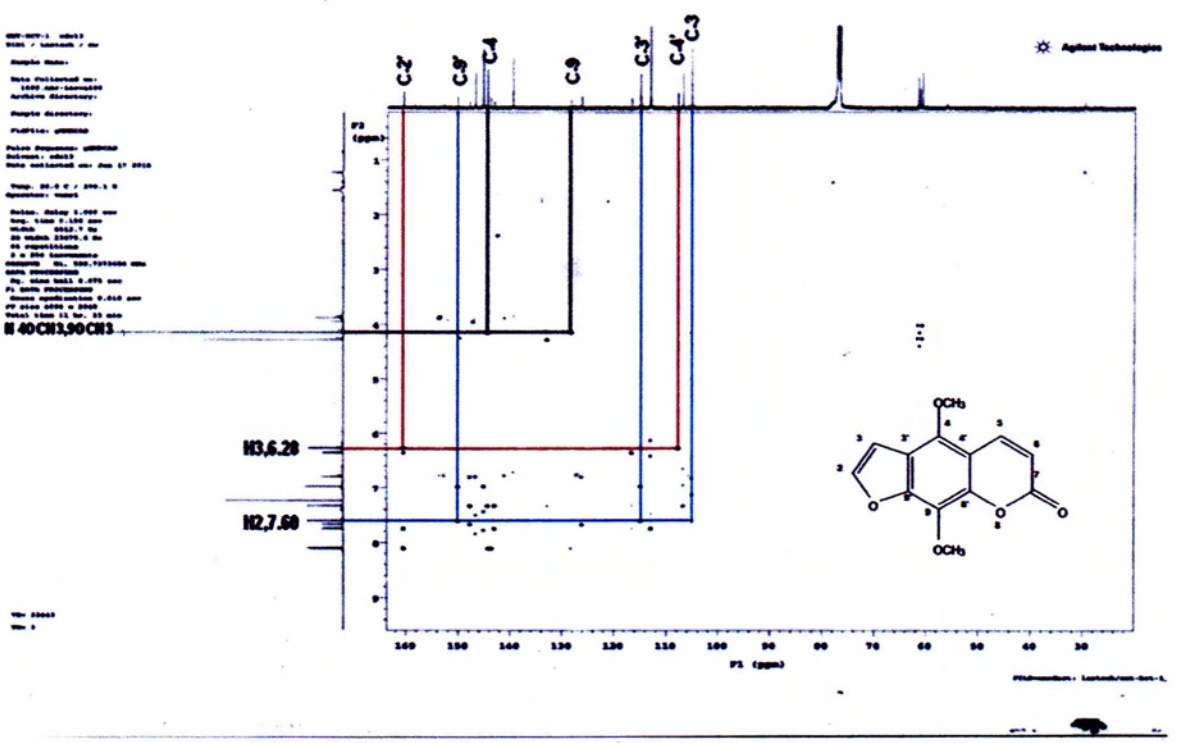
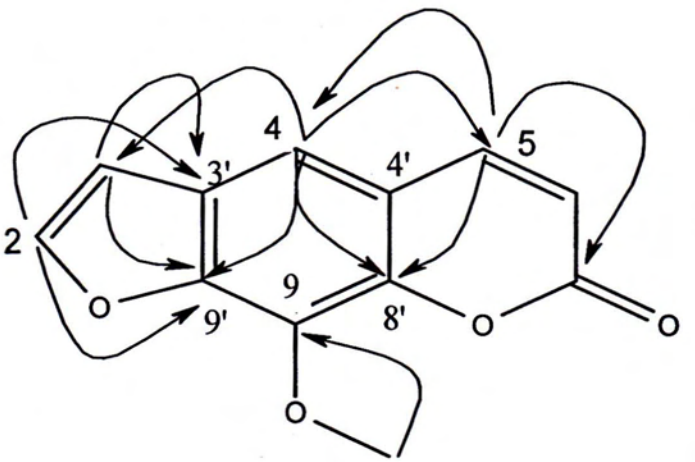
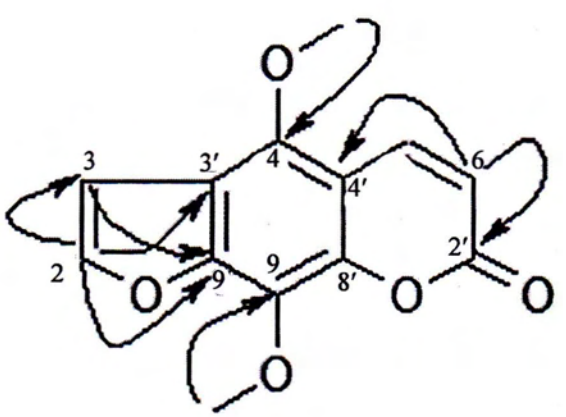


Figure 3.10 HMBC ($CDCl_3$, 600 MHz) spectrum of isolated compound II



H-2 ➡ C-3′, C-9′
H-3 ➡ C-3′, C-9′
H-4 ➡ C-3, C-5, C-8′, C-9′
H-5 ➡ C-4, C-7, C-8′
H-6 ➡ C-4, C-7
H-9OCH$_3$ ➡ C-9

Figure 3.11 Important HMBC correlations of isolated compound I



H-2 ➡ C-3, C-3′, C-9′
H-3 ➡ C-2′, C-4′
H-5 ➡ C-4, C-7, C-8′
H-6 ➡ C-2′, C-4′
H-4OCH$_3$ ➡ C-4
H-9OCH$_3$ ➡ C-9

Figure 3.12 Important HMBC correlations of isolated compound II

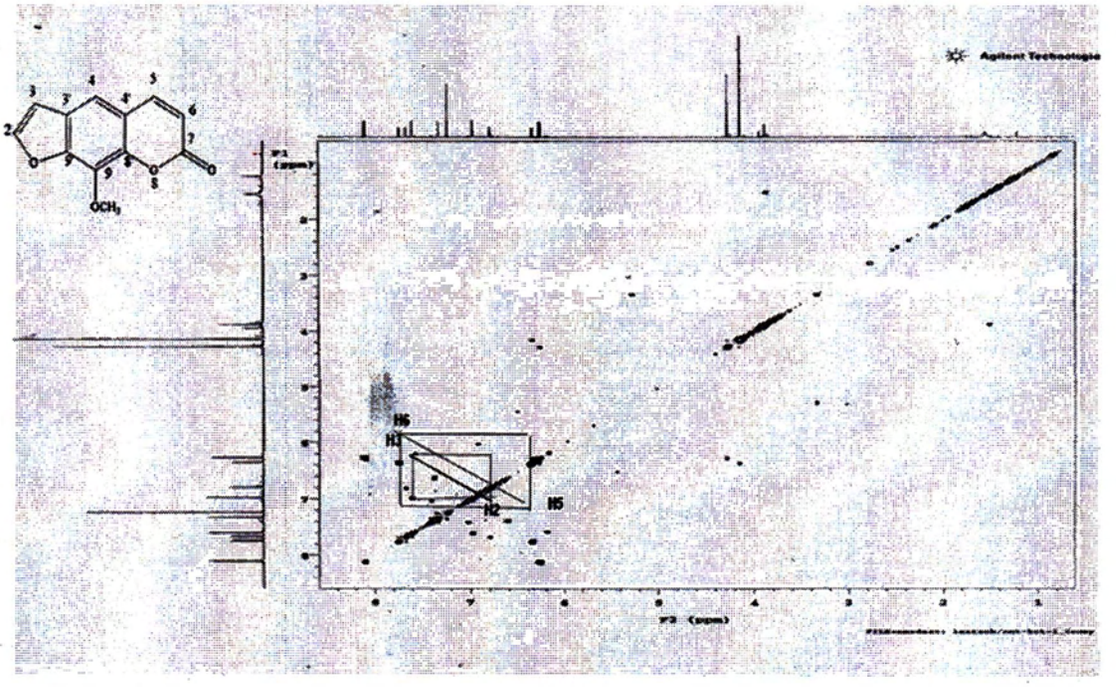


Figure 3.13 $^{1}H^{1}H$ COSY ($CDCl_3$, 600 MHz) spectrum of isolated compound I

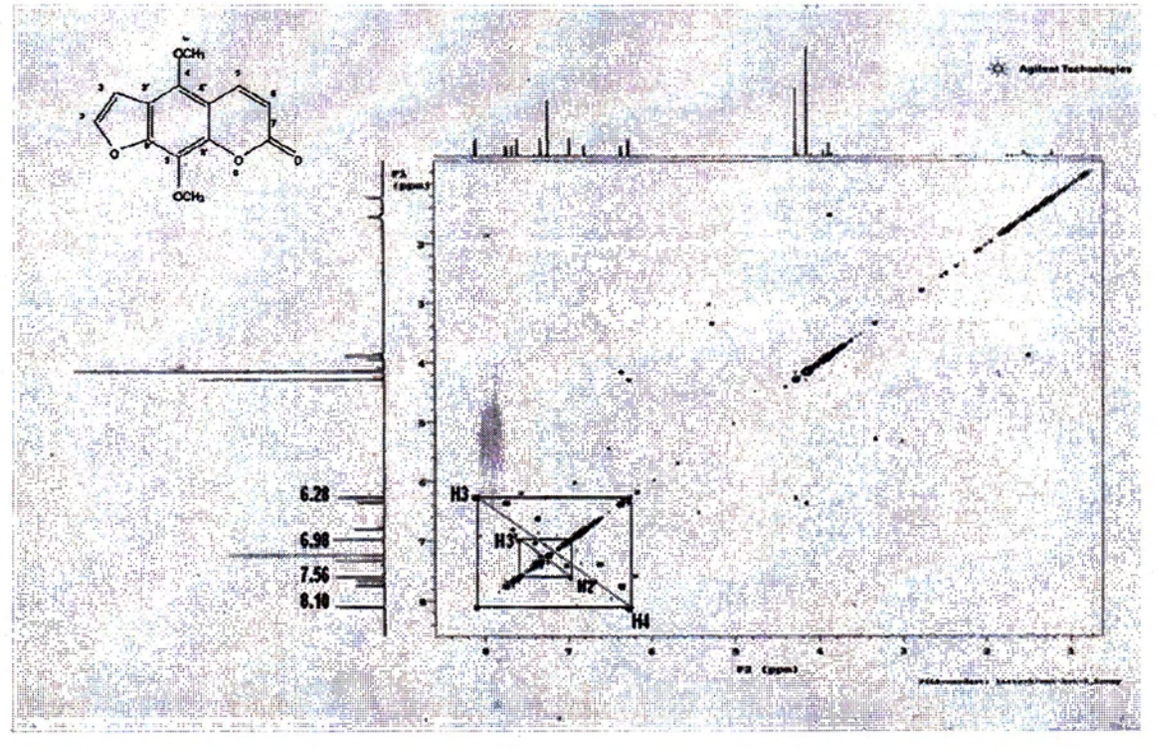


Figure 3.14 $^{1}H^{1}H$ COSY ($CDCl_3$, 600 MHz) spectrum of isolated compound II

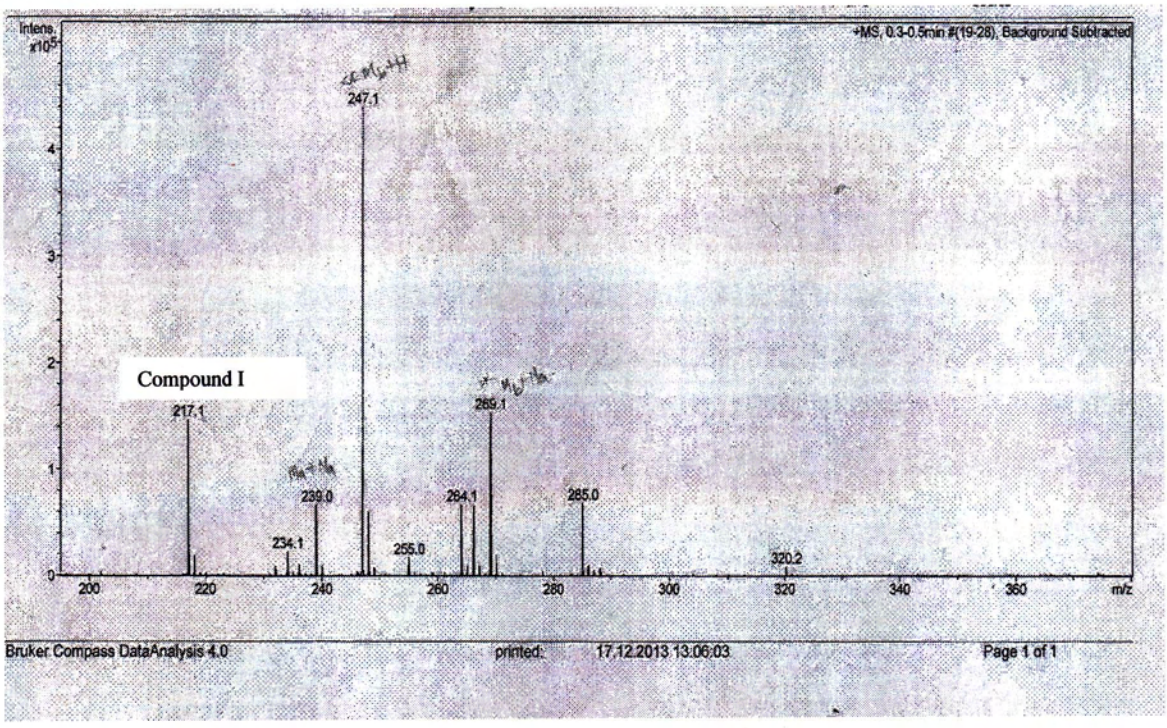


Figure 3.15 **ESI mass spectrum of isolated compound I**

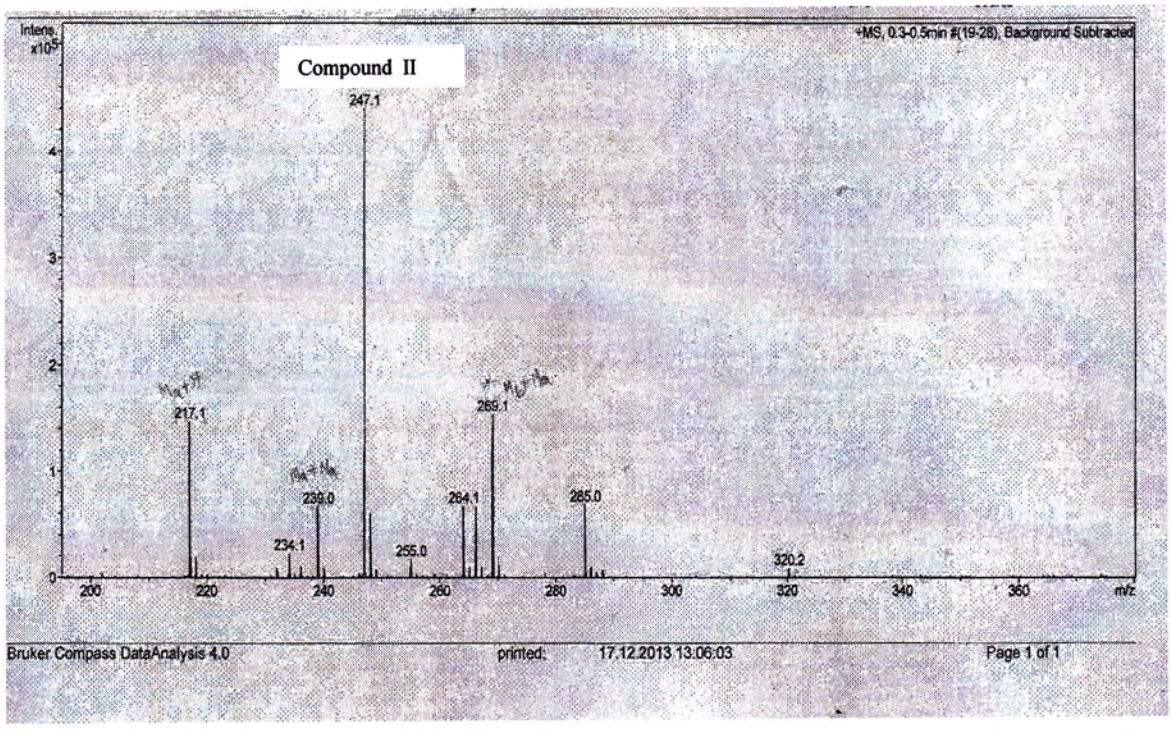


Figure 3.16 **ESI mass spectrum of isolated compound II**

3.5.2 Identification of isolated compound III

Compound III was isolated as a white needled crystals (0.026 %) from ethyl acetate extract by column chromatography on a silica gel column eluted successively with pet ether and ethyl acetate. The compound III was checked on TLC using different solvent systems. The spots on TLC were visualized firstly by viewing UV lamp. The compound absorbed both short range UV and long range UV. In addition, the color of the sport turned to dark blue when sprayed with sulphuric acid that was followed by heating at 120 ℃ for 15 minutes. The spot turn to greenish blue when treated with 10 % $FeCl_3$ solution. The R_f value on TLC was found to be 0.56 with PE: EA (1:1 v/v) solvent system.

3.5.2.1 Study on the UV spectrum of isolated compound III

The UV spectrum of compound III is described in Figure 3.17. The wavelength of maximum absorption of compound III was found to be at 227 nm and 333 nm (see Table 3.9).

3.5.2.2 Study on the FT IR spectrum of isolated compound III

The FT IR spectrum of isolated compound III (in KBr) is shown in Figure 3.18. The band assignment are presented in Table 3.10. A strong band at 3252 cm^{-1} that represented the OH stretching vibration. The stretching vibration of aliphatic C-H stretching appeared at 2917 cm^{-1}. A strong band at 1715 cm^{-1}that represented C=O stretching vibration for α, β unsaturated ketone. The peak at 1569 cm^{-1} indicating the aromatic conjugated bond. The bending vibration of aliphatic C-H group appeared at 1396 cm^{-1}. Bands at 1121 and 1229 cm^{-1} could be interpreted as C-O stretching vibration.

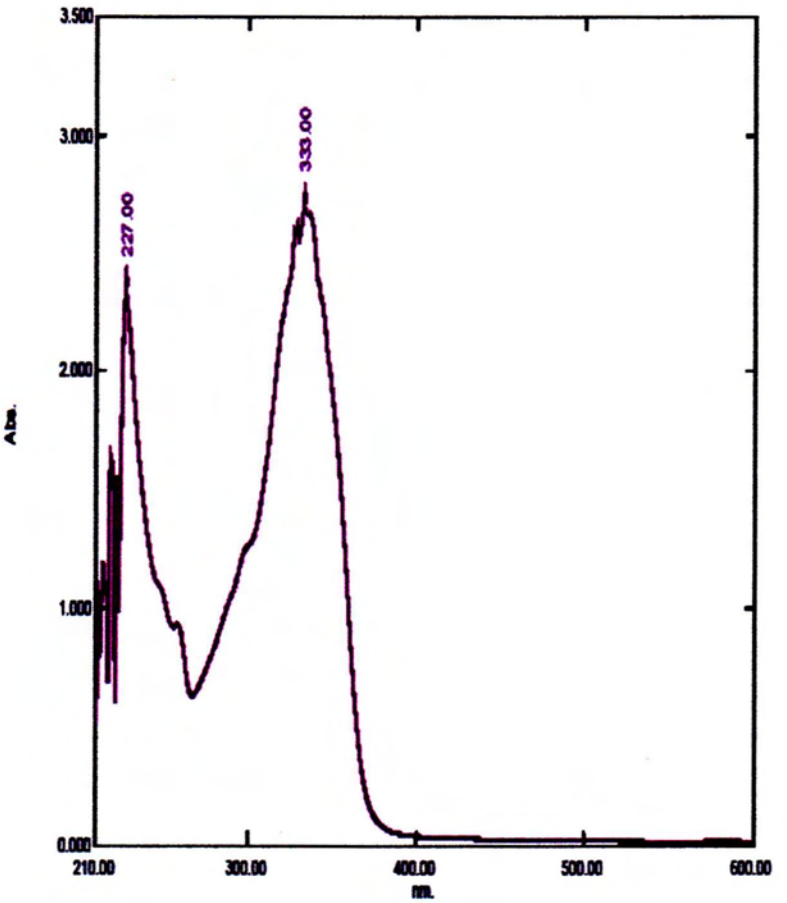


Figure 3.17 UV spectrum of isolated compound III (in MeOH)

Table 3.9 UV Spectral Data Assignment of Isolated Compound III and Reported Marmesin

Reagent	λ_{max}(nm)	
	Compound III	**Marmesin ***
MeOH	227	223
	333	322

* Wu *et al.*, 2003

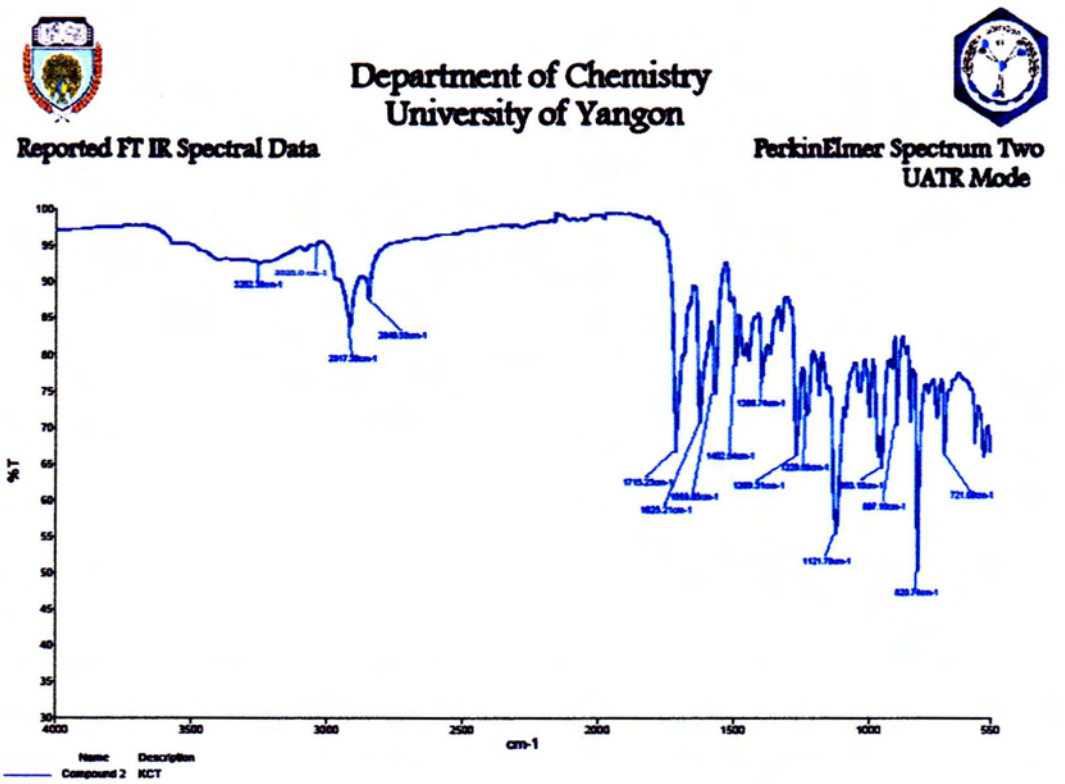


Figure 3.18 FT IR spectrum of isolated compound III (in KBr)

Table 3.10 FT IR Spectral data of Isolated Compound III

Wave number (cm^{-1})	Band assignment
3252	O-H stretching Vibration
3025	=CH stretching Vibration
2917	Aliphatic C-H Stretching Vibration
1715	C=O Stretching Vibration
1569	Aromatic Conjugated
1396	Aliphatic C-H Bending Vibration
1121	C-O Stretching Vibration
1229	

3.5.2.3 Study on the ^{1}H NMR spectrum of isolated compound III

^{1}H NMR spectrum of isolated compound III ($CDCl_3$, 300 MHz) is shown in figure 3.19. The spectrum revealed four aromatic protons. Two doublets appeared at δ 6.19 (1H, *d*, *J* = 9.5 Hz, H-3) and 7.58 (1H, *d*, *J* = 9.5 Hz, H-4) due to the characteristic peaks of α - pyrone ring. The protons on the furan ring appeared at δ 4.72 (1H, *t*, H-2′) and 3.20 (2H, *m*, H-3′). In addition, H-2′ (δ 4.72, 1H, t) would be adjacent to electronegative oxygen atom. Two singlet protons at δ 7.20 (1H, s, H-5) and 6.72 (1H, s, H-8) were assigned to benzene protons. The peak for hydroxyl group appeared at δ 1.82 (1H, broad). Two methyl singlets at δ 1.32 (3H, s, H-5′) and 1.21 (3H, s, H-6′). In fact, the presence of dihydrofuranocoumarin is common in family Rutaceae. Therefore, the compound III was assigned as marmesin (linear dihydrofuranocoumarin) and it was consistent with the NMR data of literature (Wu *et al.*, 2003).

3.5.2.4 Study on the ^{13}C NMR spectrum of isolated compound III

The ^{13}C NMR spectrum of isolated compound III ($CDCl_3$, 75MHz) is shown in figure 3.20. The ^{13}C NMR spectrum revealed 14 carbon atoms. The carbonyl proton peak was found at 163.0 ppm. The two signals at 161.9 (C-7) and 155.9 (C-10) was due to the oxygenated quaternary carbons, two aromatic quaternary carbons were observed at 124.9 (C-6) and 112.9 (C-9). In addition, the peak represent aliphatic quaternary carbon was observed at δ 71.6 (C-4′). Four aromatic methine carbons were observed at δ 143.9 (C-4), 123.6 (C-5), 112.9 (C-9) and 98.0 (C-8). Peak at δ 29.8 showed C-3′ methylene group. There are two singlets carbon at δ 26.1, 24.1 due to the presence of the two methyl groups for C-6′ and C- 5′ respectively. The data of ^{1}H NMR and ^{13}C NMR for compound III are expressed in Tables 3.11 and 3.12.

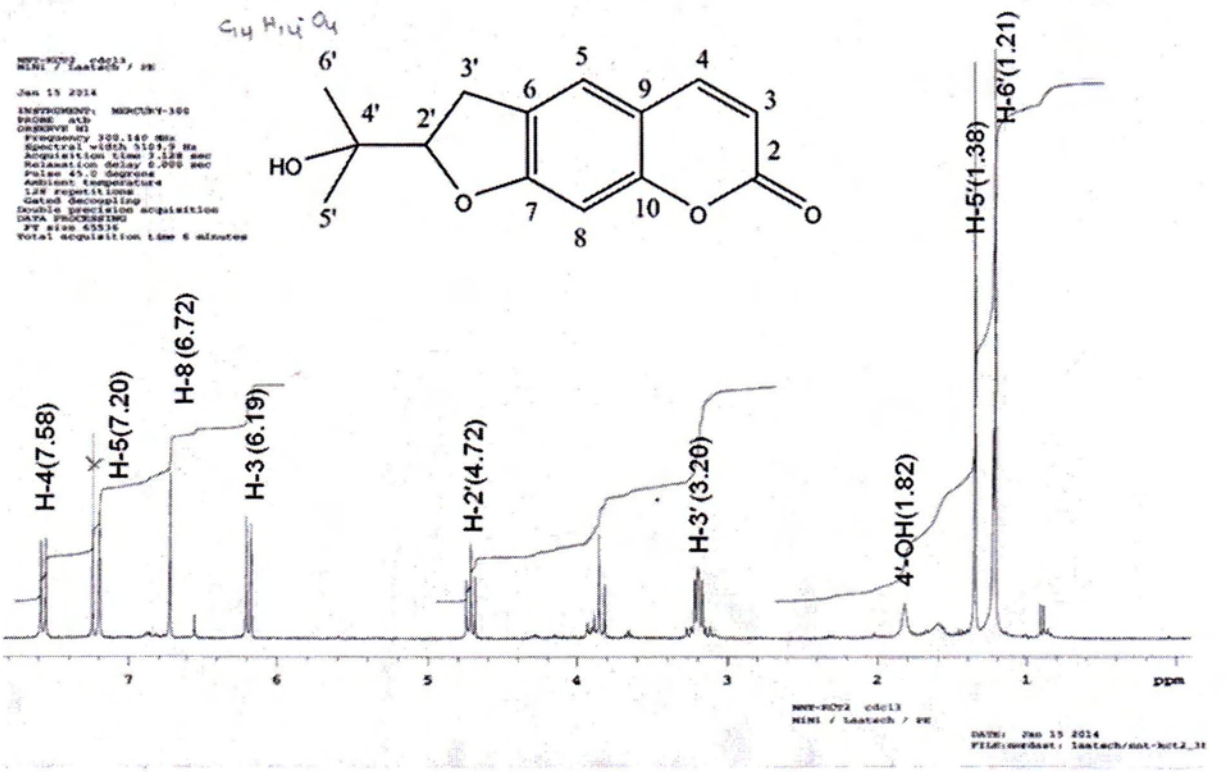


Figure 3.19 ^{1}H NMR (300 MHz, $CDCl_3$) spectrum of isolated compound III

Table 3.11 **^{1}H NMR Spectral Data of Compound III and Reported Marmesin**

Position	δ_H (ppm), J (Hz)	
	Compound III	**Marmesin***
	$CDCl_3$, 300 MHz	**$CDCl_3$, 400 MHz**
H-2′	4.72 (1H, *t*)	4.74
H-3	6.19 (1H, *d*, J = 9.5 Hz)	6.21
H-3′	3.20 (2H, *m*)	3.23
H-4	7.58 (1H, *d*, J = 9.5 Hz)	7.59
4′-OH	1.82 (1H, broad)	1.85
H-5	7.20 (1H, *s*)	7.22
H-5′	1.38 (3H, *s*)	1.37
H-6′	1.21 (3H, *s*)	1.23
H-8	6.72 (1H, *s*)	6.74

* Wu *et al.*, 2003

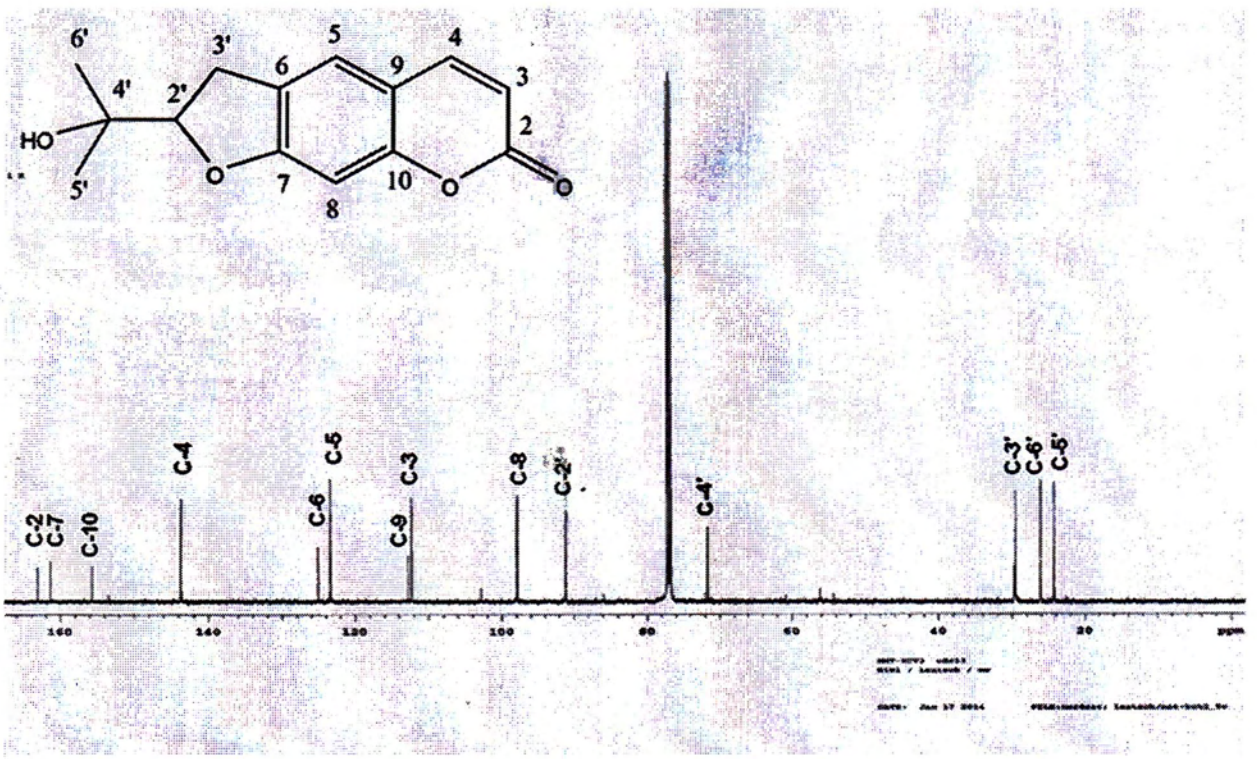


Figure 3.20 ^{13}C NMR (75 MHz, $CDCl_3$) spectrum of isolated compound III

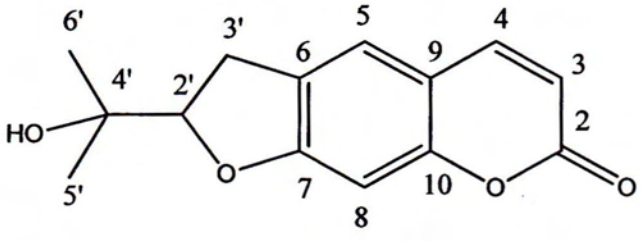
Table 3.12 **^{13}C NMR Spectral Data of Compound III and Reported Marmesin**

Position	δ_C (ppm), J (Hz)	
	Compound III	**Marmesin***
	$CDCl_3$, 125MHz	**$CDCl_3$, 100MHz**
C-2	163.0	163.1
C-2′	91.0	91.0
C-3	112.1	112.3
C-3′	29.8	29.4
C-4	143.9	143.6
C-4′	71.6	71.6
C-5	123.6	123.3
C-5′	24.1	24.2
C-6	124.9	125.0
C-6′	26.1	26.1
C-7	161.9	161.0
C-8	98.0	97.9
C-9	112.9	112.8
C-10	155.9	155.7

* Wu *et al.*, 2003

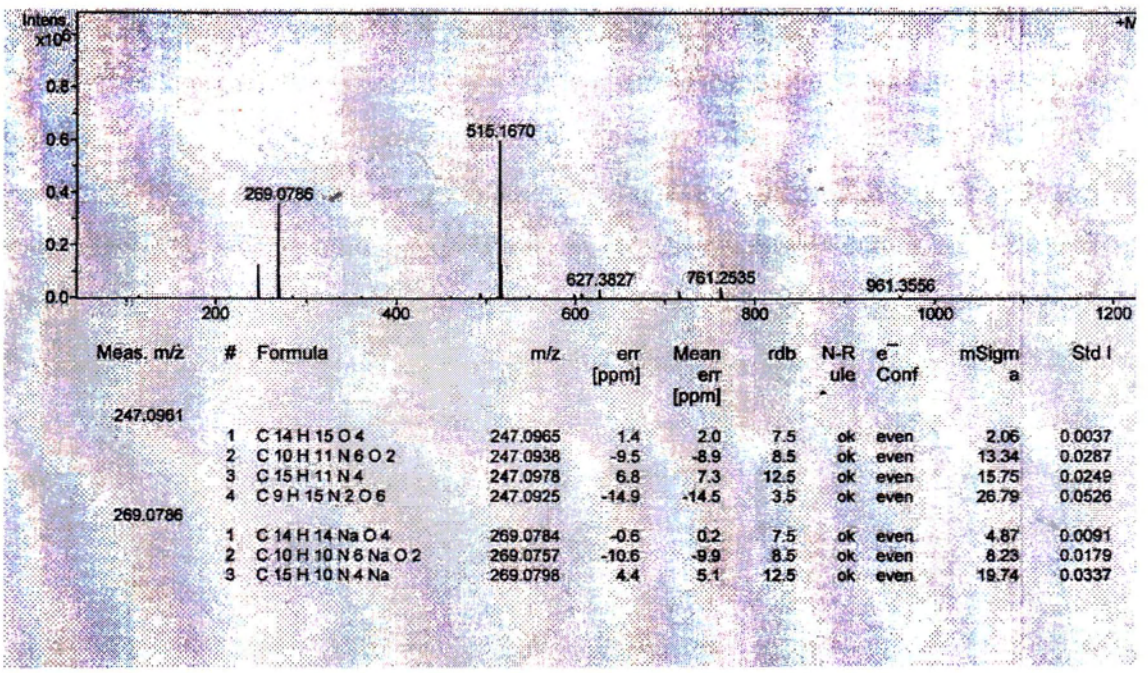
3.5.2.5 Study on the HR ESI mass spectrum of isolated compound III

According to the HR ESI mass spectrum, the molecular formula of the compound III was C_{14} H_{14} O_4 and the molecular ion peak for compound III was observed at *m/z* 269 indicating the compound III has molecular weight 269. Based on the spectral data obtained and comparison to the data reported previously, compound III was elucidated as marmesin.



Molecular formula = $C_{14}H_{14}O_4$

Isolated compound III (Marmesin)



Meas. m/z	#	Formula	m/z	err [ppm]	Mean err [ppm]	rdb	N-Rule	e⁻ Conf	mSigma	Std I
247.0961										
	1	C 14 H 15 O 4	247.0965	1.4	2.0	7.5	ok	even	2.06	0.0037
	2	C 10 H 11 N 6 O 2	247.0938	-9.5	-8.9	8.5	ok	even	13.34	0.0287
	3	C 15 H 11 N 4	247.0978	6.8	7.3	12.5	ok	even	15.75	0.0249
	4	C 9 H 15 N 2 O 6	247.0925	-14.9	-14.5	3.5	ok	even	26.79	0.0526
269.0786										
	1	C 14 H 14 Na O 4	269.0784	-0.6	0.2	7.5	ok	even	4.87	0.0091
	2	C 10 H 10 N 6 Na O 2	269.0757	-10.6	-9.9	8.5	ok	even	8.23	0.0179
	3	C 15 H 10 N 4 Na	269.0798	4.4	5.1	12.5	ok	even	19.74	0.0337

Figure 3.21 HR ESI Mass Spectrum of isolated compound III

3.5.3 Identification of Isolated Compound IV

MeOH crude extract was chromatographed by using H_2O: acetonitrile (100 : 0). Most of the compounds are found to be in the moderately polar region. There were not much non-polar compounds. Based on the data above, adequate separations needed to be done to get pure compounds as most of the compounds are very close to each other. Chromatogram of MeOH crude extract is shown in Figure 3.22.

Solvent system MeOH: H_2O (9:1 to 7:3) was done to separate the non-polar, moderately polar and polar compounds out. Non-polar fraction (hexane extract), moderate polar fraction (dichloromethane extract) and polar fraction (aqueous MeOH extract) were obtained. Based on the chromatogram, hexane extract does not have much material compared to dichloromethane extract and aqueous MeOH extracts. When comparing both dichloromethane and aqueous MeOH extracts, dichloromethane extract chromatogram shows that there is at least some separation that could be easily separated. Aqueous MeOH extract chromatogram shows that it will be difficult to separate as the compound inside are very close to one another. According to the MeOH crude extract chromatogram, majority of the compounds are found to be in the moderate polar region. Thus, dichloromethane extract was chosen to undergo VLCC as it will be easier to separate. Chromatogram of dichloromethane extract is shown in figure 3.23.

According to VLCC, six fractions (fraction A, B, C, D, E and F) were obtained. Dichloromethane: MeOH solvent system was used. According to chromatogram data, it was observed that majority of the compounds are found in the moderately polar region. Based on the chromatogram results, not much compounds are found in fraction A. Fraction B shows a much better separation. Fraction C, D, E and F chromatograms need to undergo more separation as the compounds are still found to be very close each other. However, fraction C was chosen to run FCC. Vacuum liquid column chromatogram of fraction C (Dichloromethane: MeOH 4:1 sample) is shown in figure 3.24.

According to FCC, total 35 fractions were obtained. After combining the same fractions, 11 fractions were obtained. However, the pure compounds were only obtained from fraction D and I. Comparing the chromatograms, fraction D

chromatogram is easier to obtain pure compound than I. Flash column chromatogram of fraction D is shown in figure 3.25.

All fractions ran preparative HPLC. All fractions showed that a good separation. When fraction D was eluted out at different retention time, this fraction give different pure compounds. When fraction D ran preparative HPLC, four fractions were obtained. Preparative HPLC of fraction D is shown in figure 3.26. For fraction D, it weighs less than 50mg and there were not much compounds found in the sample thus analytical column was used. The flow rate will be 10 mL/ min. Based on the analytical data, chromatogram shows that the major peak, which is important compound. This peak is for the pure compound (auraptene).

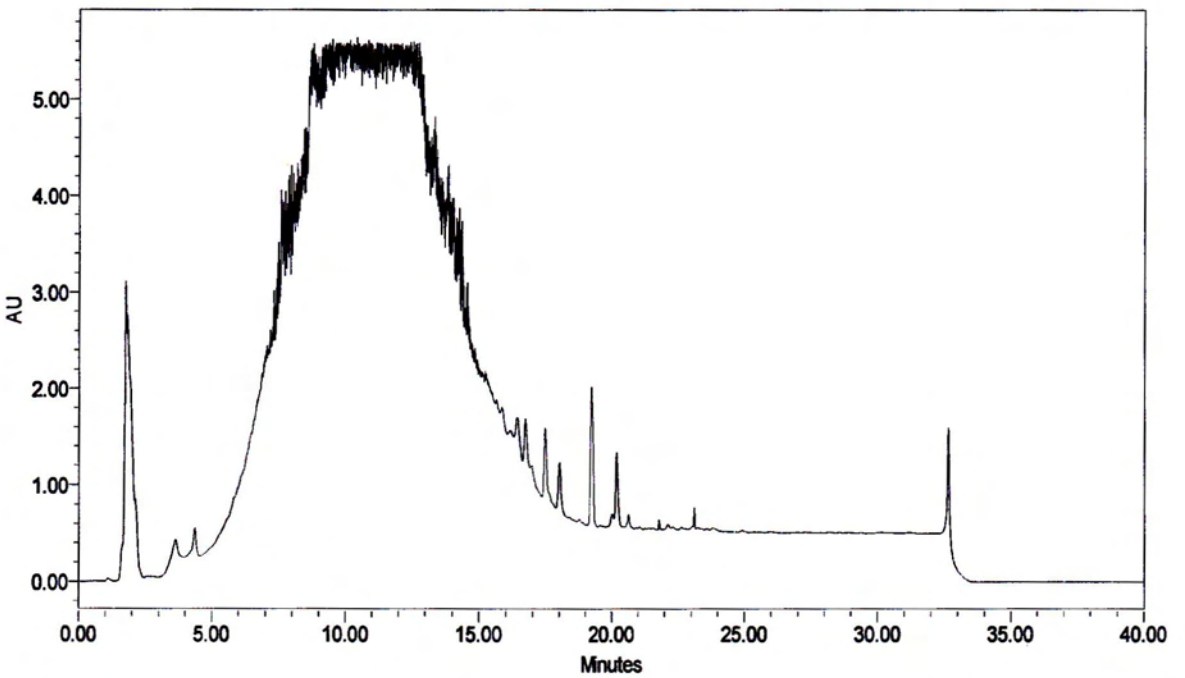


Figure 3.22 Chromatogram of MeOH crude extract

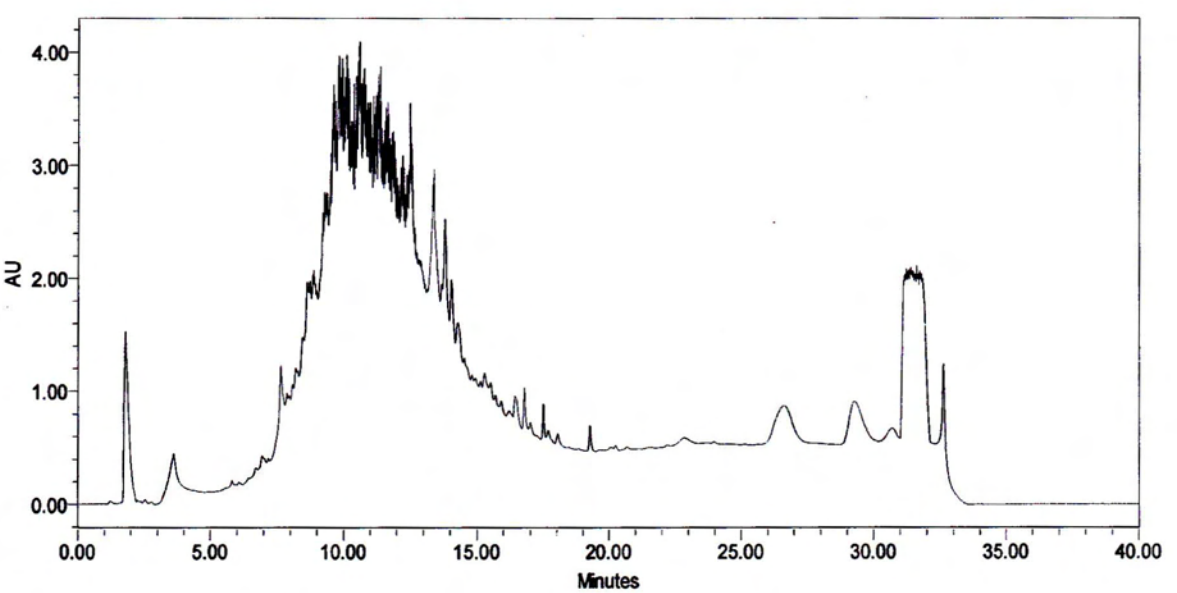


Figure 3.23 Chromatogram of dichloromethane extract

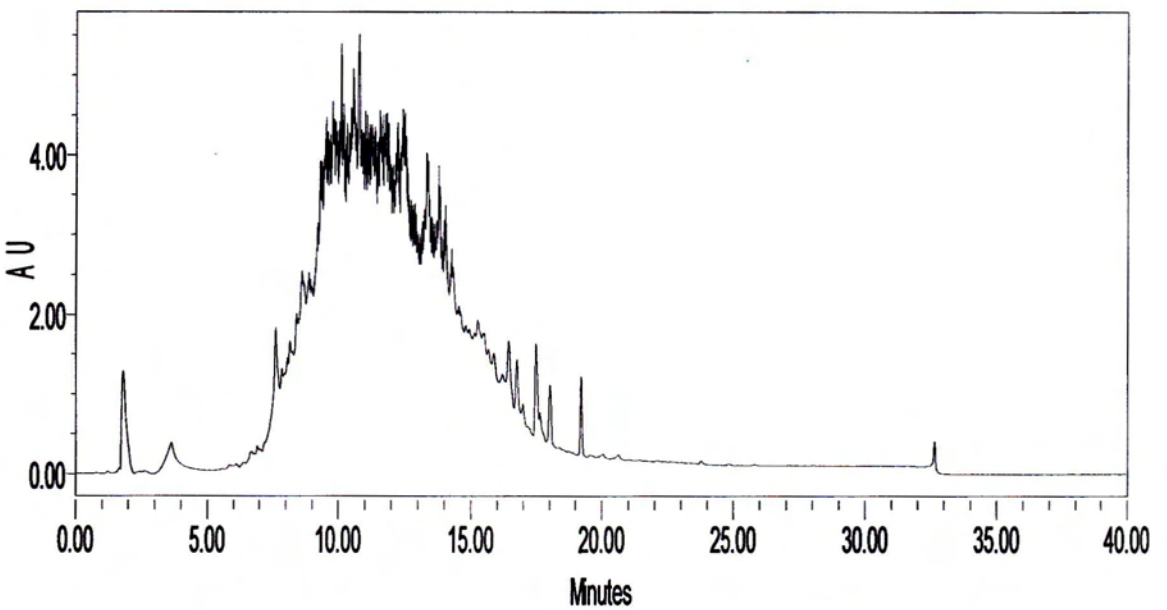


Figure 3.24 Vacuum liquid column chromatogram of fraction C (Dichloromethane: MeOH 4:1 sample)

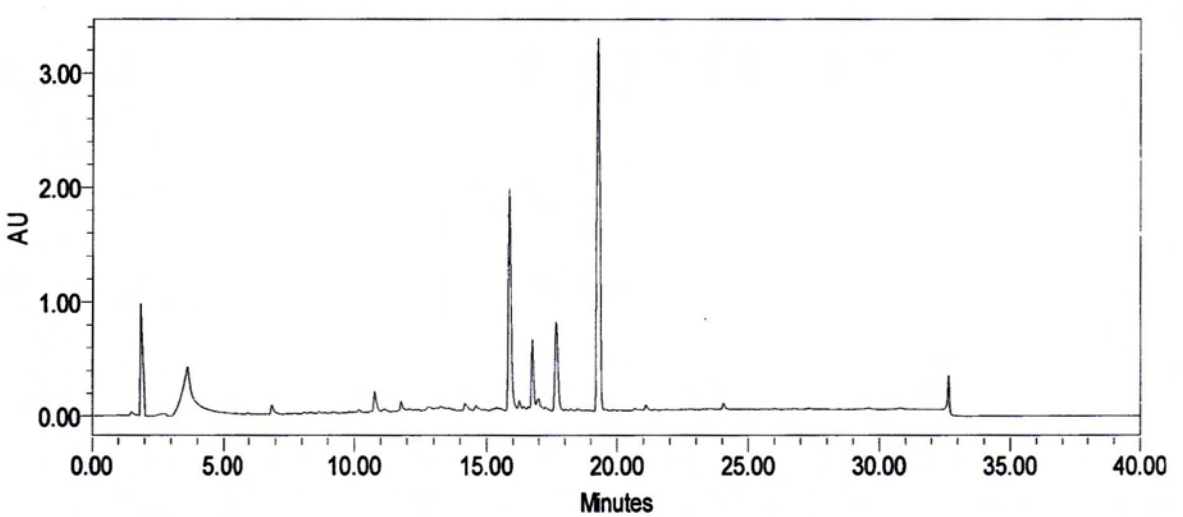


Figure 3.25 Flash column chromatogram of fraction D (Dichloromethane: MeOH 7:3 sample)

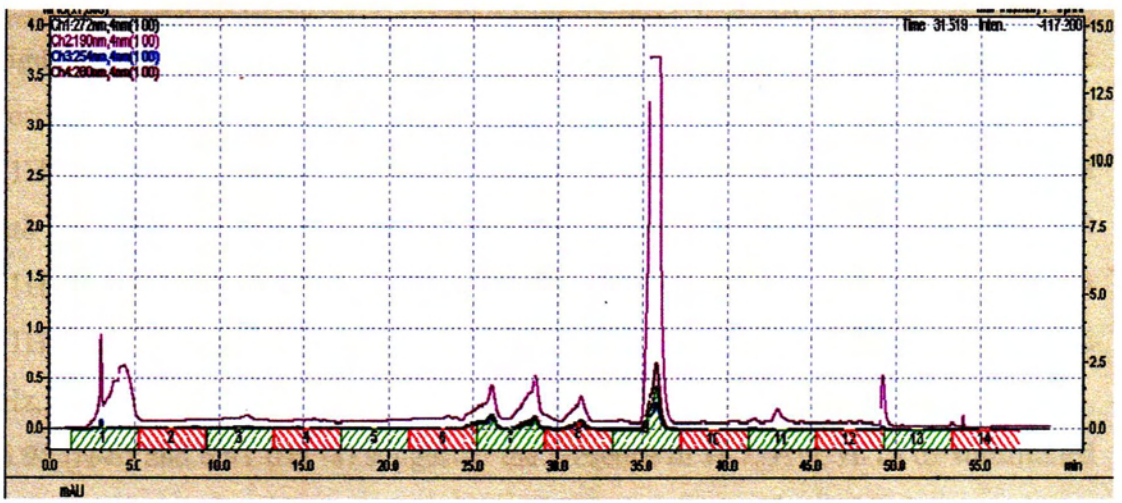


Figure 3.26 Preparative HPLC of fraction D (Dichloromethane: MeOH 7:3 sample)

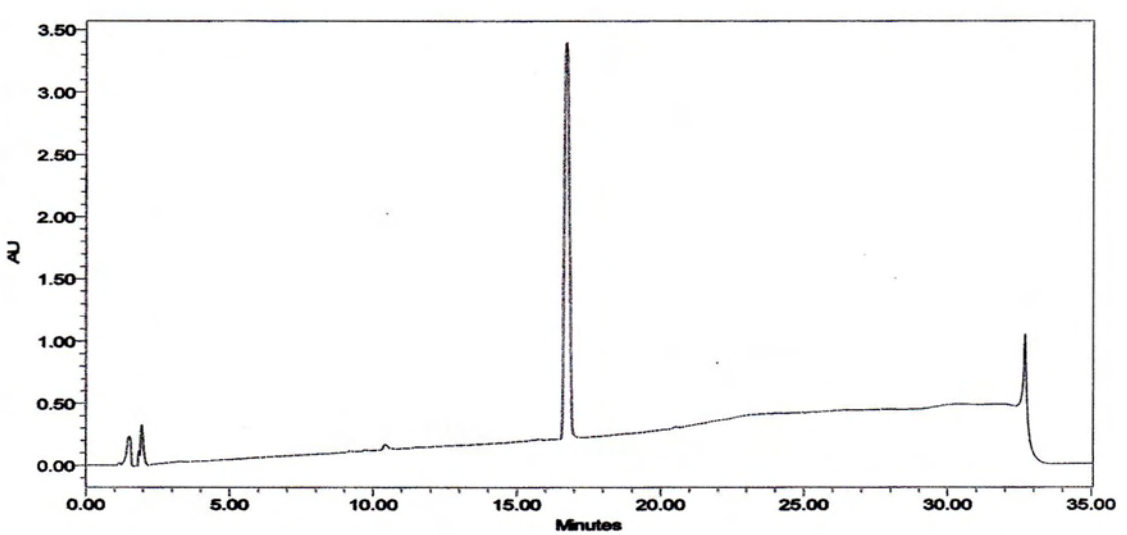


Figure 3.27 Analytical HPLC of pure compound

3.5.3.1 Study on the ^{1}H NMR spectrum of isolated compound IV

^{1}H NMR spectrum of isolated compound IV ($CDCl_3$, 400 MHz) is shown in figure 3.28. The ^{1}H NMR spectrum of isolated compound IV showed characteristic resonances for three aromatic proton signals atδ 7.58 (1H, *d*, *J* = 7.2 Hz, H-5) ; 6.94 (1H, *dd*, *J* = 7.2 Hz, H-6) and 6.91 (1H, *d*, *J* = 2.0 Hz, H-8). The three methyl singlets atδ 1.81(H-8′), 1.6 (H-9′) and 1.61 (H-10′). The seven methine resonances atδ 5.08 (1H, *q*, *J* = 7.0 Hz, H-6′), 5.47 (1H, *t*, *J* = 7.0 Hz), 6.82 (1H, *d*, *J*= 9.6 Hz, H -8), 6.24 (1H, *d*, *J* = 9.6 Hz, H-3), 6.85 (1H, *dd*, *J* = 7.2 Hz, 2.0 Hz, H-6), 7.36(1H, *d*, *J* = 7.2 Hz, H-5) and7.63 (1H, *d*, *J* = 9.6 Hz, H-4). Three aromatic protons at δ 7.58 (H-5), 6.94 (H-6) and 6.91 ((H -8) suggested the presence of H-7, H-9, H-10 tri-substituded benzene ring, which was supported by ^{13}C NMR spectrum. The ^{1}H NMR spectral data is summarized in Table 3.13.

3.5.3.2 Study on the ^{13}C NMR Spectrum of Isolated Compound IV

The ^{13}C NMR spectrum of isolated compound IV figure 3.29 showed the presence of 19 carbon signals.Two carbon signals at δ 162.2 (C-7) and 156.0 (C-9) showed characteristic oxy aryl signals which indicated that the structure was a derivative of 7- hydroxy coumarin. The ^{13}C NMR spectrum showed three methyl signals at δ 16.0(C-8′), 17.0 (C -10′), 25.0 (C-9′), two methylene signals atδ 26.5 (C-5′) and 65.3 (C-1′), seven methine signals atδ 101.3 (C-8), 112.9 (C-3), 119.2 (C-2′), 112.6 (C-6), 123.3 (C-6′), 129.2 (C-5) and 143,7 (C-4) and five quaternary carbon signals atδ 112.5 (C-10), 131.3 (C-7′), 156.0 (C-9), 141.2 (C-3′) and 162.2 (C-7), including one carbonyl function which was indicated by the downfield signal at 160.1 (C-2). The ^{13}C NMR spectral data is summarized in Table 3.14.

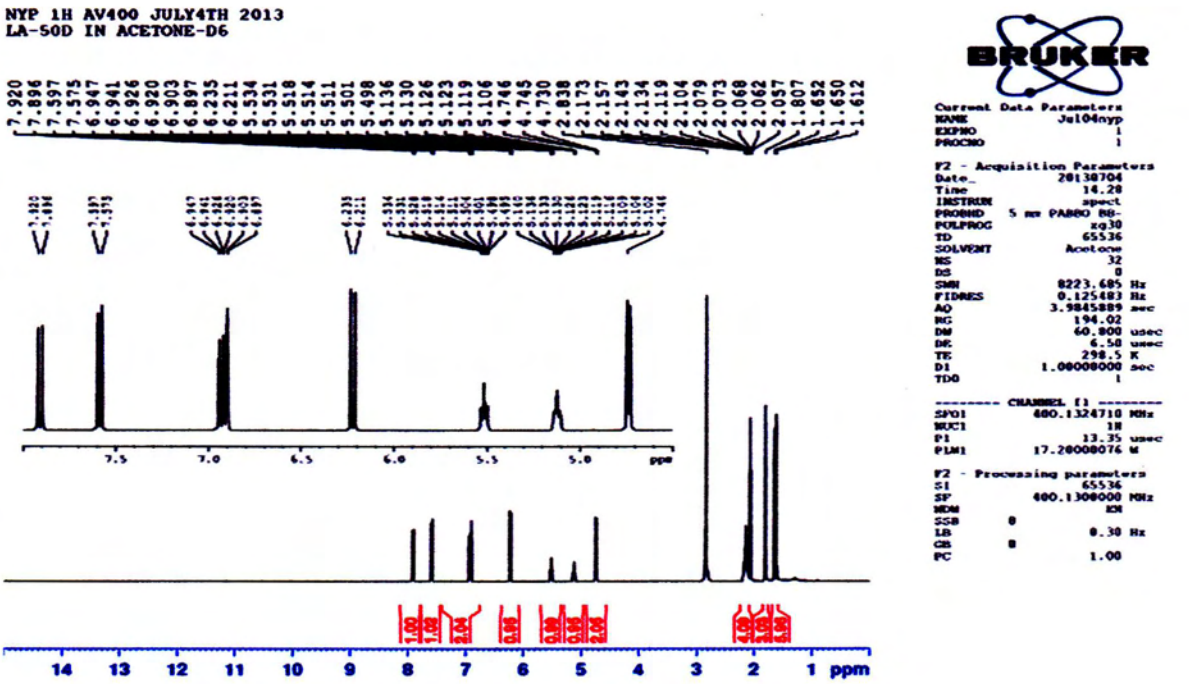


Figure 3.28 ^{1}H NMR spectrum of isolated compound IV

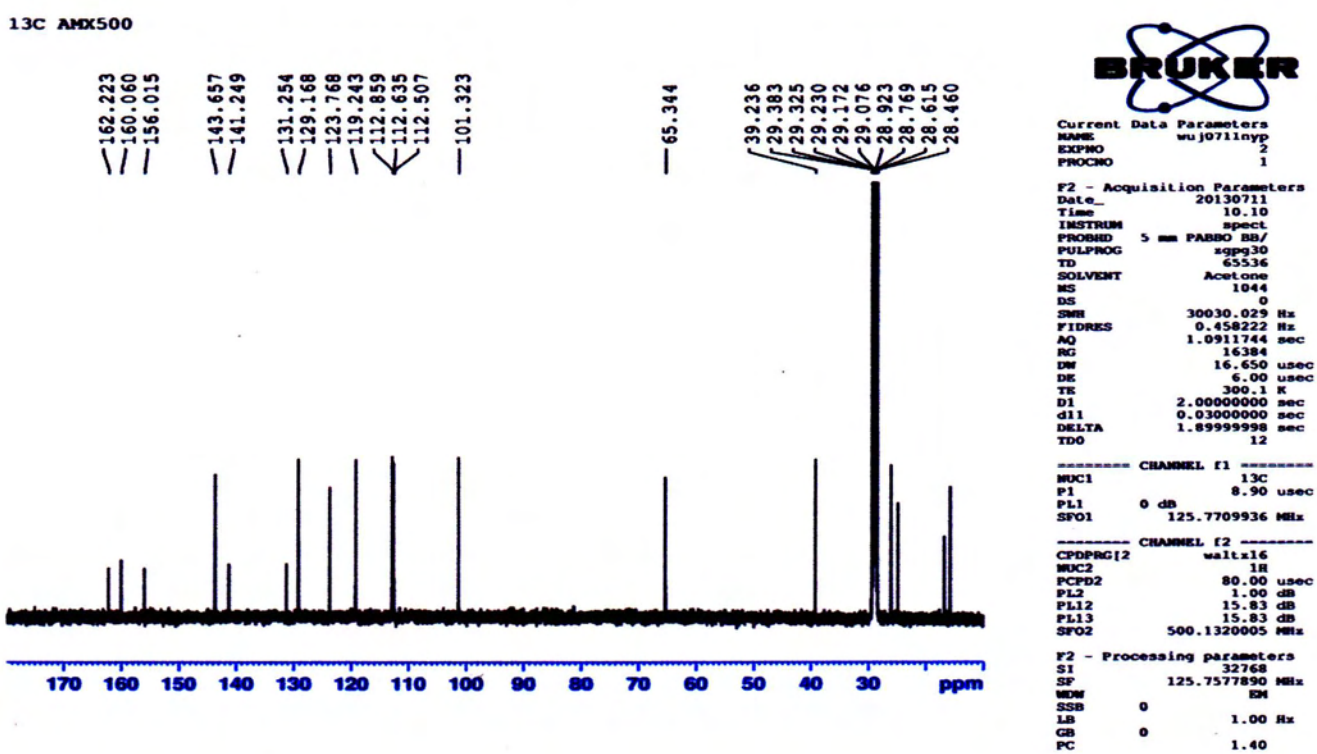


Figure 3.29 ^{13}C NMR spectrum of isolated compound IV

Table 3.13 **^{1}H NMR Spectral Data of Compound IV and Reported Auraptene**

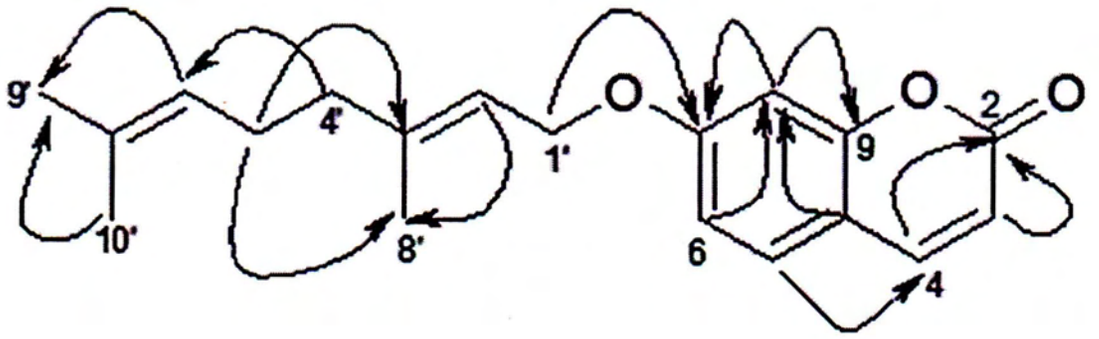
Position	δ_H (ppm), J (Hz)	
	Compound IV	**Auraptene***
	$CDCl_3$, 500 MHz	**$CDCl_3$, 400 MHz**
H-3	6.22 (1H, *d*, J = 9.6 Hz)	6.24
H-4	7.91 (1H, *d*, J = 9.6Hz)	7.63
H-5	7.58 (1H, *d*, J = 9.5 Hz)	7.36
H-6	6.94 (1H, *dd*, J = 7.2, 2.0 Hz)	6.85
H-7	-	-
H-8	6.91 (1H, *d*, J = 2.0 Hz)	6.82
H-9	-	-
H-10	-	-
H-1′	4.59 (2H, *d*, 7.0 Hz)	4.59
H-2′	5.51 (1H, t, 7.0 Hz)	5.47
H-3′	-	-
H-4′	2.13 (2H, *m*)	2.13
H-5′	2.15 (2H, *m*)	2.15
H-6′	5.11 (1H, *q*, J =7.0 Hz)	5.08
H-7′	-	-
H-8′	1.81 (3H, *m*)	1.75
H-9′	1.65 (3H, *m*)	1.65
H-10′	1.61 (3H, *q*, J =7.0 Hz)	1.59

* Tjitjik., 2014

Table 3.14 **^{13}C NMR Spectral Data of Compound IV and Reported Auraptene**

Position	δ_C (ppm), J (Hz)	
	Compound IV	**Auraptene***
	$CDCl_3$, 125MHz	**$CDCl_3$, 100MHz**
C-2	160.1	161.2
C-3	112.9	112.9
C-4	143.7	143.4
C-5	129.2	128.6
C-6	112.6	113.2
C-7	162.2	162.1
C-8	101.3	101.5
C-9	156.0	155.8
C-10	112.5	112.3
C-1′	65.3	65.4
C-2′	119.2	118.3
C-3′	141.2	142.3
C-4′	39.2	39.4
C-5′	26.5	26.4
C-6′	123.8	123.5
C-7′	131.3	131.9
C-8′	16.0	16.7
C-9′	25.0	25.6
C-10′	17.0	17.6

* Tjitjik., 2014



H-3 ➡ C-3, C-2

H-4 ➡ C-2, C-3, C-5, C-9

H-5 ➡ C-2, C-4, C-3, C-8, C-9

H-6 ➡ C-3, C-8

H-8 ➡ C-7, C-9

H-1′ ➡ C-2, C-3′, C-7

H-2′ ➡ C-1, C-4′, C-8′

H-4′ ➡ C-4′, C-2′, C-6′, C-3′

H-5′ ➡ C-8′, C-5′, C-2′, C-3′

H-6′ ➡ C-10′, C-5′, C-9′

H-8′ ➡ C-4′, C-2′, C-3′

H-9′ ➡ C-10′

H-10′ ➡ C-9′ , C-5, C-7′

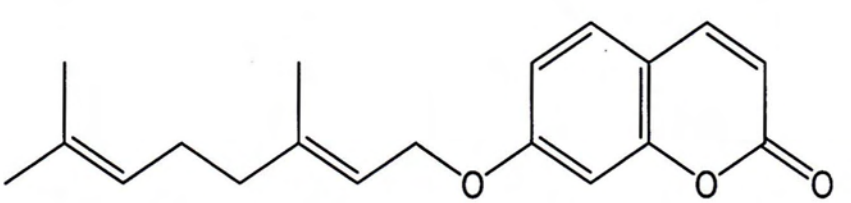
Figure 3.30 Important HMBC correlations of isolated compound IV

3.5.3.3 Study on the HMBC spectrum of isolated compound IV

The HMBC spectrum of isolated compound IV is shown in figure 3.31and the important HMBC correlations are shown in figure 3.30. In the HMBC spectrum, the presence of a geranyl group at C-7 showed long range correlation between a methylene proton signal at δ 4.58 with two quaternary atoms at δ 162.2 (C-7), 141.2 (C-3′) and one methine carbon at δ 119.2 (C- 2′). The signal for methyl proton at δ 1.61 (H-10′) correlated with methyl carbon signal δ 25.0 (C-9′) and a quaternary carbon signal at δ 131.3 (C-7′). The methine signal at δ 6.22 (H-3) was correlated with carbonyl carbon δ 160.1 (C-2) and methine carbon δ 112.9 (C-3). According to the 1D and 2D spectroscopic data and the comparison of the literature values, the compound IV was confirmed as auraptene.

3.5.3.4 Study on ESI MS spectrum of isolated compound IV

From ^{1}H and ^{13}C NMR, there are 22 hydrogen and 19 carbon respectively. The ESI mass spectrum of isolated compound IV is shown in figure 3.32. The data from ESI mass spectrum showed that the molecular weight is 298. Based on the spectral data indicated and comparison to the data reported previously, the compound IV was consistent with the molecular formula $C_{19}H_{22}O_3$.



Molecular formula = $C_{19}H_{22}O3$

Isolated compound IV (Auraptene)

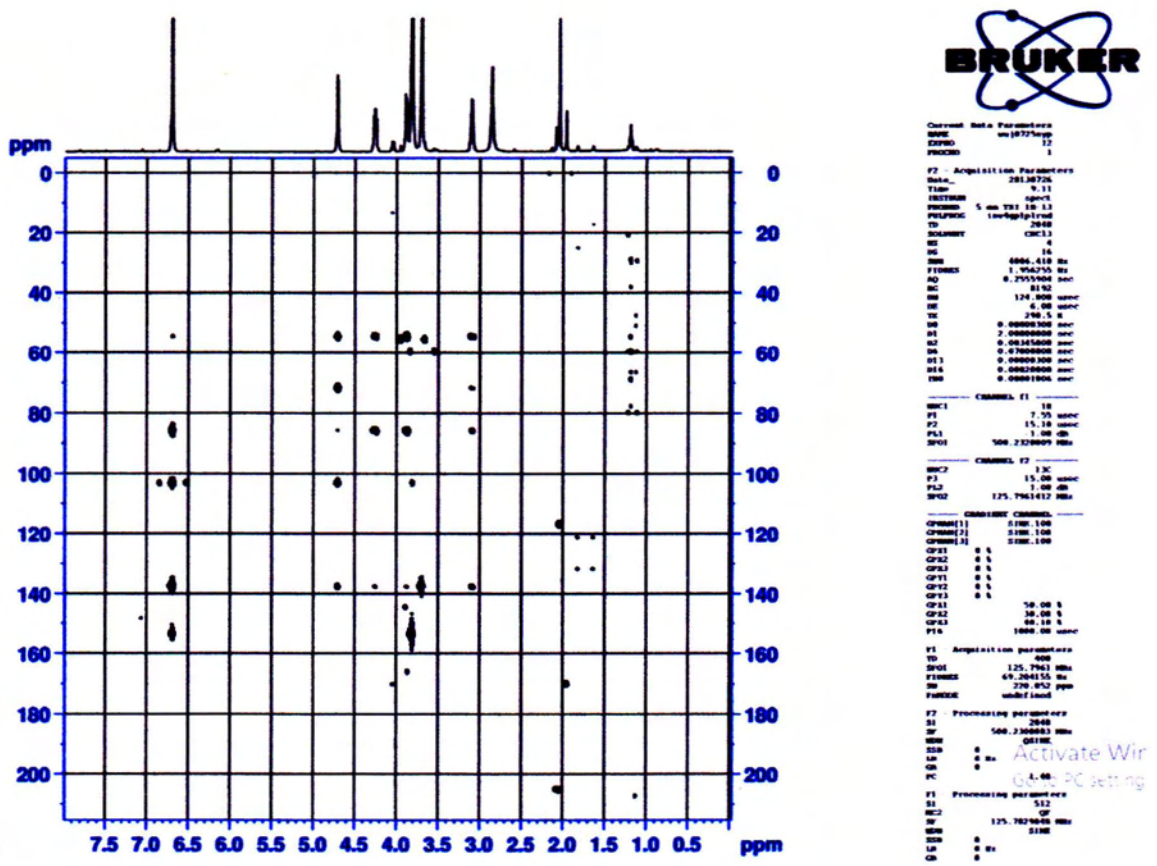


Figure 3.31 HMBC spectrum of isolated compound IV

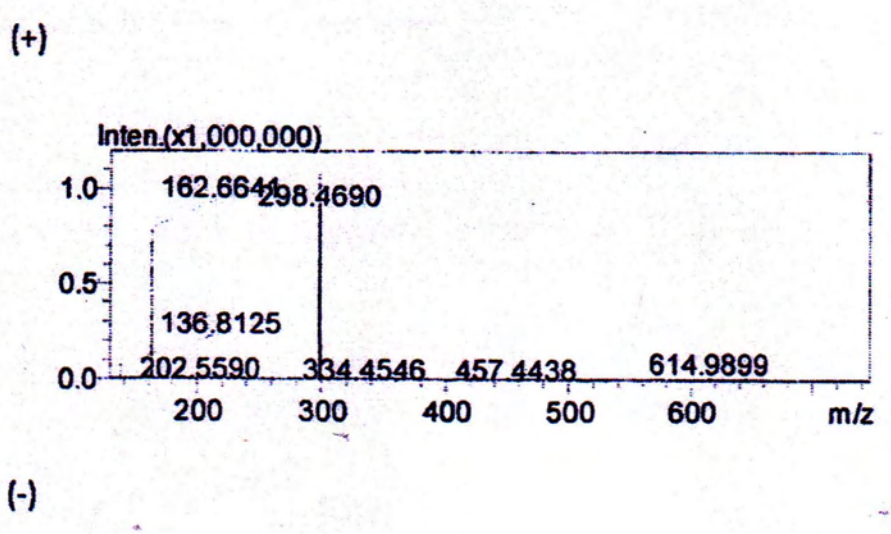


Figure 3.32 ESI MS spectrum of isolated compound IV

3.6 Study on Pharmacological Activities of Selected Plant Samples

3.6.1 Screening of antimicrobial activity of the bark Extract, fruit pulp extract and isolated compounds by agar well diffusion method

Screening of antimicrobial activity of plant extracts and isolated compounds were carried out on different strains of microorganisms by agar well diffusion method. The measurable zone diameter of growth inhibition reflects the degree of antimicrobial activities. Various extracts of stem bark, fruit pulp, mixture of compound I and II and compound III were investigated on six strains microorganisms of which include *Bacillus subtilis, Staphylococcus aureus, Pseudomonas aeruginosa, Bacillus pumilus, Escherichia coli* and *Candida albicans* by agar well diffusion method.

The antimicrobial activities were estimated by measuring the diameter of inhibition zone in terms of millimeter. The larger the diameter of clear zone, the more potent the antimicrobial activity. The results of antimicrobial activities on plant crude extracts, mixture of compound I and II and compound III of are summarized in Tables 3.14 and 3.15. The photographs showing the inhibition zone provided by crude extracts and isolated compounds against six species of microorganisms are presented in Figures 3.33, 3.34 and 3.35.

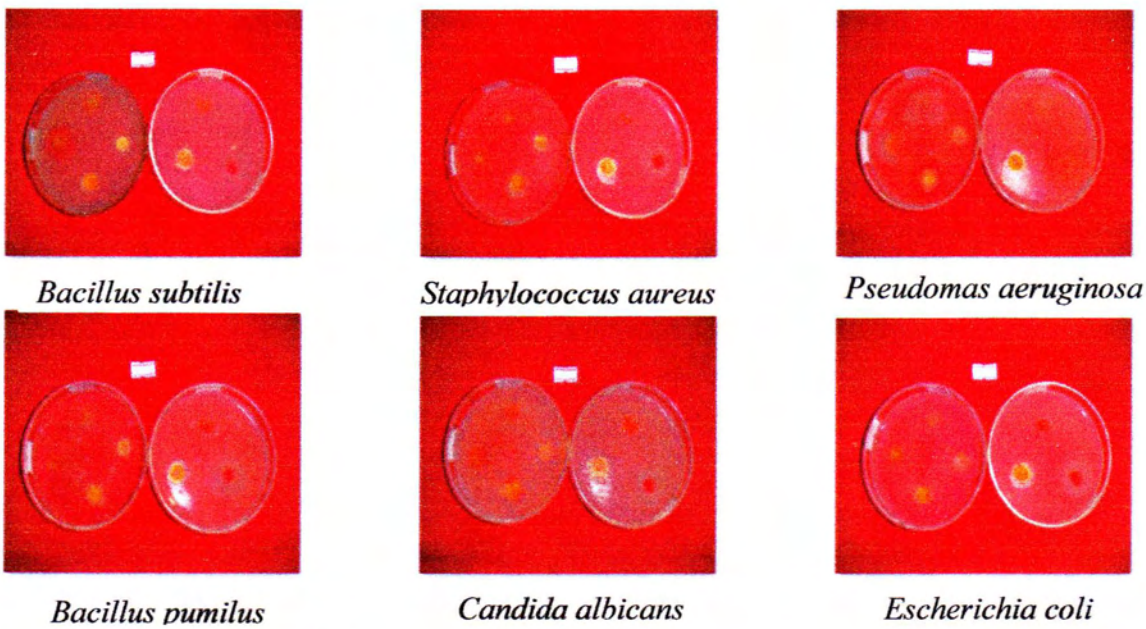
It was observed all extracts showed moderate antimicrobial activity against the organisms tested. Among these extracts, MeOH extract show more potent antimicrobial activity than other extracts.

Table 3.14 Results of Antimicrobial Activity of Various Crude Extracts from Bark of *L. acidissima* (Thee) by Agar Well Diffusion Method

Organisms used	Diameter of inhibition zone (mm) of various extracts from Thee bark						
	PE	$CHCl_3$	MeOH	CH_3OCH_3	EtOAc	EtOH	H_2O
Bacillus subtilis	15	20	18	17	12	15	-
Staphylococcus aureus	15	13	15	14	13	16	14
Pseudomonas aeruginosa	-	-	25	14	-	14	-
Bacillus pumilus	14	13	18	14	12	15	13
Candida albicans	-	13	14	12	13	14	-
Escherchia coli	17	14	18	16	12	15	13

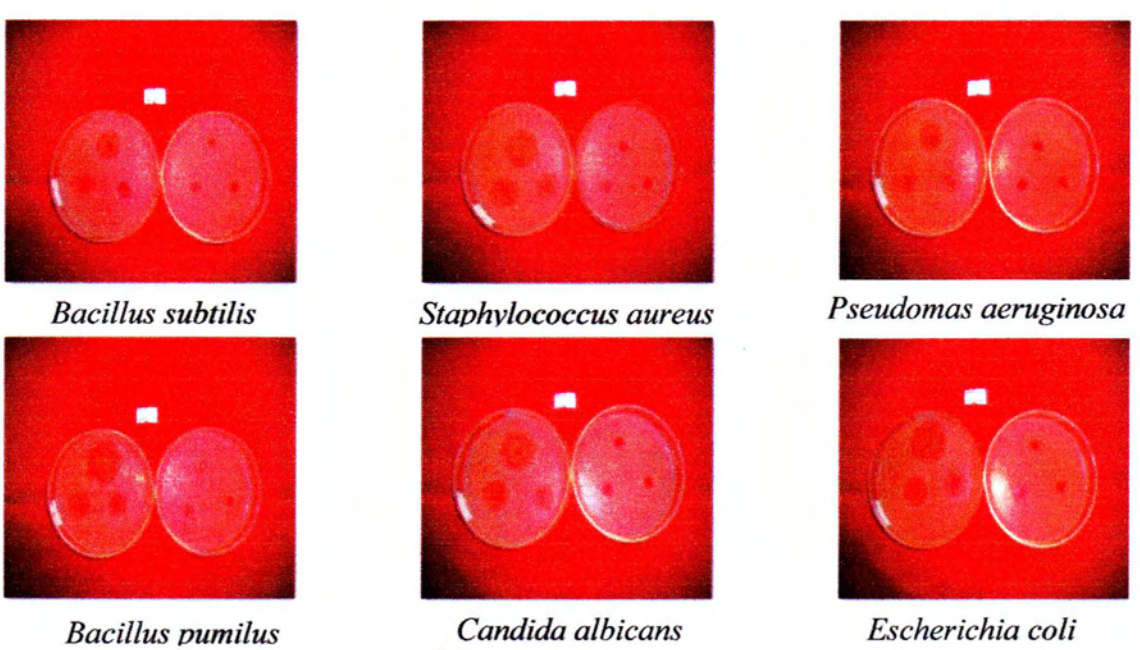
Table 3.15 Results of Antimicrobial Activity of Various Crude Extracts from Fruit Pulp and Isolated Compounds of *L. acidissima* (Thee) by Agar Well Diffusion Method

Organisms used	Diameter of inhibition zone (mm) of various extracts from Thee fruit pulp extracts and isolated compounds				
	MeOH	EtOAc	EtOH	Mixture of Compound I and II	Compound III
Bacillus subtilis	20	18	15	15	18
Staphylococcus aureus	28	23	19	15	19
Pseudomonas aeruginosa	17	14	14	-	-
Bacillus pumilus	30	21	18	18	15
Candida albicans	30	22	18	13	17
Escherchia coli	23	21	19	17	17



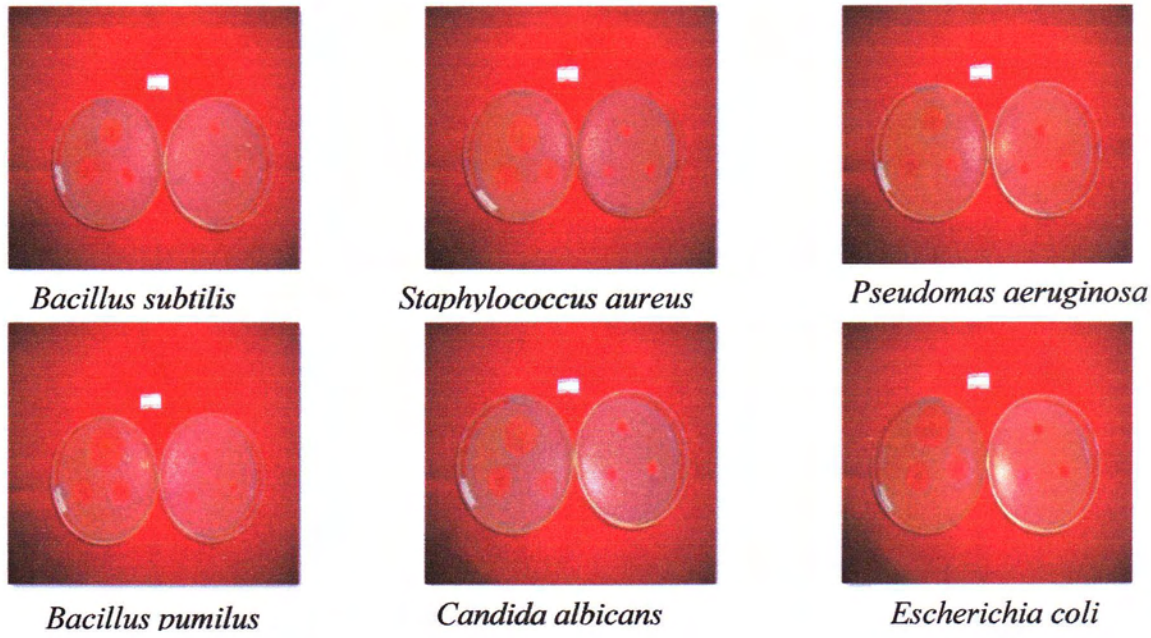
Diameter of agar well = 10 mm

Figure 3.33 Inhibition zones of various crude extracts from bark of *L. acidissima* (Thee) against six microorganisms



Diameter of agar well = 10 mm

Figure 3.34 Inhibition zones of various crude extracts from fruit pulp of *L. acidissima* (Thee) against six microorganisms



Diameter of agar well = 10 mm

Figure 3.35 Inhibition zones of isolated compounds from bark of *L. acidissima* (Thee) against six microorganisms

3.7 Acute toxicity of 95 % ethanolic extract from the bark of *L. acidissima* (Thee)

The medium lethal activity and calculation of LD_{50} of the 95% ethanolic extract was done according to the method of Litchfield and Wilcoxon (1949). Since the route of administration selected should be the intended route for administration of the tested drug given to the human during therapy, the oral route was chosen for this test. Both sexes of 50 albino mice, weighing (20-30) g were used in this study.

Mice were separated into 5 groups and each group contained 10 mice. Each group was placed separately in the 5 mice cages. Food was with the help for the period of 18 hours before administration of drugs.

At first, the mice were individually marked with picric acid staining on the parts of the body and weighing and the required doses were calculated. Group I served as control group and administered 0.1 ml/10g distilled water. Group II-V were administered orally with four doses 95% ethanolic extract. The given doses of 95 % ethanolic extract were 2 g/kg, 4 g/kg, 8 g/kg and 16 g/kg weight respectively. After giving the extracts orally, each group of mice was kept in 5 mice cages with free access to water and food. They were observed carefully for 24 hours.

Any mortality within the groups was recorded with 24 hours, survivors were observed for 14 days. All the animals were observed to be remained alive and did not show any visible symptoms of toxicity like restlessness, respiratory disorders, convulsions, aggressive activities, coma and death at the doses tested. According to the result as shown in Table 3.16, no lethality of the mice was observed up to fourteen days, even with the dose up to 16 g/kg body weight of extract. From these results, it was found that the plant extract was free from acute toxic effect under condition.

Table 3.16 Results Obtained from Acute Toxicity of 95 % EtOH Extract of Thee Bark on Mice Model after Two Weeks Treatment

Group	Drug administration	Dosage g/kg (b.wt)	No. of death per tested mice	Lethality (%)
1	95 % EtOH	16	0/10	0
2	extract	8	0/10	0

Note : Each group contains 10 no. of mice

: Medium lethal dose LD 50 > 16 g/kg body weight

3.7.1 Antioxidant activity of crude extracts from bark of *L. acidissima* (Thee)

Many aromatic plants have been known to support various biological activities such as antimicrobial and antioxidant properties. The radical scavenging effects (percentage of quenched radicals) were determined for the PE, EtOAc and EtOH extracts prepared from the bark and fruit pulp of *L. acidissima* by free radical scavenging for the PE, EtOAc and EtOH extracts by DPPH free radical scavenging assay as mentioned in Section 2.8.3.

DPPH (1, 1-diphenyl-2-picryl hydrazyl) free radical method is the most widely reported method for screening of antioxidant activity on many plants and it is also a simple and acceptable method to evaluate the antioxidative activity of antioxidant. This method associates with the change in absorption, which could be followed spectroscopically. This method is based on the reduction of colored free radical DPPH in ethanolic solution by different concentration of the samples. The bark extracts or their constituents decolorized, when mixed with DPPH due to hydrogen donating ability. The antioxidant activity was expressed as 50 % oxidative inhibitory concentration (IC_{50}).

Ethanol extract of bark of *L. acidissima* (Thee) at different concentrations were prepared for the screening of radical scavenging activity. Determination of absorbance was carried out at wavelength 517 nm using UV visible spectrophotometer. Each experiments were done triplicate.

The percent oxidative inhibition values of crude extracts were measured at different concentrations and the results are summarized in Table 3.17 and Figure 3.36. From these results, it was found that as the concentrations increased, the absorbance values decreased, i.e increased in radical scavenging activity of crude extracts that is usually expressed in term of % inhibition. From the average value of % inhibition, IC_{50} (50 % inhibition concentration) values in μg/mL were calculated by linear regressive excel program.

In the case of screening for antioxidant activity, the ethanol crude extract showed the highest free radical scavenging activity with IC_{50} value 16.74 μg/mL. At the same time, pet ether and ethyl acetate extracts also exhibited antioxidant potential having IC_{50} value of 155.74 and 64.54 μg/mL. Comparing standard ascorbic acid

(IC_{50} = 28.4 µg/mL), ethanolic extract of bark of *L. acidissima* (Thee) possesses mild antioxidant activity, showed it has ability to trap free radical that generate in the skin. Therefore, the use of this bark on skin hopes individuals to protect from photoaging.

Table 3.17 Percent Inhibition and IC_{50} Values of some Crude Extracts of *L. acidissima* (Thee) Bark in Various Concentrations

Sample	% RSA ± SD at Different Concentration (µg/mL)					
	12.5	**25**	**50**	**100**	**200**	**400**
PE	9.98 ± 0.41	12.05 ± 0.81	21.75 ± 1.52	36.84 ± 3.04	60.58 ± 1.01	90.12 ± 0.20
EA	15.76 ± 1.21	26.44 ± 1.56	42.99 ± 2.49	67.27 ± 1.25	87.17 ± 0.42	91.67 ± 0.21
EtOH	44.11 ± 0.65	71.28 ± 1.82	84.62 ± 1.03	87.69 ± 0.56	86.67 ± 2.12	89.44 ± 1.31
AA	23.10 ± 0.11	44.03 ± 2.52	82.51 ± 1.49	89.31 ± 0.69	89.97 ± 0.46	89.57 ± 0.11

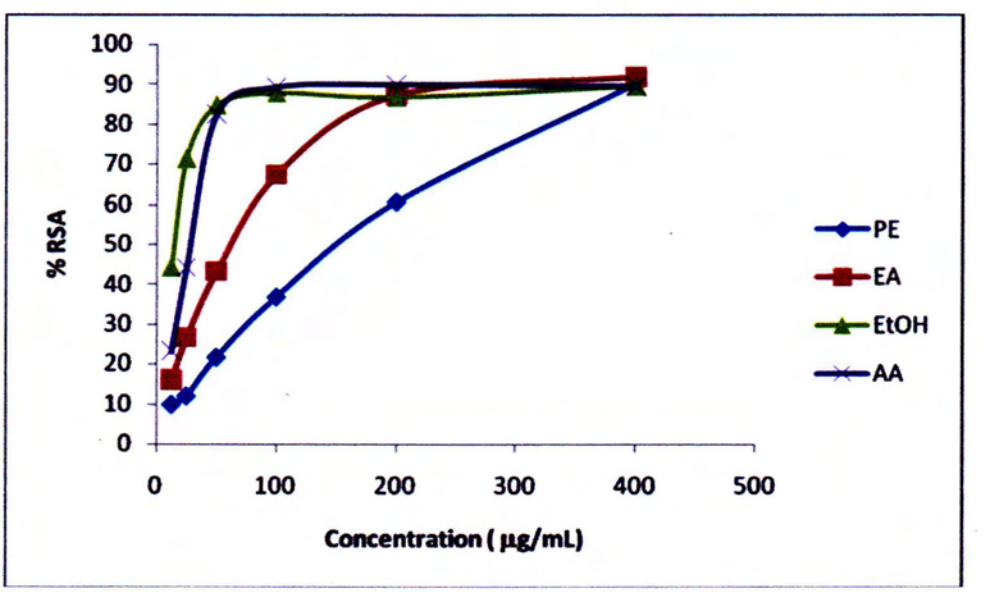


Figure 3.36 Percent radical scavenging activity of some crude extracts of *L. acidissima* (Thee) bark

3.8 Formulation of Sunscreen Lotion from Crude Extracts of *L. acidissima* (Thee)

Natural substances extracted from plants have recently been considered as potential sunscreen resources owing to high ultraviolet ray absorption and antioxidant activity. Recently, the development of sunscreens possessing broad spectrum anti-UV effectiveness with reduced concentration of chemical UV filters and bioactive products have been the focus of several researchers due to ecological issues, minimum ambient impact and for safe utilization. Various synthetic agents are used as photoprotectives, but their use is limited because of their potential toxicity in humans and their ability to interfere in certain selected pathways of multistage process of carcinogenesis. Phytoconstituents are gaining popularity as ingredients in cosmetic formulations as they can protect the skin against exogenous and endogenous harmful agents and can help remedy many skin conditions.

The present study attempts to develop skin lotion, *L. acidissima* (Thee) was selected as a bioactive agent. The procedure for the sunscreen lotion is presented in Section 2.9. Topical application of *L. acidissima* (Thee) lotion reduces UV-induced skin erythema in healthy human volunteer. Topical application of *L. acidissima* (Thee) has also been found to decrease the number of UV-induced sun burn cells in hairless mice skin.

3.9 Evaluation of Sunscreen Lotions from Bark and Fruit Pulp of *L. acidissima* (Thee)

Formulated sunscreen lotions were determined by *in-vivo* method using UV visible spectrophotometer. There are very few single chemical substances that have absorbance over full range of UV. Plant extracts, due to containing a wide range of natural compounds, usually cover this full range of UV wavelengths. One approach to protecting the body from harmful effects of UV irradiation is to use active photoprotectives. The absorbance value of stem bark sunscreen lotion was at 204.6 nm, 209.2 nm, 258.8 nm and 342.2 nm. And then, fruit pulp sunscreen lotion showed the absorbance at 215.2 nm, 259.0 nm and 342.0 nm.

3.9.1 Irritation test

Skin irritation is defined as a locally arising, non-immunogenic inflammatory reaction, which appears shortly after stimulation and usually disappears during a few days and represents the most common adverse effects in humans. Skin irritation test was also observed in all experimental groups. At the end of each day of experimentation, all animals were removed from the rat holders and their skin was observed and photographed. The full experiment comprised 3 days of application. After the third (last) day, all the groups did not present significant differences among themselves. The skin irritation test performed in Wister albino rats showed no signs of sensitivity, erythema and edema (Table 3.18). So the formulated sunscreen lotions were considered to be safe.

Table 3.18 Safety of Sunscreens

Group	Reactions	
	Erythema	**Edema**
G1	-	-
G2	-	-
G3	-	-
G4	-	-
G5	-	-

- = No visible reaction

G1 - Control group

G2 - The group tested with Thee bark sunscreen lotion (F1)

G3 - The group tested with Thee fruit pulp sunscreen lotion (F2)

G4 - The group tested with marketed sunscreen lotion (SPF 22)

G5 - The group tested with marketed sunscreen lotion (SPF 50)

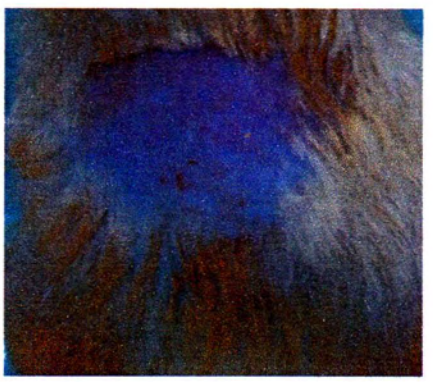
3.9.2 Study on the skin whitening effects of sunscreen lotions on the albino rats

Skin whitening effect was studied by adopting the method reported by Yusutami, *et al.,* 2004 with some modification. In this method, inhibitory effects of sunscreen lotions of bark and fruit pulp of *L. acidissima* (Thee) on the hyperpigmentation induced by sunlight was investigated by using albino rat models *in vivo*. Albino rat was chosen as the experimental animal not only because its skin is similar to human skin histologically and biochemically, furthermore, it also has the same natural history as human nevi and previously being described as robust small laboratory animal model for solar-stimulated light experimentation (Menzies *et al.,* 1998).

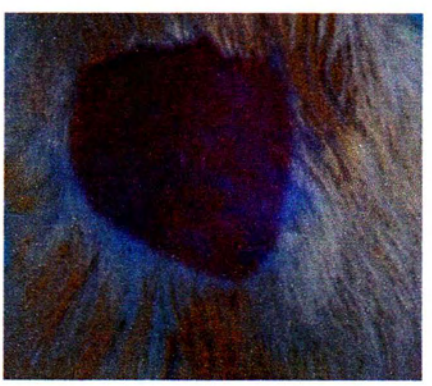
The hair was cleanly shaved on the back (2cm x 2 cm) area and the shaven skin was exposed to sunlight daily (10-11 AM and 2-3 PM, 2 hr/ day) for one week. After the sunlight induced on the hyperpigmented skin of the albino rats, the sunscreen lotions of the bark and fruit pulp of *L. acidissima* (Thee) were applied twice a-day for two successive weeks. Finally, the whitening effect of the samples on hyperpigmented skin was evaluated by viewing the naked eyes while comparing with control area.

Figures 3.37 and 3.38 represent photographs showing whitening effect on sunlight induced hyperpigmentation after two weeks of application. It can be seen from the figures that the skin of albino rats receiving treatment was appeared to be whiter than control. The extent of whitening effect of bark sunscreen lotion was seemed to be more effect than the fruit pulp sunscreen lotion.

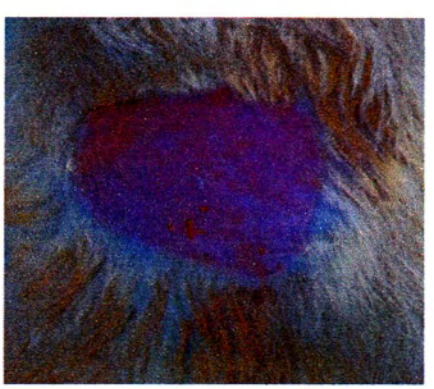
From this finding, it can be inferred that the bark of *L. acidissima* (Thee) possessed the ability to balance hyperpigmentation of the skin and to protect from photoaging.



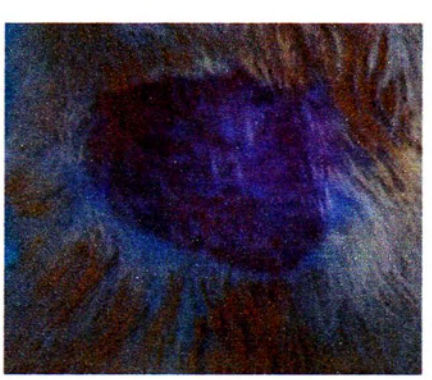
Before sun light induced



Sun light induced hyperpigmented skin

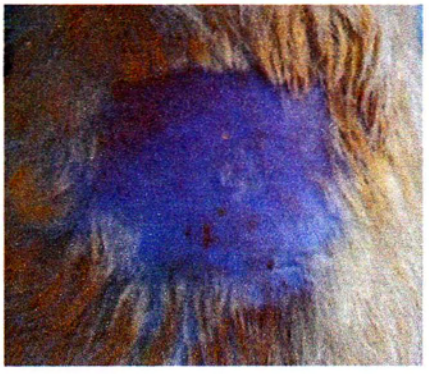


Whitening result after 1 week successive treatment

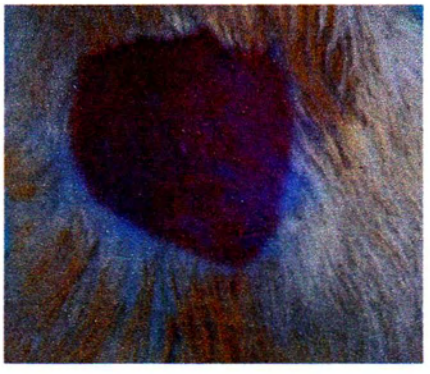


Whitening result after 2 weeks successive treatment

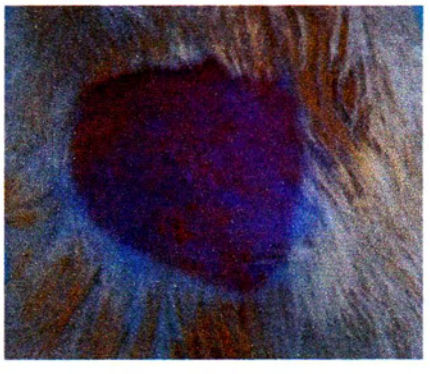
Figure 3.37 Whitening effect of Thee bark lotion on sun light induced hyperpigmented skin



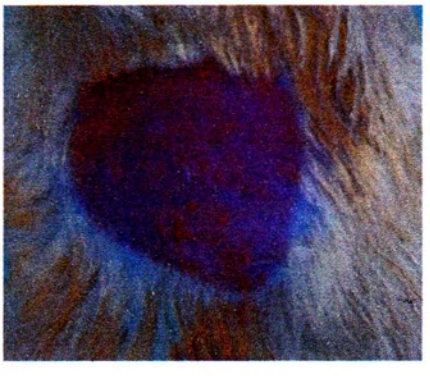
Before sun light induced



Sun light induced hyperpigmented skin



Whitening result after 1 week successive treatment



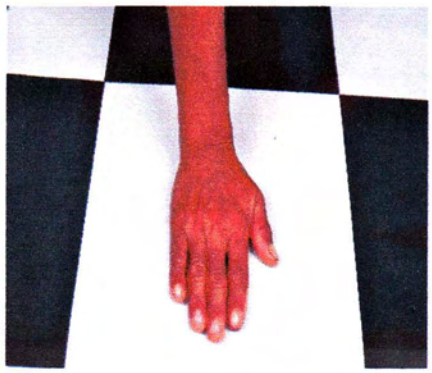
Whitening result after 2 weeks successive treatment

Figure 3.38 Whitening effect of fruit pulp of Thee lotion on sun light induced hyperpigmented skin

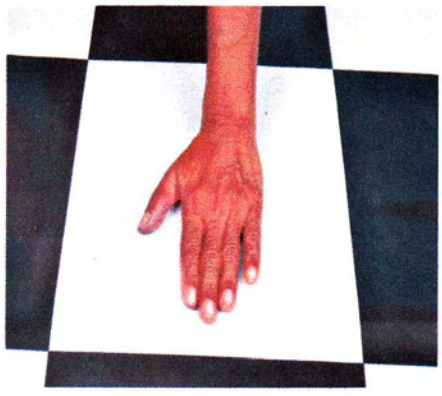
3.9.3 Study on the skin whitening effect of sunscreen lotions on human skin

As the skin irritation test was performed with the albino rats, the sunscreen lotions showed no signs of sensitivity, erythema and edema. Therefore, the sunscreen lotions were considered to be safe. The people working on the paddy field feel the skin redness and other skin diseases from the sun burning. Therefore, the sunscreen lotions of the bark and fruit pulp of the *L. acidissima* (Thee) were topically applied on the UV-induced skin erythema in healthy human volunteers. 5 mL lotions of the bark and fruit pulp of *L. acidissima* (Thee) were applied twice a-day for two weeks.

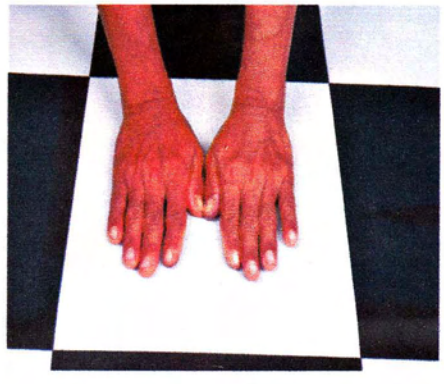
Topically application of sunscreen lotions has proven to be a significant protection against UV-induced skin erythema. The study on the topical application of sunscreen showed that the sunscreen lotions were good fed regarding handling, firmness and glowness of skin. The glow effect of sunscreen lotions were found to be capable to block the UV-radiation and protect the skin from sunburn. Figures 3.39 and 3.40 show the photographs showing whitening effect on UV-induced skin of human. On viewing the naked eyes, the whitening effect of bark sunscreen lotion was seemed to be more effect than fruit pulp sunscreen lotion.



Control

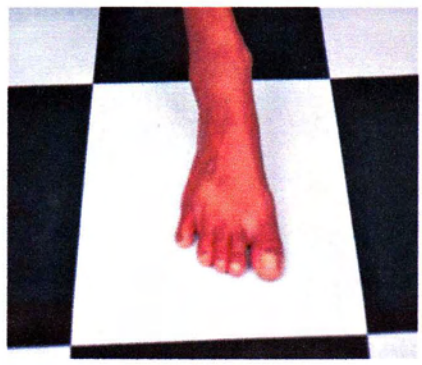


After application for two weeks

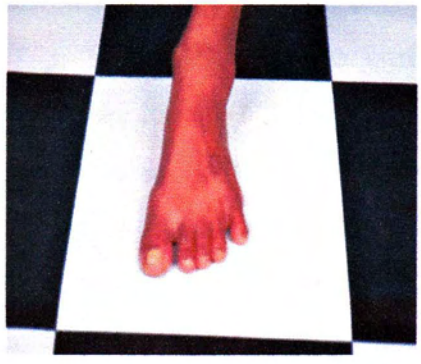


Comparing with control and using lotion

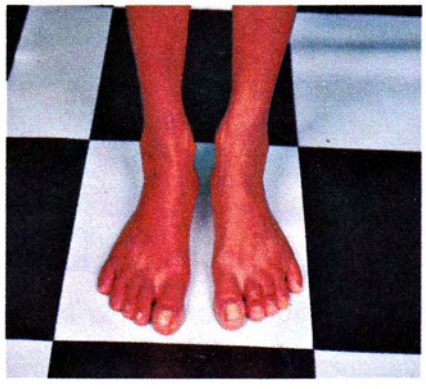
Figure 3.39 Whitening effect of Thee bark lotion on human skin



Control



After application for two weeks



Comparing with control and using lotion

Figure 3.40 Whitening effect of Thee fruit pulp lotion on human skin

3.10 Determination of Sun Protection Factor (SPF) of Sunscreen Lotions by Ultraviolet Spectrophotometry

In this research, bark and fruit pulp of *L. acidissima* (Thee) sunscreen lotions and two selected commercial sunscreen lotions were evaluated by UV spectrophotometry applying Mansur mathematical equation (Mansur et al., 1986). Two marketed formulations having known SPF 50 and 22 were considered for comparative evaluation with that of the formulated sunscreen lotions. The absorbance values at various wavelengths (λ_{max}) from 290 nm to 320 nm of both formulated and marketed sunscreen lotions are given in Table 3.19. The SPF values of formulations F1, F2 and the two marketed sunscreen lotions were calculated and presented in Table 3.20. The results showed that F1 (sunscreen lotion of bark of *L. acidissima*) have high SPF of 22.531 ± 1.7 which may be attributed to the presence of furocoumarin. Fruit pulp sunscreen lotion (F2) presented a calculated SPF value is smaller than the bark sunscreen lotion (F1). SPF value of F2 is 12.357 ± 1.0. The formulation F1 showed medium SPF (as sunscreen lotions with SPF ranging 15-20 are considered to be medium protection sunscreen lotions) which is sufficient for protection against sun burn for a period of about 3 hours. The presence of photo shielding flavonoids such as furocoumarin, which quench the production of free radicals in the skin, makes it possible for the formulations to get protection from UV solar radiation (Arun Rasheed, 2012).

Data variation can be due to the use of non- validated spectrophotometric methodology being used for determination of the absorption characteristics of sunscreen agents. However, there are many factors affecting the determination of SPF values, as for example, the use of different solvents in which the sunscreen lotions are dissolved; the combination and concentration of the sunscreen lotions; the type of emulsion; the effects of interactions of vehicle components, such as esters, emollients and emulsifiers used in the formulation; the interaction of the vehicle with the skin; the addition of the other active ingredients; the pH system and the emulsion rheological properties, among other factors, which can increase or decrease UV absorption of each sunscreen. The effect that different solvents and emollients have upon the wavelength of maximum absorbance and upon the UV absorbance of several

sunscreen lotions. Excipients and other active ingredients can also produce UV absorption bands, thus interfering with those of UVA and UVB sunscreen lotions. The effect of a solvent is only realized at high percentages. A high SPF values are more difficult to measure. A high SPF normally leads to a greater uncertainty also in the final *in vivo* result, due to the biological variations of the volunteers. Therefore, to develop sunscreen lotions with better safety and high SPF, the formulator must understand the physicochemical principle, not only the UV absorbance of the actives, but also vehicle components, such as esters, emollients and emulsifiers used in the formulation, since sunscreen lotions can interact with other components of vehicle, and these interactions can affect sunscreen lotions efficacy (Dutra, 2004).

Table3.19 Absorbance Values of Formulated and Marketed Sunscreen Lotions

Wavelength (nm)	F1	F2	Marketed Sunscreen	
			(SPF 50)	(SPF 22)
290	2.207	1.947	6.521	2.942
295	2.157	1.842	6.112	2.587
300	1.976	1.611	5.873	2.131
305	1.285	1.203	5.180	1.960
310	1.214	0.881	4.902	1.380
315	1.185	0.380	4.767	1.151
320	1.164	0.154	4.154	0.894

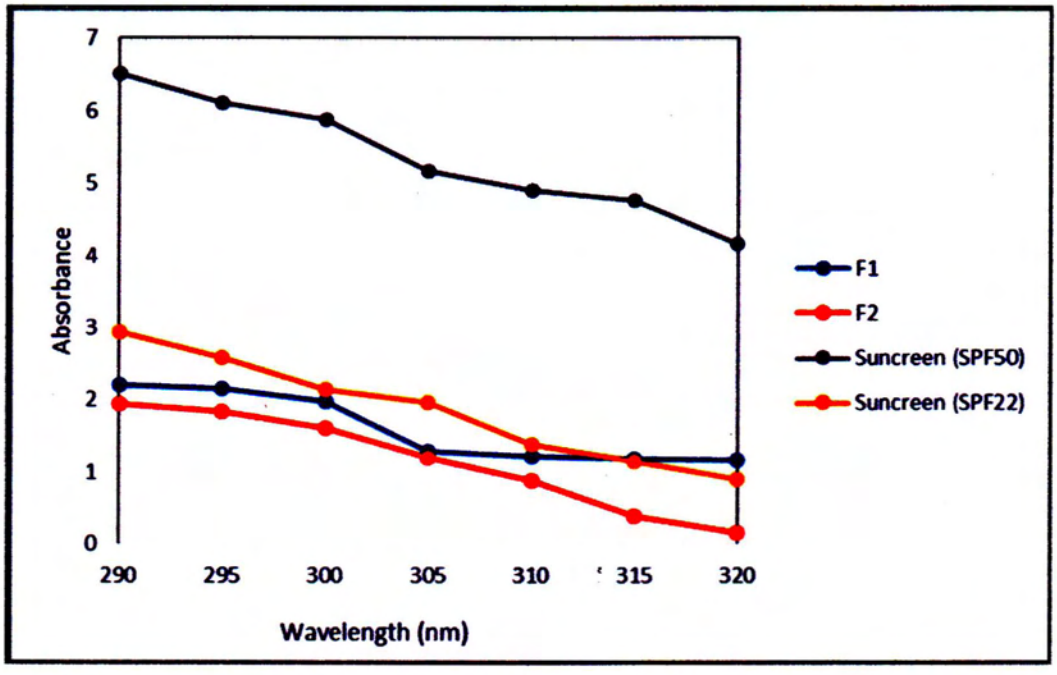


Figure 3.41 Absorbance values of formulated and marketed sunscreen lotions

Table 3.20 SPF of the Formulated and Marketed Sunscreen Lotions

Sr. No	Sunscreen Lotions	SPF
1.	F1	22.531 ± 1.7
2.	F2	12.357 ± 1.3
3.	Marketed Formulation with SPF50	48.685 ± 2.0
4.	Marketed Formulation with SPF22	20.631 ± 1.0

CHAPTER IV

4. CONCLUSION

From the overall assessment concerning with the chemical and biological activity investigation on *Limonia acidissima* L. (Thee), following inferences may be deduced.

- Preliminary phytochemical investigation by test tube method on bark and fruit pulp of *L. acidissima* indicated the presence of α-amino acids, alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, starch, steroids and tannins.
- In the determination of some heavy toxic metals and macronutrient mineral elements Na (0.742 ppm), K (14.90 ppm), Zn (0.134 ppm) and Mn (0.211ppm) were found to be present in bark and Na (1.12 ppm), K (14.80 ppm), Zn (0.2015 ppm) and Mn (0.163ppm) were found to be present in fruit pulp of *L. acidissima* by AAS method.
- In the determination of nutritional values, 9.25 % of moisture, 6.72 % of ash, 55.20 % of fibre, 0.48 % of fat, 5.01 % of protein, 23.07 % of carbohydrate and 121 kcal of energy value were found to be present in the bark of *L. acidissima* by AOAC methods.
- Compound I (xanthotoxin, 0.025 %), compound II (isopimpinellin, 0.025 %), compound III (marmesin, 0.0026 %) and compound IV (auraptene) were isolated from the bark of *L. acidissima*. Their structures were identified by modern spectroscopic technique.
- Antimicrobial activity of some crude extracts such as PE, $CHCl_3$, MeOH, CH_3COCH_3, EtOAc, EtOH and watery and extracts of Thee bark and MeOH,EtOAc andEtOH extracts of Thee fruit pulp were screened by using Agar Well Diffusion Method. Among the tested extracts, MeOH extract was the more potent than other extracts.
- *In vivo* examination of acute toxicity of Thee bark using albino mice model. Since the 95 % EtOH extract of Thee bark did not exhibit toxic effect with the

dose of 16g/kg body weight mice, it may be inferred that Thee bark may be used safely.

- Antioxidant activity test assessed by DPPH radical scavenging activity assay revealed Thee bark possess mild radical scavenging activity. EtOH extract of Thee bark (IC_{50} = 16.74 µg/mL) was more potent than other extracts: EtOAc (IC_{50} = 64.74 µg/mL) and PE (IC_{50} = 155.74µg/mL) comparing with standard ascorbic acid.
- The anti-solar property of Thee bark and fruit pulp sunscreen lotions were evaluated by using UV spectrophotometer. The result indicated that the sunscreen lotions absorbed in the UV B range.
- Skin irritation test of sunscreen lotions was performed on albino rats. From irritation test, it showed no signs of sensitivity, erythema and edema. Therefore, the formulated sunscreen lotions were considered to be safe.
- Determining of skin whitening effect was tested by using albino rat model and human skin. Both sunscreen lotions (containing Thee bark and fruit pulp) provided skin whitening effect. Thee bark sunscreen lotion was more whitening effect than fruit pulp lotion.
- Determination of SPF value of Thee bark and fruit pulp sunscreen lotion was performed by using UV spectrophotometer. By using the Mansaur mathematical equation, SPF value of Thee bark and fruit pulp sunscreen lotions were calculated. The SPF value of Thee bark sunscreen lotion was 24 and that of fruit pulp sunscreen lotion was 13. Therefore, Thee bark sunscreen lotion was found to be more stable with high SPF value proving a better sunscreen lotion.

The study attempted to develop herbal sunscreen lotion using extracts of *L.acidissima* and examined their efficacy for preventing sun burn. As the formulations with *L.acidissima* extracts were found to possess SPF in the range of 13 to 24, they can be used for normal skin to prevent sun burns. It can be concluded that the present research could bring advancement in the treatment of sun burns caused by exposure to UV rays. The present study can therefore assist the scientific organization and manufacturers in developing uniform standards for herbal sunscreens lotions.

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APPENDICES

APPENDIX I

1. 1 % Ferric Chloride Solution

Ferric chloride (1 g) was dissolved in distilled water and the volume made up of to 100 cm^3 in a volumetric flask.

2. Dragendroff's Reagent

5 cm^3 of 1.6 g of bismuth (III) nitrate in 20 % acetic acid and 5 cm^3 of 40 % aqueous solution of potassium iodide was added.

3. Sodium Picrate Solution

Picric acid (Ca.7.2 g) was quickly dried between filter paper, weighed quickly and treated with about 100 cm^3 distilled water. The solution was then neutralized with 1 M sodium hydroxide (20 cm^3). The resulting solution was found to be rather alkaline, this saturated picric acid solution (7.2 g in 100 cm^3) was added until it was slightly acidic.

4. Mayer's Reagent

Mercuric chloride (1.3 g) was dissolved in 60 cm^3 of water. It was added to a solution of 5 g of potassium iodide in 20 cm^3 of water, mixed and sufficient water was added to make up to 100 cm^3.

5. 5 % H_2SO_4 Solution

5 % H_2SO_4 solution was prepared by adding 5 cm^3 of concentrated H_2SO_4 to 100 cm^3 of distilled water.

6. 10 % lead Acetate Solution

Lead acetate (10 g) was accurately weighed and dissolved in 100 cm^3 of distilled water.

7. Ninhydrin Reagent

Ninhydrin (0.2 g) was dissolved in 100 cm^3 of acetone (0.2 % w/v) (Wagner and Bladt, 1996).

8. 1 % HCl Solution

1 % HCl solution was prepared by the mixing of 10 cm^3 of HCl and 428.4 cm^3 distilled water.

9. 1% Iodine Solution

1 % Iodine solution was prepared by the mixing of 1 g of iodine solution and 100 cm^3 distilled water.

10. 1% Potassium Ferricyanide Solution

Accurately weighed $K_3Fe(CN)_6$ (1 g) was dissolved in distilled water (100 cm^3).

APPENDIX II

1. Moisture Content

$$\text{Moisture / percent} = \frac{\text{Weight of moisture (g) x 100}}{\text{Weight of stem bark powdered sample (g)}}$$

2. Ash Content

$$\text{\% of ash} = \frac{\text{Weight of ash (g) x 100}}{\text{Weight of stem bark powdered sample (g)}}$$

3. Fibre Content

$$\text{\% of fibre content} = \frac{\text{Weight of fibre (g) x 100}}{\text{Weight of sample (g)}}$$

4. Fat Content

$$\text{\% of fat content} = \frac{\text{Weight of extracted fat (g) x 100}}{\text{Weight of stem bark powdered sample (g)}}$$

5. Protein Content

$$\text{Protein (\%)} = \frac{(V_s - V_b) \times M \times 0.014 \times 100 \times 6.25}{W}$$

V_s = volume of standard acid (mL) in required for sample titration

V_b = volume of standard acid (mL) in required for blank titration

M = molarity of standard acid solution in mol dm^{-3}

W = weight of the sample in gram

6. Carbohydrate Content

Carbohydrate % = 100 - (moisture + fat + ash + protein + fibre)

7. Energy Value

Energy value = [Protein (g) x 4 + Fat (g) x 9 + Carbohydrate (g)x 4]

(kcal/100 g)

Where,

1 g of protein = 4 kcal

1 g of fat = 9 kcal

1 g of carbohydrate = 4 kcal

APPENDIX III

Calculation of Sun Protection Factor (SPF)*

$$SPF = CF \times \Sigma_{290}^{320} EE(\lambda) \times I(\lambda) \times Abs(\lambda)$$

EE (λ) – erythemal effect spectrum

I (λ) – solar intensity spectrum

Abs (λ) – absorbance of sunscreen product

CF – correction factor (10)

* Sayre *et al.*, (1979)

Table 1. Normalized Product Function used in the Calculation of SPF

Wavelength (λ nm)	EE x I (normalized)
290	0.0150
295	0.0817
300	0.2874
305	0.3278
310	0.1864
315	0.0839
320	0.0180

CREDIT SEMINARS COMPLETED

Seminar I

Title : Preliminary Phytochemical Investigation and Elemental Analysis of Bark and Fruit Pulp of *Limonia acidissima* L.(Thee)

Date : 5.12.2012

Venue : Chemistry Theatre (S-17), Chemistry Department, University of Yangon

Seminar II

Title : A Study on the Phytochemical Constituents and Acute Toxicity and Antioxidant Activities of *Limonia acidissima* L. (Thee)

Date : 12.12.2013

Venue : Chemistry Theatre (S-17), Chemistry Department, University of Yangon

Seminar III

Title : Structural Elucidation of Isolated Compounds and Study of Anti-solar Activity of *Limonia acidissima* L. (Thee)

Date : 17.2.2015

Venue : Chemistry Theatre (S-17), Chemistry Department, University of Yangon

Seminar IV

Title : Isolation and Identification of Bioactive Phytoconstituents and their Biological Activities of *Limonia acidissima* L. (Thee)

Date : 27.5.2016

Venue : Chemistry Library, Chemistry Department, University of Yangon

PRELIMINARY PUBLICATION

1. Isolation and Identification of Bioactive Phytoconstituents and their Biological Activities of *Limonia acidissima* L. (Thee)

   (Chemical Bulletin, Issue No. (12), May, 2016, Department of Chemistry, University of Yangon)

2. Structural Elucidation of Isolated Compounds from Bark of *Limonia acidissima* L. (Thee)

   (Myanmar academy of arts and science, 15th Research Conference, October 2015, Department of Chemistry, University of Yangon)

CURRICULUM VITAE

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Title of M.Res	:	Investigation of Chemical Constituents and Screening of Antimicrobial Activity from Bark of *Michelia champaca* Linn. (Sa Ga War)
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External Examiner's Report
for PhD Dissertation submitted by Ma Khin Chan Thar (4-PhD-Chem-12)

27-05-2016

I have read carefully PhD Dissertation Title with "Isolation and Identification of Bioactive Photoconstituents and Their Biological Activities of *Limonia acidissima* L. (thee)" submitted by Ma Khin Chan Thar (4-PhD-Chem-12).

This Dissertation consists of phytochemical investigation, determination of nutritional values and biological activities studies such as antimicrobial activity, acute toxicity and antioxidant activity of the selected medicinal plant from medicinal point of view.

Further more, the identification of isolated compounds by tlc method and modern spectroscopic methods including 2D NMR spectroscopy and HR ESI mass spectrometry have also completed for organic chemistry point of view.

The special study which determines the sun protection factor of sunscreen lotion of the selected medicinal plant is very interested for medicinal point of view.

Instead of a little typing error was found in this dissertation Viva voce examination is also satisfactory.

Therefore, I am glad to recommend Ma Khin Chan Thar (4-PhD-Chem-12) for the Degree of Doctor of Philosophy in Chemistry.

Than Soe

Dr Than Soe
External Examiner
Rector
Myitkyina University

Referee's Report on Khin Chan Tha's PhD Dissertation

Date- 27.5.2016

I have gone through the PhD Dissertation submitted by **Khin Chan Tha** (4-PhD-Chem-12) for the award of PhD Degree entitled "Isolation and Identification of Bioactive Phyto - constituents and their Biological Activities of ***Limonia Acidissima*** L. (Thee)

In her dissertation , she described the Preliminary phytochemical investigation by test tube method on bark and fruit pulp of *L. acidissima* indicated the presence of amino acids, alkaloids, carbohydrates, flavonoids, glycosides, phenolic compounds, reducing sugars, starch, steroids and tannins.

In her dertermination of some heavy toxic metals and macronutrient mineral elements Na (0.742 ppm), K (14.90 ppm), Zn (0.134 ppm) and Mn (0.211 ppm) were found to be present in bark and Na (1.12 ppm), K (14.80 ppm), Zn (0.2015 ppm) and Mn (0.163 ppm) were found to be present in fruit pulp of *L. acidissima* by AAS method.

In her determination of nutritional values, 9.25% of moisture, 6.72% of ash, 55.20% of fibre, 0.48% of fat, 5.01% of protein, 23.07% of carbohydrate and 121 kcal of energy value were found to be present in the bark of *L. acidissima* by AOAC methods.

She isolated Compound I (xanthotoxin, 0.025%), Compound II (isopimpinellin, 0.025%), Compound III (marmesin, 0.0026%) and compound IV (auraptene) from the bark of *L. acidissima*.

Antimicrobial activity of some crude extracts such as PE, $CHCI_3$, MeOH, CH_3COCH_3, EtOAc, EtOH and watery and extracts of Thee bark and MeOH, EtOAc and EtOH extracts of Thee furit pulp were screened by using Agar Well Diffusion Method. Among the tested exracts, she found that MeOH extract was the more potent than other extracts.

Antioxidant activity test assessed by DPPH radical scavenging activity assay revealed Thee bark possess mind radical scavenging activity. From her results she observed that EtOH extract of Thee bark (IC_{50} = 16.74μg/mL) was the more potent than other extracts: EtOAc (IC_{50} = 64.74μg/mL) and PE (IC_{50} = 155.74μg/mL) comparing with standard ascorbic acid.

In her *vivo* examination acute toxicity of Thee bark using albino mice model. Since the 95% EtOH extract of Thee bark did not exhibit toxic effect with the dose of 65 g/kg body weight mice, it may be inferred that Thee bark may be used safely.

She evaluated the anti – solar property of Thee bark and fruit pulp sunscreen lotions by using UV spectrophotometer. The result indicated that the sunscreen lotions absorbed in the UV B range.

She made skin irritation test of sunscreen lotions was performed on albino rats. From irritation test, it showed no signs of sensitivity, erythema and edema. Therefore, the formulated sunscreen lotions were considered to be safe.

She tested the determining of skin whitening effect using albino rat model and human skin. Both sunscreen lotions (containing Thee bark and fruit pulp) provided skin whitening effect. She got the result, Thee bark sunscreen lotion was more whitening effect than fruit pulp lotion.

She determined the SPF value of Thee bark and fruit pulp sunscreen lotion was performed by using UV spectrophotometer. By using the Mansaur mathematical equation, SPF value of Thee bark and fruit pulp sunscreen lotion was 24 and that of fruit pulp sunscreen lotion was 13. Therefore, she found that Thee bark sunscreen lotion was stable with high SPF value proving a better sunscreen lotion.

I am glad to recommend her research work and I deeply recommend that she be awarded the degree of Doctor of Philosophy in Chemistry by University of Yangon.

Dr Khin Myo Naung

Professor and Head

Department of Chemistry

Yangon University of Distance Education