การประเมินฤทธิ์ทางชีวภาพและองค์ประกอบทางพฤกษเคมีของสิ่งสกัดด้วยแอลกอฮอล์จากพืชบางชนิดในวงศ์กระถินของไทย

นางสาวซาลฟารินา รามลิ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต

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BIOACTIVITY EVALUATIONS AND PHYTOCHEMICAL CHARACTERIZATIONS OF ETHANOLIC EXTRACTS FROM SELECTED MIMOSACEOUS PLANTS ENDEMIC TO THAILAND

Miss Salfarina Ramli

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Research for Health Development
(Interdisciplinary Program)
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Field of Study	Research for Health Development
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ชาลฟารินา รามลิ : การประเมินฤทธิ์ทางชีวภาพและองค์ประกอบทางพฤกษเคมีของสิ่งสกัดด้วยเอลกอฮอล์จากพืชบางชนิดในวงศ์กระถินของไทย (Bioactivity evaluations and phytochemical characterizations of ethanolic extracts from selected Mimosaceous plants endemic to Thailand) อ. ที่ปรึกษาวิทยานิพนธ์หลัก : รศ. ดร. นิจศิริ เรืองรังษี, 133 หน้า.

จากการศึกษาฤทธิ์ทางชีวภาพของสารสกัดเอทานอลจากส่วนต่างๆของพืช ได้แก่ ฝัก ใบ และเปลือกของพืช 20 ชนิดในวงศ์ Mimosaceae โดยประเมินความเป็นพิษต่อเซลล์ ฤทธิ์ด้านจุลชีพ และฤทธิ์ด้านออกซิเดชัน ผลการศึกษาพบว่าสารสกัดเอทานอลจำนวน 13 ชนิด แสดงความเป็นพิษต่อเซลล์ โดยทดสอบการตายของไรทะเล และพบว่าสารสกัดเอทานอลจากเปลือกต้นของมะขามเทศ มีความเป็นพิษต่อเซลล์สูงสุด โดยมีค่า LC_{50}=3.45 ไมโครกรัมต่อมิลลิลิตร การศึกษาฤทธิ์ยับยั้งเชื้อแบคทีเรียและเชื้อรา โดยวิธี disc diffusion method พบว่า สารสกัดเอทานอลจากใบของก้ามปู และสารสกัดจากเนื้อผลของเนียง มีความสามารถยับยั้งการเจริญเติบโตสูงสุดต่อเชื้อ *E. coli* and *S. Aureus* โดยมีค่า zone inhibition=20.67 ± 0.55มิลลิเมตร และ 13.35 ± 0.45 มิลลิเมตร ตามลำดับ ผลการศึกษาฤทธิ์ด้านเชื้อแบคทีเรียและเชื้อรา โดยวิธี broth microdilution พบว่าสารสกัดจากเปลือกของสะบ้ามอญแสดงค่าต่ำสุดในการต้านเชื้อ *S. aureus* มีค่า MIC=0.2 มิลลิกรัมต่อมิลลิลิตร และฤทธิ์ต้านเชื้อ *B. subtilis* มีค่า MIC=0.1 มิลลิกรัมต่อมิลลิลิตร สารสกัดจากใบของระกำป่ามีค่า MIC=0.8 มิลลิกรัมต่อมิลลิลิตร และสารสกัดจากใบของต้นไม้แดงมีค่า MIC=0.8 มิลลิกรัมต่อมิลลิลิตรในการยับยั้งเชื้อ *E. coli* ในขณะที่สารสกัดส่วนมากไม่มีฤทธิ์ในการยับยั้งเชื้อ *E. coli.* ด้วยวิธี broth microdilution เมื่อทำการศึกษาเปรียบเทียบฤทธิ์ด้านแบคทีเรีย และฤทธิ์ด้านเชื้อราของพืชวงศ์ Mimosaceae พบว่ามีส่วนน้อยที่แสดงฤทธิ์ด้านเชื้อรา มีเพียงสารสกัดจากใบของต้นก้ามปูและสารสกัดจากใบของจามจุรีสีทองแสดงฤทธิ์ยับยั้งสูงสุดต่อเชื้อ *Saccharomyces cerevisiae* และ *Candida albicans* โดยมีค่า MFC=0.25 มิลลิกรัมต่อมิลลิลิตร และ 2.5 มิลลิกรัมต่อมิลลิลิตร ตามลำดับ จากการศึกษาคุณสมบัติในการต้านออกซิเดชันในสารสกัดเอทานอลของพืชในวงศ์ Mimosaceae พบว่าสารสกัดจากใบของต้นไม้แดงมีความสามารถสามารถในการจับกับอนุมูลของดีพีพีเอชสูงสุด โดยมีค่า IC_{50}=0.01±0.001 มิลลิกรัมต่อมิลลิลิตร สารสกัดจากใบของต้นข่างมีความสามารถในการจับกับไนตริกออกไซด์สูงสุด โดยมีค่า IC_{50}=2.81±0.15 มิลลิกรัมต่อมิลลิลิตร สารสกัดจากใบของต้นระกำป่ามีความสามารถในการรีดิวซ์สูงสุด โดยมีค่า IC_{50}=12.0±1.00 µg/ml ไมโครกรัมต่อมิลลิลิตร และสารสกัดจากกิ่งของกระถินมีความสามารถในการคีเลทโลหะสูงสุด โดยมีค่า IC_{50}=1.365±0.034 มิลลิกรัมต่อมิลลิลิตร นอกจากนี้สารสกัดจากเนื้อผลของลูกเหนียงและสารสกัดจากใบของต้นไม้แดงแสดงฤทธิ์สูงสุดในการยับยั้งเอนไซม์ไทโรซิเนสอีกด้วย การศึกษาองค์ประกอบทางพฤกษเคมีของสารสกัดดังกล่าว โดยการวิเคราะห์หาปริมาณของสารฟีโนลิคและการวิเคราะห์ด้วยเทคนิค HPLC-PDA และได้นำสารสกัดที่แสดงฤทธิ์ทางชีวภาพสูงสุดมาวิเคราะห์ด้วยเทคนิค HPLC-ESI-MS ซึ่งจากผลการศึกษามีความเป็นไปได้ว่าฤทธิ์ทางชีวภาพของสารสกัดเอทานอลจากพืชวงศ์ Mimosaceae เป็นผลเนื่องมาจาก สารกลุ่ม ฟลาโวนอยด์ไกลโคไซด์ และโปรแอนโทรไซยานิดินที่พบตรวจพบในสารสกัดของพืชด้วยวิธีดังกล่าวข้างต้น

สาขาวิชา.....วิจัยเพื่อพัฒนาสุขภาพ..... ลายมือชื่อนิสิต.....

ปีการศึกษา.....2553..... ลายมือชื่อ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

5087764620: MAJOR RESEARCH FOR HEALTH DEVELOPMENT
KEYWORDS: CYTOTOXICITY/ ANTIMICROBIAL/ ANTIOXIDANT PROPERTIES / HPLC-ESI-MS / POLYPHENOLS

SALFARINA RAMLI: BIOACTIVITY EVALUATIONS AND PHYTOCHEMICAL CHARACTERIZATIONS OF ETHANOLIC EXTRACTS FROM SELECTED MIMOSACEOUS PLANTS ENDEMIC TO THAILAND. THESIS ADVISOR: ASSOC. PROF NIJSIRI RUANGRUNGSI, Ph.D. THESIS 133 pp

A total of 35 extracts from 20 species of Mimosaceae plants consisted of selected materials as pods, leaves and bark were studied. The biological activities as cytoxicity, antimicrobial, and antioxidant of the ethanolic extracts prepared by successive extraction in soxhlet apparatus were evaluated. Thirteen extracts showed cytotoxicity in the brine shrimp lethality test with the extract of *Pithecellobium dulce* stem bark was the most toxic (LC50=3.45µg/ml). In the paper disc diffusion method, extracts of *Samanea saman* leaves (zone inhibition=20.67 ± 0.55 mm) and extract of *Archidendron jiringa* pericarp (zone inhibition =13.35 ± 0.45 mm) showed the highest growth inhibition against *E.coli* and *S. aureus*, respectively. The antibacterial and antifungal activity of Mimosaceae ethanolic extracts were also being evaluated by broth microdilution method. Different results on antibacterial activity from broth microdilution method were obtained compared to the paper disc diffusion method. In the broth microdilution method, extract of *Entada rheedii* seed coat exhibited the lowest minimum inhibition concentration (MIC) against *S. aureus* (MIC=0.2 mg/ml) and *B. subtilis* (MIC=0.1 mg/ml). *E. coli* showed resistant to most of the extract except for the extract of *Cathormion umbellatum* (MIC=0.8 mg/ml) and *Xylia xylocarpa* leaves (MIC=0.8 mg/ml). On the antifungal activity, extract of *Samanea saman* leaves (MFC=0.25 mg/ml) and *Albizzia lebbeck* leaves (MFC=2.5 mg/ml) acted as fungicide to *Saccharomyces cerevisiae* and *Candida albicans,* respectively in the broth microdilution method. The antioxidant properties of the Mimosaceae plants ethanolic extract were evaluated from the ability to scavenge DPPH radical, scavenge nitric oxide, reducing power and metal chelating ability. The extract of *Xylia xylocarpa* leaves (IC_{50}=0.01±0.001 mg/ml), *Albizzia lebbeckoides* leaves (IC_{50}=2.81±0.15 mg/ml), *Cathormion umbellatum* leaves (IC_{50}=12.0±1.00 µg/ml) and *Leucaena glauca* twig (IC_{50}=1.365±0.034mg/ml) exhibited the highest activity in each antioxidant assays, respectively. In addition, extracts of *Archidendron jiringa* pericarp and *Xylia xylocarpa* leaves showed the highest inhibition in the *in vitro* tyrosinase enzyme inhibition property with L-Tyrosine and L-DOPA as substrates, respectively. The phytochemical content of the extracts were analysed by quantifying the total phenolic content and the analysis with HPLC-PDA. The extracts which showed the highest activity in the biological assays were further characterized by HPLC-ESI-MS. It was plausible that the biological activities of Mimosaceae plants ethanolic extracts were contributed by the tentatively characterized flavonoids glycoside and proanthocyanidins from the extracts.

Field of Study: Research for Health Development Student's signature .....

Academic Year: 2010 Advisor's signature.. Nijsiri Ruangrungsi

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LIST OF ABBREVIATIONS

HPLC	High performance liquid chromatography
PDA	Photo diode array
ESI	Electrospray ionization
MS	Mass spectrometer
LC/MS	Liquid chromatography/Mass spectrometer
STD	Standard deviation
DPPH	1,1-diphenyl-2-picryhydrazyl
DMSO	Dimethylsulfoxide
EDTA	Ethylenediamineteraacetic acid
L-DOPA	L-3,4-diydroxyphenylalanine
NO	Nitric oxide
SNP	Sodium nitroperusside

CHAPTER I

INTRODUCTION

Background and Significance of the Study

Since time immemorial, plants have been undeniable sources for both medicine and food. Currently, the health care approach using plant-based medicines has been an option or working as a complimentary treatment to modern medications. Being practised by numerous societies from different part around the world, selected plants are consumed for the taste and to treat ailments. For instance, the most influential ancient plant based medications in Asia are Ayurveda, Jamu, Kampo and traditional Chinese medicine which have been originated from India, Indonesia, Japan and China, respectively. Therefore, the recognition from consumers has been expected which in turn has escalating the demands and encouraging more scientific studies on the plant-based medicine. The study of such plants not only has provided insight into the earliest knowledge on the nutritional and toxicity of the plants used, but also a fascinating evidence of a geographical or environmental influences on the establishment of plants to the customs or traditions of a society. The archaeological documentations recorded on medicinal plants, clarified that dependencies to the plants were irrefutable although the underlying pharmacological properties were not fully understood. The selection of medicinal plants was unconscious and the discoveries of beneficial plants were purely serendipitous at that time [1].

Apparently, various chemical compounds are synthesized as results of both primary and secondary metabolic pathways in plants. These plants derived metabolic products known as phytochemicals have been found responsible for the nutritive and pharmacological effects of plants in preventing and treating ailments, whereas some compounds are constitutively expressed or activated upon threats as cell damage and competition. For instance, the ability of plant to defend from diseases or microbial attack is contributed by types of secondary metabolites; such compounds are at particular consideration to be screened as antimicrobial agents. Therefore, the studies of chemistry and biological activities of extract from traditional medicinal and food plants have been at most interest to support the claimed beneficial consumption and ethnopharmacological

effects [2]. In the most successful breakthrough, pure compounds from plants have been isolated and are further investigated to be developed as functional food, drugs or poisons. The increasing interest has consequently, diversifying the studies dealing with the health effects of plant extract and its phytochemicals. Several terminologies have been applied to define the field of research (Table 1).

Table 1 Several terminologies applied in the research of plant extracts and phytochemicals [3]

Terminology	**Definition**	**Example**
Pharmacognosy	Study of nature derived pharmaceuticals and poisons. The field also involves the standardization and quality control of the herbal drug.	
Nutraceutical	Study of food and food plants for its beneficial effects on health	• Carotenoids from tomatoes • Herbs and spices: garlic, rosemary
Phytotheraphy	Herbal medicine and their clinical use	• Leaves of *Ginkgo biloba* for cognitive deficiencies
Ethnopharmacology	Study of plants traditionally employed as medicine, food and toxin	
Natural product chemistry/ phytochemistry	Research involving the isolation and elucidation of pure chemical entities. Some were then developed into medicines or chemically modified for medicinal use	• Morphine • Salicilin: derivatized to yield salicylic acid.

Regardless the definition, the studies share the same objective which is to investigate the plants as prospect of finding and exploiting the sources to increase the quality of life. In most instances of plant extract investigation, biological assays are carried out to identify the promising plant extract, to guide the separation and isolation, finally to evaluate the active compounds. The application of these assays to plant extracts has been the earliest step in the discovery of bioactive compounds. Principally, a bioassay is any *in vitro* or *in vivo* system used to detect the biological activity of an extract or a pure substance from a living organism. In recent years, a large number of *in vitro* assays have been developed. Simple and inexpensive assays are suitable for a rapid and primary screening of extracts in the typical laboratory. Biochemical screenings that employ chromophores as the end-products are suitable for spectrophotometric detection. The spectrophotometric approach also offers the advantage of carrying out assays in 96-well microplates, thus able to maximize the number of extracts and using only small amount of extract to be evaluated in a time. In the most frequent investigations, the antioxidant and antimicrobial properties has been representing a starting point for health beneficial discovery of an extract [4]. However, to be most of valued, the *in vitro* bioassays have been designed on the basis of the disease or closely related to the possible underlying causes of diseases (Table 2).

Table 2 Bioassays for evaluation of plant extracts *in vitro*.

Model	**Example**	**Bioactivity evaluation**
Cell lines	**Cancerous cells** [5]: MCF-7 human metastatic mammary carcinoma cell line HepG2 cell lines Caco-2 cells HeLa cell lines Malignant and non-malignant cells (L929) ML-1 human acute myeloblastic leukaemia	Anticancer properties [5]: Cell cytotoxicity assay, Cell viability assay, cell proliferation inhibition assay

	Fibroblast: Swiss 3T3 albino mouse fibroblasts [6] human dermal fibroblasts [7-9]	wound healing potential [6] procollagen production promotion [7] cytotoxicity [8] free radical scavenging activity [9] antioxidant, anti-photo aging activity [10]
Tissue cultures	Mice brain tissue [11] Rat hepatocyte [12]	Neuroprotection from inflammatory damage [11] Lipid peroxidation inhibition [12]
Microorganisms	**Bacteria:** *Helicobacter pylori* [13] *Bacillus subtilis* [14] *Staphylococcus aureus* [15] *Escherichia coli* [16] *Salmonella typhimurium* [17] **Fungus:** *Candida albicans* [18] *Saccharomyces cerevisiae* [18]	gastrointestinal disorders protection [13] antibacterial screening [14-16] mutagenicity and antimutagenicity [17] antifungal screening [18]
Invertebrate	Brine shrimp (*Artemia salina)* [19]	Cytotoxicity activity [19]
Biochemical screening based on spectrophotometric detections	Free radical scavenging activity • DPPH radical • Reactive oxygen radicals (OH, O_2-) Lipid peroxidation assay TBARS (Thiobarbituric Acid Reaction)	Antioxidant properties [20]

Crude extract, which is the mixture of both active and non active constituents, frequently exhibited a wide range of biological activities. Therefore, preparation of an extract is a crucial process to successfully extracting the useful or targeted phytochemicals. Alcoholic extracts as methanol and ethanol have been recurrently reported showing promising biological activities. Technical advances in analytical techniques assist the identification and quantification of phytochemicals composition of extracts. Development and optimization of high performance liquid chromatography (HPLC) coupled with mass spectrometry facilitate the detection and characterization of active constituents in the extract. Results from chemical characterizations are often able to provide reasons to the biological properties of an extract and a short cut to the isolation of active constituents [21]. For example, phenolic compounds have been reported responsible for the antioxidant and antibacterial activity of plant extracts, therefore the quantification and characterization of phenolic compounds from extract have been frequently reported. Throughout optimised studies and advances in technologies, both biological activities evaluation and chemical characterization have been part of drug design before a drug enters a market (Figure 1).

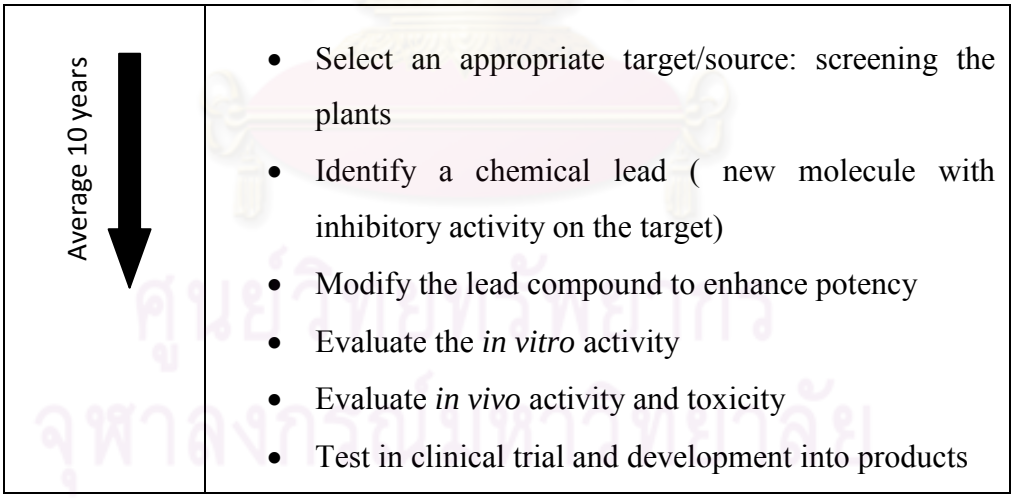


Figure 1 Stages in designing of a drug [21].

Although some plants with health beneficial reputations have been successfully developed into drugs and functional food, it is encouraging to exploit the diversity of plants offered by nature. Nevertheless, it has been a setback to make an investigation from number of plant species still unexamined. However, the fact that some plant

families are more represented indicated that some taxonomic group are pre-adapted with the social community as medicinal plants and food. A biological activity noted in a member of particular family also has been ascribed to the phytochemicals that prevalence to the family. Consequently, was postulated as extracts which contain similar compounds are expected to show similar bioactivities [22].

The principle of botany and systematic has been an important tool to study the available sources in an organisable means and reducing the inconsistency between the ethnomedical uses and the study of the other members from the same family due to the lack of botanical knowledge among the traditional practitioner. Thus, a taxonomic approach is appropriate to overcome the setback. Endemism is a taxa restricted to specified geographic region. Study on endemism allows identification and prioritising the natural sources. Selection of materials for scientific evaluation has often based on the application of the species as medicinal plants [23] traditional food [24] or simply by randomly selecting the species. However, the selection of plants for scientific study is more systematic if it is based on their ethnopharmacological values such as food and traditional medicine plants as that these materials have already been long utilized and showing reliable beneficial effects.

Thailand has long history of plants application both as medicine and food, thus there are numbers of potential plants to start with. Mimosaceous plants in Thailand are known to the Thais as plant for food and medicine. Mimosoideae is one the sub-family of Leguminosae or Fabaceae. This sub-family containing about 40 genera and encompasses numbers of trees and shrubs. Identified from its regular flower;calyx usually gamosepalous;stamens equal in number to the petals or twice as numerous [1]. Some that very common as food are fruit of *Parkia speciosa, Archidendron jiringa, Pithecellobium dulce*, Shoot tip of *Acacia pennata*, bud of *Leucaena glauca*, and leaves of *Neptunia oleracea* [25]. These species are familiar to the Thais and are available in the market. Although the consumption of Mimosaceous plant fruits causes pungent smell in breath and urine, the plants are familiar in local dishes not only in Thailand but also in the other countries of the South East Asia region. Other than being known as food, Mimosaceous plants are also applied in the Thai traditional medicine to treat various ailments [26].

Although there studies carried out on the Mimosaceous plants, only those that are commonly consume and known for its pharmacological values have been reported. There are numbers of Mimosaceous plants of Thailand which have not yet been investigated mainly due to underutilized. In contrary with the previous works, 20 species of Mimosaceous plants endemic to Thailand consisted of some which has never been reported previously were selected. Since it is imperative to successfully exploit the available natural sources, the antioxidant, antimicrobial and toxicity properties of the extracts prepared from the bark, stem, leaves, seed coat, pericarp, cotyledon and seed of Mimosaceous plants which have not been reported for pharmacological properties will be investigated. In addition, the phenolic content quantification and the characterization of phenolic compounds from Mimosaceous plants extract by liquid chromatography coupled with mass spectrometry (LC/MS) were also carried out. Thus, the outcome from this study will be able to guide, add value and provide information to validate the potential of the plants from this subfamily.

Objectives

1. To evaluate the biological activities of the Mimosaceous ethanolic extract by the selected *in vitro* bioassays
2. To characterise the constituents in the ethanolic extract of Mimosaceous plants

Scopes of the Study

1. Preparation of ethanolic extract from 20 species of selected Mimosaceous plants
2. Evaluation of biological activity of the ethanolic extracts, namely:
    a. Toxicity property
    b. Antimicrobial properties
        i. Antibacterial activity
        ii. Antifungal activity
    c. Antioxidant activities:
        i. Free radical scavenging
        ii. Metal chelating

iii. Reducing ability

d. Tyrosinase enzyme inhibition

3. Characterization of extract constituents
    a. Determination of total phenolic content by Folin-Ciocalteu reagent
    b. Detection of constituents from extract by HPLC (High performance liquid chromatography)
    c. Characterisation of constituent by LC-ESI-MS (Liquid chromatography coupled with negative electrospray ionization mass spectrometry)

Expected Benefits

1. Despite the other studies carried out previously on the extract of Mimosaceous plants, this study will provide systematic information on bioactivities of the ethanolic extract evaluated by selected bioassays.
2. This study also reports the phytochemical composition of the Mimosaceous ethanolic extract obtained from HPLC-DAD and LC-ESI-MS.
3. The assays selected in this study are appropriate to evaluate the potential of each extract and able assist the characterization of extracts thus provide a basis of the structure activity relationship of the extracts *in vivo*.
4. Generally, the results are expected to raise the appreciation to the species of the family which currently undervalued for their unknown biological properties.

CHAPTER II

LITERATURE REVIEWS

Biological activities of plants origin

It is indisputable that plants are unrivalled in producing natural compounds that both biologically active for medicinal and toxicological purposes, however it is the dosage of natural plants preparation that determined whether the extracts will be showing medicinal or toxicity. Therefore, before these natural extracts can be applied or utilized, their biological activities have to be assessed not only to evaluate their potential but also to ensure their safety. Testing large number of extracts to determine the biochemical or cellular effects has been the first steps in the discovery of bioactive compounds.

In principle, a biological assay or bioassay is any *in vitro* or *in vivo* system used to detect the biological activity of an extract or a pure substance from a living organism. Currently, extracts from plants have been tested for various biological activities, for instance; antimicrobial, antiviral, antidiabetic, antimutagenic, cytotoxicity, antioxidant properties to evaluate and provide scientific reports on the claimed therapeutic and toxicity potentials of plant extracts [4]. The application of these assays to monitor the presence of a bioactive compound during the isolation process is called bioassay-guided fractionation or isolation. Thus, all fractions generated are tested for biological activity, and those giving a positive test are further processed until the bioactive agent is obtained in a pure form. There are a large number of bioassays available that differ in a degree of sophistication. Although there is no satisfactory assay available, the investigations on plant extract are often based on the toxicological and pharmaceutical properties.

Cytotoxicity activity

Many compounds of botanical origin are assumed to be natural and safe. However, this concept is largely circumstantial, as the medical property derived from their chemical structures also contributes to their toxicity effects. Thus, other than determining the pharmacological property of plant extract, the toxicity is indispensable to consider the extract is safe [27]. Adverse effects of plant-derived remedies have been

reported which contributed by many factors that increasing the frequency of adverse reactions. For instance, the toxicity of herbal treatment has been reported [28]. Gastrointestinal effects such as nausea and diarrhea, and skin reactions are common to ingested products. Herbal treatment as herbal tea are considered as food products, therefore unlike all medications prescribed, herbal supplementation are not subjected to randomised clinical trial testing for efficacy. Which means the manufacturing standard and the regulation of quality are not required lawfully. Therefore, the toxicities of these plants were unaware and poorly understood [29].

Toxicity tests are needed to assess the associated toxicity risks clinical significances of this potential supplementation and its medical interaction. Generally, toxicity involves studies of toxic agents and the untoward consequences that affect biological systems. No single method is expected to cover the complexity of plant extract toxicity to humans but a prediction can be made based on the results from available assays. In most frequently studies, animals, cell lines and microorganisms are the model employed to observe the toxicology effects of plant extract. Any exposure of plant extract to the studied organism that equal or more than 24 hours is regarded as acute. In some experiment settings, 72 hours may still considered as acute exposure. Sub-acute refers to continuous or repeated exposure to a chemical for more than 72 hours but less than 1 month. Chronic is any relative time period for which continuous or repeated exposure beyond the acute phase is required for the same chemical to induce a toxic response. Traditional subcronic is 1-3 months [30]. Such study provides scientific information on the safety, efficacy and quality control of the plants. The results are also useful for the elaboration of official monograph of the plants in pharmacopeia.

Brine shrimp lethality assay

Substituting the use of laboratory animals in toxicological tests due to the high cost for chemicals and animal suffering caused by the toxicological tests have been proposed [31]. The bioassay utilizing brine shrimp or *Artemia salina* (Artemiidae) for predicting the toxicity of plant extracts has been employed as an alternative. In this test, the tiny crustacean *Artemia salina* is used. The eggs are readily available commercially, can be maintained indefinitely in the laboratory in their cyst form for years if refrigerated

are easy to hatch. The eggs hatch within 48 hours to provide large numbers of larvae (*nauplii*) in the artificial sea water. Initially, *A. salina nauplii* are among the best live prey for first feeding larvae to increase the normal growth and high survival ratio of fish larvae in aquaculture. Previously, a study was done to enrich the fatty acid content in *A. salina* thereby, improving the nutritional values of *A. salina* for feeding of larval fish [32].

A wide variety of biologically active chemical compounds, in particular cytotoxic agents, are found toxic to brine shrimp. Thus, the death of brine shrimp during an exposure to varying concentrations of these compounds forms the basis of a toxicity test. Bioactive compounds are nearly always toxic in high concentrations and, as toxicology can be described as pharmacology at higher doses, this principle has been applied to the screening of medicinal plant extracts in the brine shrimp toxicity test. The test has been used to access the toxicity such as algal extract, aldehydes and fatty acid [33]. Furthermore, toxicity study put confidence to the potential treatment or drugs and has become a part of pharmacological evaluation to corroborate the employment of medicinal plants in ethnomedical practices [34]. In many toxicological studies, brine shrimp assay has been a complementary assay to other toxicity assay. For instance, an extract which exhibited an absence of genotoxic response against HepG2 cells and low mortality of brine shrimp, was suggested as a safe extract to be developed as an anticarcinogen agent [35].

In the absence of toxic compound, *A. salina* able to survive up to 48 hours without sensitivity to hunger because of the fact that the nauplii derives enough nutrition from the egg. The suitable age to use *A. salina* for toxicology evaluation was proposed at the age of 48 to 72 hours [36]. Frequently, the compounds or extracts are tested at initial concentrations of 10, 100 and 1000 µg/ml containing 5ml of artificial seawater, 10 brine shrimps and 5 replicates. After exposure of 24 hours the survivors are counted. Increase of mortality which proportional to the increase of concentration provided linearity in the dose-effect relationship of every extract and determination of the LC_{50}. The results can be expressed as; not toxic (LD50>1000), weak toxicity (LD50 500-1000 µg/ml) toxic (LD 50 100-500 µg/ml) and very toxic (LD50 <100 µg/ml). In one of the previously study, it

was reported that the LD_{50} values of extracts with ≤100 µg/ml was considered toxically active and those with <20 µg/ml were considered to be very toxically active [37].

The brine shrimp lethality technique is rapid which required around 24 hour of exposure, simple, no aseptic techniques are required, easily mastered, inexpensive and uses small amounts of test materials. It offers advantages against other toxicity assays. Consequently, the brine shrimp lethality assay has become a general bioassay that suitable for crude extract with the aim to provide a frontline screen for predicting the cytotoxicity and pesticidal activity of crude extract. The assay is considered a useful tool for preliminary assessment of toxicity. Since it was first introduced, the assay has been used in the isolation of *in vivo* active antitumor agents and has been part of protocols for bioassay guided fractionation [35].

The reliability of brine shrimp lethality test have been evaluated by comparing the assay with toxicity assay using mice to determine the oral acute toxicity of plant extract. Previously, the toxic effect of 20 plant extracts was evaluated by both animal and brine shrimp assay. A good correlation ($r=0.85$, $p<0.05$) between both assays was observed [38]. In another study, the extract which was toxic for brine shrimp was found toxic to mice and showed toxic background to the *Salmonella* strains although it was not mutagenic [39]. Thus, suggesting the assay as a useful alternative model. The brine shrimp lethality assay can also be carried out to select the non toxic concentration of plant extract.

Due to its simplicity, the assay has been extended not only to evaluate a cytotoxicity property. The brine shrimp lethality bioassay has been reported employed in other pharmacological evaluations such as antimicrobial, pesticidal and antitumor activities of the natural compounds [40].

Previously, brine shrimp lethality assay was applied to investigate the effect of antioxidant and reactive oxygen species to cell. It was carried out as an attempt to expand the utility of brine shrimp lethality assay. Result showed that 4-methylcoumarins compound that possessed antioxidant activity was significantly nontoxic to the larvae viability at concentrations up to 500 mM, which is similar to or higher than those shown

capable to induce significant antioxidant effects [41]. This result supported the study that due to the lipophilicity of the compounds which allow it to enter the cell, the exposure of compounds which involve with superoxide generation as hydrogen peroxide, paraquat resulted in damage by superoxide or other reactive oxygen species was toxic to *A. salina*. It was suggested the toxicity effect was generated by reducing the natural ability of *A. salina* to protect itself using endogenously generated antioxidant enzyme SOD whereas a compound that have protective effects by virtue of its ability to scavenge superoxide displayed protective activity to the brine shrimp. Furthermore, an inhibitor of endogenous SOD increased the toxicity of superoxide mediated toxicity; a menadione bisulfite, thus lending some credence to the proposed mechanism and utilization of the brine shrimp lethality assay [42].

Antimicrobial

The evolution of antibiotic-resistant bacteria has stimulated the search for potent antibacterial agents from natural sources. Plant extracts are of interest as antiseptics and antimicrobial agents. Due to the fact that plants are able to synthesize toxic substances to defend against infections, insects and herbivores in nature, natural antimicrobials derived from the barks, stems, leaves, seed and flowers of plants have been increasingly studied [43]. The continuous needs for discovery of antibacterial drugs are resulted from the incidence of current infectious diseases and the occurrences of bacteria resistance to the existing antibiotics. Along with the utilization as preservatives in food or cosmetic, the search of new antibacterial agents has been escalating. The substances that can either inhibit or kill the pathogens have been considered as potential candidates in the development of antimicrobial agents.

Different methods available to detect antibacterial activity have been contributed to variations of observed antimicrobial effects of plant extract. For instances, paper disc assay has been an approach to study potential antibacterial compounds. By quantifying the size of the inhibition zone the effectiveness of the extract is considerately evaluated compared to visibly evaluate the microbial growth by eyes in the broth microdilution method. The values of MIC (Minimum Inhibition Concentration) and MBC (Minimum

Bactericidal Concentration) indicate the bacteriostatic or bactericidal property of an antibacterial agent, respectively. The MIC is defined as the lowest concentration of an antimicrobial agent that will inhibit the visible growth of a microorganism after overnight incubation. MIC has been often used as a research tool to determine the *in vitro* activity of potential antimicrobial; whereas minimum bactericidal concentration (MBC) is the lowest concentration of an antimicrobial agent that will prevent the growth of bacteria after being subcultured on the antibiotic free media. Some agents kill bacteria (bactericidal) while others only inhibit the growth (bacteriostatic). The bactericidal is irreversible while bacteriostatic is reversible. Nevertheless, the bacteriostatic agents are successful in the treatment of infections because they prevent the bacterial populations from increasing and host defence mechanisms can consequently cope with the static population although it is less effective for immunocompromised patients. Some agents also are bactericidal some species, but are only bacteriostatic for others [44]. The concept of MBC has been extended to the antifungal agent as minimum fungicidal concentration (MFC).

Many antimicrobial screening studies have shown that gram-positive bacteria are more sensitive than gram-negative bacteria and fungi to plant extracts. However, resistant strains of the gram-negative bacteria *Escherichia coli* and the gram-positive *Staphylococcus aureus*, along with coagulase-negative staphylococci are responsible for over 50% of hospital bloodstream infections. Thus, there is an urgent need for novel chemical entities against both gram-negative, gram-positive bacteria and fungi [45]. From the collected evidences, antimicrobial activity of natural products have been at great interest and showing a huge prospective for medical proposes [46]. In addition, the development of antimicrobial resistant strains is a growing cause of concern. These drawbacks justify further research and development of natural antimicrobial agents. The use of plant extracts with medicinal properties represents a concrete alternative for the treatment of different pathological states. However, in the absence of a scientific basis, such practices may generate serious adverse effects.

Scientific strategies incorporate the evaluation of both bacteriostatic and bactericidal activities of possible candidates with the identification of the compounds

responsible from the plants. In most frequently reported studies, alcoholic extract as methanol [47] and ethanol have been showing promising antibacterial activities. Nevertheless, along with the solvents and extraction procedures [48], and different methods available to detect antibacterial activity have also been suggested contribute to the variation of observed antimicrobial effects of plant extract.

Previously, a stem bark decoction of *Albizia gummifera* the members of Mimosoideae subfamily which traditionally used for the treatment of hepatitis, parasites and other infectious diseases in Cameroon were tested *in vitro* for their antimicrobial activity. The ethyl acetate extract of the stem showed antimicrobial activity against 5 species of Gram-positive bacteria, 4 species of Gram-negative bacteria and 2 pathogenic yeasts species [49]. Therefore, there is a potential to find the antimicrobial agents from Mimosaceous plant in Thailand. Furthermore, the antimicrobial properties reported will contribute to the appreciation to the plants before a significant improvement in managing the natural product for health disorders is possible.

Antioxidant activity

Free radicals and lipid peroxidation

Compelling evidences that oxidation and free radical have been contributing to the deterioration of food, implication in human health, onset of diseases, along with preference of consumers, have been driven extensive studies toward evaluation of natural sources with high antioxidant properties. Free radical has been described as atoms or compounds which contain an unpaired electron due to their odd number of electrons. They exist independently which is free from any support from other species and are very reactive. The free radicals can be stabilized by losing the unpaired electron (oxidation process) or pairing another electron to the unpaired electron (reduction process), however, by doing so the free radicals are engaged in a chain reaction that eventually breaks apart important biology molecules as lipid and protein [50].

One of the important free radical is molecule oxygen. It is a biradical which contain two unpaired electrons. The oxygen derived intermediates such as superoxide radical ($O_2^{-\bullet}$), hydroperoxyl radical ($HOO^{\bullet}$), hydrogen peroxide (H_2O_2) and hydroxyl

radical ($OH^{\bullet}$) are collectively called as reactive oxygen species or ROS. Hydrogen peroxide is not radical but it is able to generate hydroxyl radical. ROS is highly reactive species due to their unstable electron configuration, thus they attract electron of other molecules to form another free radical that is also unstable and capable of reacting with another molecule. This sustainable formation of free radicals is called chain reaction. Molecule oxygen slowly oxidises organic compound at moderate temperatures by reacting with the double bonds of the unsaturated fatty acid to produce an ultimately a complex mixture of volatile, rancid smelling; short chain carboxylic acid products [51].

The deterioration of fats, oils and foodstuff which are rich in polyunsaturated fat in the presence of oxygen or termed as auto-oxidation have prompted the design of antioxidant substances or inhibitors to slow down and even stop the undesirable auto-oxidation reaction in the food processing industry. An antioxidant is a substance that acts as a reducing agent which donates electron or hydrogen to the free radical to form a stable molecule. The antioxidant substances have been defined from its property to scavenge free radical, reducing capacity, metal chelating which all are important in the inhibition of free radical generation and lipid peroxidation in the ezymatically or nonenzymatically conditions [52].

In the body, oxygen radicals are derived from mitochondria which are the energy factories of cell. Polyunsaturated fatty acid (PUFA, is a lipid of cellular membrane that confer the fluidity, flexibility and selective permeability of cellular membrane which essential in sustaining the cellular functions. High concentration of PUFA results continuous oxidative challenge to the cellular membranes. Fatty acid with no double bond or with one double bond can undergo oxidation but not lipid peroxidation, therefore PUFA exhibited the highest sensitivity to the oxidation due to the number of double bond present per fatty acid, whereas monosaturated and saturated fatty acids are much less reactive and do not participate in the lipid peroxidation [53].

Lipid hydroperoxide is the first product of lipid peroxidation which is relatively stable, however stimulation from iron furthered lipid peroxidation and accelerates peroxidation by decomposing lipid hydroperoxides into peroxyl and alkoxyl radical that perpetuate chain reaction of lipid peroxidation. Iron is among essential element for living

organism. Iron exists as ferrous (Fe^{2+}) and ferric (Fe^{3+}) ion. The Fe^{3+} is biologically inactive however after being reduced to Fe^{2+}, it involves in the production of hydroxyl radical ($OH^{\bullet}$). Due to the presence of metal ion, a large variety of products as short and long chain aldehyde are also produced during lipid peroxidation [54]. For instance, it was observed that the incubation of rat liver slices rich in protein with iron ($FeSO_4$), generated aldehydes, oxidized protein and showed increased in cell death [55].

For such reason, metal chelating capacity of antioxidant substances is important to reduce the concentration of the catalysing transition metals involve in lipid peroxidation [56]. Chelators are small organic compounds that form a complex with a metal ion. The generation of free radical mediated by metal is depending on the binding of metal with the chelator, if the binding is loose, metal ion will be released and react with hydrogen peroxide or lipid hydroperoxide. Therefore, metal chelating capacity was reported as secondary antioxidants by stabilizing the oxidized form of the metal ion and considered as prevention mechanism against free radical generations [53].

Antioxidant substances

Identified roles of free radicals led to identification of dietary antioxidants that may regulate or combat free radical tissue damage. Supplementation of antioxidants as vitamin C, vitamin E and diet rich in antioxidant has been recommended to the athletes for a reason that strenuous exercise has been found generating more free radical in the body. The supplementation was not meant to increase the performance of the athlete but to replenish the loss of indigenous antioxidant enzymes resulted from acute exercises, thus antioxidant work as preventer of inflammation to the muscles [57].

Primarily, an antioxidant was not considered as an essential nutrient, and the importance was unknown. However, consuming antioxidants has been observed able to slow the pace of free radical damage to the body. The scourge to sailors described as scurvy demonstrated the consequence in deficiency of vitamin C; a water soluble antioxidant which is abundant in the citrus fruits [58]. A number of exogenous antioxidant derived from diet has been identified and practically all diets contain at least

some antioxidant. Some of the nutritive antioxidants and their dietary sources are shown in table 3.

Table 3 Antioxidants from dietary sources [59]

Antioxidant substances	Dietary sources	Biological functions
Alpha tocopherol (Vitamin E)	vegetable oil, secondary sources are liver, eggs, cereal, and legumes	Preventing oxidative degradation of cellular constituents, contributes to membrane repair, block the formation of nitrosamine, protect human LDL and reduced exercise-induce oxidative damage.
Ascorbic acid (Vitamin C)	fruits and vegetables	Has been regarded as the most efficient water soluble free radical chain breaking antioxidant in human tissues and plasma. Increased ascorbic acid in serum enhanced immune function.
Conjugated dienoic isomers of linoleic acid (CLA).	Dairy product and ruminant tissue. CLA is produced via microbial biohygdrogenation and the thermal isomerozation of linoleic acid.	
Peptides and amino acids as dipeptides carnosine, B-alanyl-mathylhistidine,	skeletal muscle tissue of most vertebrate	Histidine residue plays critical role in peptide antioxidant activity by

and anserine (b-alanyl-L-1-methylhistidine)	chelating the transition metal.

Dietary antioxidant helps keeping the balance of free radical and endogenous antioxidant in the body, which has profound effect to the health. The French paradox; is the low incidence of mortality levels provoked by coronary artery disease in France than other industrialised countries [60]. Although the consumption of saturated fats in France is much the same and the other factors associated with risk of coronary artery disease as arterial blood pressure, body weight and smoking are no lower in France compared to other countries [61], studies carried out on the phenomenon of French paradox successfully associated the protective effects of wine consumption that rich in antioxidant polyphenols with the low risk of coronary heart disease [62]. Supplementation of vitamin E resulted a decreased in platelet aggregation among healthy volunteer suggested a protection from development of atherosclerosis compared to healthy subjects with low antioxidant consumption [63], whereas the consumption of polyphenol-rich cocoa beverage protected against human erythrocyte hemolysis *in vitro* [64].

Some antioxidants have been recognised responsible for the medical and nutritive properties of plants. For instance, the rhizome of turmeric (*Cucurma longa*), rhizome of ginger and chilli fruit which are well known in the Asian culinary were reported to act as antitumor promoter. The property was contributed by the pronounced antioxidant activity of cucurmin, gingerol, capsaicin that present in the plants respectively [65].

Consequently, the study of antioxidants and free radicals have lead to popular claimed as antioxidant vitamins or dietary supplements protect from diseases and maintain the beauty. Considerable interests have been directed toward identification of plants with antioxidant ability. The quantification of antioxidant from commonly consumed food and beverages has been intensively carried out. The aims of such the study are to provide information as estimation on the intake of antioxidant phytochemicals and the potential sources. For instance, due to abundance of vitamins, phenolic compounds and carotenoids, a study showed that fruit juices contributes

(approximately 5-6%) to the total antioxidant in the Spanish diets [66]. Study on 28 plant extracts consisted of commonly consumed crops as flaxseed, berry, potato and vegetables revealed the ability of these extracts against oxidation [67].

Similar study conducted have suggested the advantageous consumption of guava [68] and spinach [69], whereas results from the antioxidant screening of fruits, vegetables, spices, cereals and herbs of Finnish origin suggested berries and apples as high in antioxidant [70]. Red wine extract showed a potent scavenger of free radical and the activity was relative to the abundance of polyphenol in the extract. The polyphenols in wine possess structure that confers them with an antioxidant property which can inhibit the processes leading in the long term to atherosclerosis and arterial thrombosis [71]. Results from such studies are able to encourage, educate and give consumers more choices of good and nutritious food to promote good health. Other than being consumed as antioxidant supplement, some of phytochemical and plant extract are in a consideration to be formulated into skin care products to provide protection against generation of free radical induced by exposure to the ultraviolet rays [72]. Table 4 shows some classes of phytochemicals with antioxidant activity.

Table 4 Classes of phytochemicals known for antioxidant properties [73]

Class of phytochemicals	**Example of compounds**
Carotenoids	Lycopene, lutein,astaxanthin
Bioflavonoids	Genistein, diadzein
Phytosterols	Sitosterol,stigmasterol, oryzanol
Tannins	Phenolic, polyphenols
Terpenoids	Limonine, limonene
Allylic compounds	Diallyl sulphide and disulfide
Indoles	Indole-3-carbinol

The practical role of antioxidant as food additive to delay the rancidity of food and increasing the product shelf life should not be neglected. The addition of antioxidant is a method of increasing the shelf life, especially to fat and vegetable oil. Antioxidants also have been added to fortify the food and to act as nutritional supplement. For

instance, spices as rosemary, thyme, oregano and curry leaves which are abundant with antioxidant substances have been added to preserve the freshness of foods. However, synthetic antioxidants such as Butylated hydroxytoluene (BHT) and Butylated hydroxyanisole (BHA) are much preferred in the food industry. Nevertheless, recent health concern over the usage of the synthetic antioxidant and their undesired medical symptoms as allergy and intolerance has increased demand on antioxidant from natural sources [74].

Therefore, from previously studies antioxidants are considered important and the search of its source especially from plant origin has been increasing greatly. Antioxidant activity is ubiquitous in plant kingdom which is sensible because the absorption of photons from the sun during the photosynthesis involves oxidation process. Therefore, plants are loaded with molecular antioxidant compounds. Source of antioxidant from the non edible part of the plant are at immense consideration. The by product from industry have been overlooked, however recent studies showed that the by product exhibited a promising antioxidant activity.

The extract from by product of wine and juice making industries as stalk, skin, seed and marc of grape, apple and vegetables by products as green tea, artichoke, cauliflower, onion, carrot and celery [75] have been investigated for their antioxidant properties. A similar study was also carried out to the cocoa shell, mango peel, roselle seed and by product of pink guava juice processing [76]. The by-products are rich with phenolic compounds which acknowledged for the antioxidant properties. Therefore utilization of by-product industry as a low cost natural antioxidant sources have been suggested. Further studies have been carried out to optimize the extraction of antioxidant substances from these materials. Among that frequently discussed were the optimization of solvent extraction, time and temperature [77].

Well developed antioxidant methods to evaluate antioxidant activity both *in vivo* and *in vitro* along the mechanisms underlying the assays have been widely published. Antioxidants inhibit or retard oxidation in two ways: first as a free radical scavenger by donating electron to the unpaired electron of free radical, hence interrupting the free

radical chain process. Vitamin E (tocopherol) and phenolic compounds are the examples of free radical scavenger. Whereas, the second mechanism does not involve direct scavenging of free radicals such as binding of metal ions, scavenging oxygen, converting hydroperoxides to non-radical species, absorbing UV radiation or deactivating singlet oxygen [78]. Depending upon the reactions involved, there are numbers of reliable antioxidant assay to choose from which are based on the mechanisms of antioxidants as inhibition of lipid peroxidation, binding to transition metal ion, reducing capacity and scavenging ability [79].

Tyrosinase inhibition activity

Tyrosinase is the copper containing enzyme known responsible for the browning of a cut fruits and involve in the formation of melanin in human skin. The enzyme acted upon two substrates; L-tyrosine and L-3,4-dihydroxyphenylalanine or DOPA. Tyrosinase catalyzes the hydroxylation of tyrosine to DOPA activity and the oxidation of DOPA to dopaquinone known as monophenolase and diphenolase activity, respectively [80]. Melanin protects human skin from UV radiation and the synthesis takes place in the melanocytes. However, further melanin synthesis caused hyperpigmentation and other dermatological disorders as melasma, freckles and age spot. Apart of avoiding the UV exposure, application of tyrosinase inhibitors help to maintain the skin fairness [81].

The control of tyrosinase activity also is of great importance in preventing the browning of vegetables and fruits other than the accumulation of an excessive level of epidermal pigmentation in animals. So far, many efforts have been spent in the search for effective and safe tyrosinase inhibitors, and a large number of naturally-occurring and synthetic tyrosinase inhibitors have already been reported [82]. However, some of them are either not potent enough to be considered of practical use or not compatible with safety regulations for food and cosmetic additives. For instance, some of the tyrosinase inhibitors for skin lightening products suffer from limitations such as low activity, allergy and insufficient penetrative ability [81]. So an urgent effort to discover and develop novel and potent tyrosinase inhibitors has become increasingly important in the food industry [83] as well as in medicinal and cosmetic products [84].

Natural sources offer the best selections for the discovery of tyrosinase inhibitors. Kojic acid and arbutin are the examples of natural tyosinase inhibitors which have been incorporated into skin lightening products and clinically used to treat melasma [81]. Study on 25 selected Chinese herbal medicines for cosmetic purpose, suggested the potential of herbal extracts to be incorporated in the cosmetic products for skin whitening effects due to their tyrosine inhibition property [85]. Screening of plant extracts using the *in vitro* inhibition of mushroom tyrosinase enzyme was carried out to preliminary evaluated the potential whitening agent for cosmetic purposes [86].

From previously studies, lavender [87], soybean [88], saffron flower [89] and licorice [90] have been suggested as potent whitening agents due to their tyrosinase inhibition property that were attributed by their phenolic compounds; predominantly the flavonoids. In addition to their well known antioxidant properties, the attribution of phenolic compounds as tyrosinase inhibitors have been previously reported [91]. It was suggested that the antioxidant substances able to inhibit enzymes tyrosinase by inhibiting the oxidation of substrate. Some antioxidant substances block the tyrosinase activity by becoming the substrate for the tyrosinase enzyme, leading to the displacement of tyrosine or DOPA in a lock-and-key model [90].

An *in vitro* anti-tyrosinase screening of 67 tropical plants belonging to 38 families against Arbutin and Kojic acid was carried out spectrophotometrically. The bark of *Entada Africana*; a Mimosaceous plants from Brazil showed over 90% inhibition of tyrosinase. The extract made of the propylene glycol/deionised water (50:50) will be characterised to identify the phytochemicals and further investigation on human melanocytes will be done to verify the activity [92]. The results were encouraging to evaluate the tyrosinase inhibition properties of Mimosaceous plants from Thailand which have not been reported previously.

Analysis of chemical constituents from plant extracts

Preparation of a plant extract frequently started with the collection of potential plant material derived from those used in the traditional medication such as root, leaves or seed, plants of culinary as herbs, vegetable and spices or commonly consumed crops as

grain and fruits. The fresh or dried materials are extracted with an appropriate solvent either by allowing the solvent and sample to stand overnight at room temperature or exhaustive extraction by soxhlet apparatus at chosen temperature [93]. In most of the studies, solvent extraction has been a preferable method in preparing the extract for antioxidant evaluation although other method as supercritical carbon dioxide extraction was also proposed [94]. Selection of the solvent extraction is very important as certain compounds may have high solubility in particular solvents, which later influence the biological properties of the extract.

The influence of solvent polarity to the constituents of the extract was carried out to *Amaranthus lividus*; a popular leafy vegetable consumed in west Black region of Turkey. The extract of stems along with leaves and flowers of *A. lividus* were prepared by 3 different solvents. The methanol and ethyl acetate extracts were prepared by successive 4 hours extraction with sohxlet apparatus while the water extract was prepared by heating the powdered *A. lividus* with distilled water for 30 minutes. Total phenolic, β-carotene, α-tocopherol and ascorbic acid content of each extracts were determined. High content of phenolic compound was found in water extract, but β-carotene and α-tocopherol were not detected, whereas the lowest phenolic content and the absence of ascorbic acid was reported from the ethyl acetate extract [95].

It was also observed that plant extracts prepared by different solvents exhibited various responses to the employed bioassays. For instance, a study was carried out on differently prepared extract of *Ecklonia cava*; a well known brown seaweed in Korea. The *E. cava* fraction of 70% methanol, absolute hexane, chloroform, ethyl acetate, aqueous hexane, chloroform and ethyl acetate were prepared and each fraction were evaluated for their antioxidant activities and compared with the activity of BHT and α-tocopherol. From the results, the fraction of absolute chloroform was good in DPPH scavenging activity which was comparable with the scavenging activity of α-tocopherol but poor in chelating metal assay, whereas the 70% methanol fraction exhibited reasonable antioxidant activities in all assays. Therefore, different solvents with different polarity influenced the antioxidant activity of all *E. Cava* extracts [96].

Solvent influence on biological activity was also observed from a strong electron donating ability of 95% ethanolic extract from *Fructus lycii* fruit. The activity of *Fructus lycii* was evaluated from the ability of extract to reduce the oxidizing agent; a ferricyanide $Fe^{3+}(CN^-)_6$ to $Fe^{2+}(CN^-)_6$ in the assay. Flavonoids as myricetin, quercetin and kaempferol were detected in the extract by reversed phase HPLC and the identity of flavonoids were confirmed by LC-ESI-MS in positive mode ionisation. Thus, flavonoids were found responsible for the electron donating activity of the extract [97]. A 95% ethanol solvent was also employed to the extract flower of *Cassia siamea*. The extract of various concentrations (50, 100, 250 and 500 μg/ml) showed highest scavenging activity of DPPH radical, superoxide anion, hydrogen peroxide and nitric oxide at 50 μg/ml. High polyphenol content was also quantified from the extract. No correlation was made but the polyphenols were suggested responsible for the antioxidant activities of the flower [98].

Due to the type of plant, varieties of polarity, and chemical composition of the extract, there are no certain standard or satisfactory solvents that able to prepare an extract. However, it was observed that in the assays that evaluate the antioxidant substances by the ability to reduce or donate hydrogen or electron, such as scavenging DPPH radical or reducing capacity assay, good results will be obtained from the alcoholic extracts.

From previously studies, both methanolic and ethanolic extracts have been the favourable solvents to extract active compounds from plants material. For example, higher amounts of polyphenols were reported in both methanol and ethanol extracts. Although methanol extract showed a better solvent for polyphenols than ethanol, the differences were insignificant and suggested ethanol as more preferable solvent due to absent of toxicity [99]. However, for the assays that employ cell culture and the inhibition of oxidation enzyme, it is important to prepare extracts that able to be dissolved in the solvent such as DMSO or phosphate buffer in a value that is not irritating or causing toxicity to the cell or interfere with the enzyme activity [100].

Based on the results of bioassays, a number of studies have concluded polyphenol as the underlying reason of the observed biological activity of the alcoholic extract. Polyphenols are the member of benzene derivatives with one or more hydroxyl

groups associated to the aromatic ring called phenol group (Figure 2). Polyphenols encompasses wide range of chemical structure, from the simplest to the most complicated structures (Table 5). A well known polyphenols for their antioxidant and antimicrobial properties are flavonoids, phenolic acid, and proanthocyanidins.

Polyphenols possess multifaceted antioxidant properties such as chelating metal or directly reacting with radicals to form less reactive product [79]. For instance, caffeic acid (3,4-dihydrocynamic acid); a phenolic acid has ability to inhibit peroxidation of linoleic acid emulsion, reducing power, DPPH radical scavenging, superoxide anion radical scavenging and metal chelating activity that comparable with synthetic antioxidant as BHA, BHT and other antioxidant as α-tocopherol and trolox; a water soluble analogue of tocopherol [101].

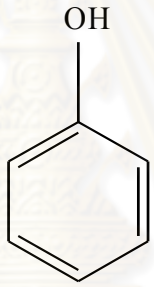


Figure 2 Phenol group [102]

Table 5 Basic skeleton and classes of phenolics [102]

No of carbon atoms	Basic skeleton	Class
6	C_6	Simple phenols, benzoquinones,
7	C_6-C_1	Phenolic acids
8	C_6-C_2	Acetophenones, phenylacetic acid
9	C_6-C_3 (phenylpropanoid)	Hydroxycinnamic acid, phenylpropenes, coumarins, isocoumarins
10	C_6-C_4	Naphthoquinones

11	C_6-C_1-C_6	Xanthones
13	C_6-C_2-C_6	Stilbenes
15	C_6-C_3-C_6	Flavonoids, isoflavonoids
18	$(C_6$-$C_3)_2$	Lignan
30	$(C_6$-C_3-$C_6)_2$	Biflavonoids
n	$(C_6$-$C_3)_n$	Lignins
	$(C_6)_n$	Catechol melanin
	$(C_6$-C_3-$C_6)_n$	Flavolans (condensed tannin)

Although the polyphenols have been suggested responsible for the biological activity of ethanolic extract, the extract is still consisted of mixtures of compounds with identical polarity. Therefore, identifying the chemical structure of the constituents will be able to describe the effectiveness, synergistic and antagonistic of compounds presented by the biological activity. Reversed phase High Performance Liquid Chromatography (HPLC) with UV detection or diode array detection (DAD) has been widely used to detect constituents present in plant extracts. Basically, in chromatography, the compounds in extract are separated by differential in polarity of the compound between stationary phase and mobile phase. HPLC is the vogue and frequently used for the separation and isolation of phytochemical.

HPLC is an adsorption chromatography, which employ small particle sizes of stationary phase in the column that results in fairly large backpressure when the mobile phase is passed through the stationary phase. Pump systems is applied to achieve flow of the mobile phase. Typically HPLC system consists of the following components: solvent reservoir, injection system, column, pump and a computer serving as data station to collect information (Figure 3). The most widely used stationary phase is C_{18} reversed phase chromatography with water/acetonitrile or water/methanol mixtures as mobile phase. The column sizes are typically 25cm in length and 4.6 mm diameter and the common flow rates for such columns are 1ml/min. However, for column with larger diameter increase in flow rate is needed thus HPLC pumps with larger pumping capabilities are also required. The separation process is typically accomplished by probing various functional groups in the molecules to be separated. The variation of

mobile phase is very important as separation of molecules is focused on the hydrophobic part. Almost every HPLC is equipped with a UV detector to detect compound of interest. Traditionally the UV detector was single-wavelength detector, but photodiode-array (PDA or DAD) permits scanning of the full UV-visible range (210-650 nm) [103].

The maximum absorption (λ_{max}) provided by HPLC with multiple wavelength detector or photodiode array (PDA) detector is useful for early classification. For instance, phenolic compounds contain at least one aromatic ring thus efficiently absorb UV light. Different class of phenolics showed distinguishable λ_{max} with UV. The λ_{max} for the single ring phenolic acids other than hydroxybenzoic acids was found near 320nm, hydroxybenzoic acids, flavan-3-ol (including dimers) and hydroxychalcones showed λ_{max} near 280nm, flavonols λ_{max} was 360nm, whereas the anthocyanins showed λ_{max} near 520nm. The UV characteristic has been used to present the subclass of flavonoids aglycone. However, the substitution groups such as methyl, methoxy, glycosides and acyl residues only give minor changes in the absorption maxima due to poor chromophores. Nevertheless, the HPLC-PDA has been a complementary tool during structural characterization of phenolic compound, by comparing the retention time and maximum absorption of studied compound with the known standard [104].

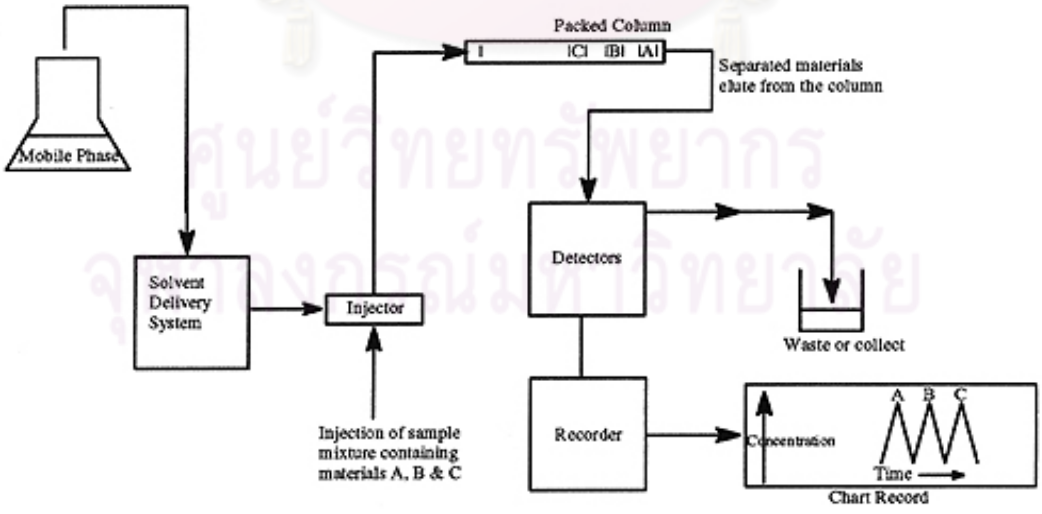


Figure 3 Components of HPLC instrument **[**103**]**

The advent of electrospray ionisation (ESI) combined with mass spectrometry (MS) and tandem mass spectrometry (MS/MS) permitted determination of the constituents even a low concentration in extract. This technique is suitable for polar compound and has great sensitivity, and specificity. It is a method of soft ionisation which produces a molecular ion that is useful for the determination of the molecular weight determination of large biological compounds that are difficult to vaporize or ionize. Ionization performed in the negative mode resulted loss of one or several protons and formation of as many charges [104].

The combination of HPLC and mass spectrometer has facilitated the characterization of phytochemicals in the studied extract (Figure 4). There are numbers of ionization of analytes method available such as fast atom bombardment (FAB), thermospray, electrospray ionization and matrix-assisted laser desorption ionization (MALDI). The most common ionization technique in polyphenols study is electrospray ionization which employs the combination of high voltage field (3-5 kV) that produces nebulization of the column effluent in a narrow-bore capillary or electrospray needle which resulting in charged droplets that are focussed towards the mass analyser. An ion trap mass-spectrometer has capability to produce MS^n data that important in structural elucidation [103].

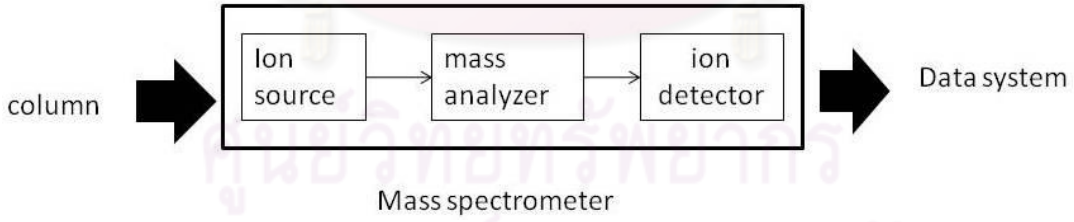


Figure 4 component of mass spectrometer [103]

Optimising condition for both HLPC and LCMS mobile phase system is important to get well separated peaks in the chromatogram. In a reversed phase column, the mobile phase solvent system consists of both organic and water. The organic solvents as methanol or acetonitrile paired with water have been a preferred choice for most of researcher in polyphenols studies. Methanol has suitable polarity to extract polyphenols

whereas acetonitrile known for a better resolution. Additives as base (phosphate), acid (formic acid, acetic acid, phosphoric acid) and salt (sodium acetate, ammonium acetate) have been added to mobile phases to improve the separation. The retention behaviour of polyphenols on the reversed phase HPLC column was significantly affected by the pH of mobile phase. Study on *Radix Paeoniae Rubra* extract showed the addition of 0.1% (v/v) phosphoric acid showed a good baseline and satisfactory resolution of peaks. However, for the HPLC coupled with MS it is important to choose the volatile additive to avoid the pollution or damage to the ion source. Therefore additives as phosphate, phosphoric acid was avoided in HPLC/MS study [105].

Numbers of polyphenols from etholic extract plants extract have been successfully characterised and the results have been satisfying to associate the presence and influence of polyphenols structure to the extract responses in antioxidant assays. Twenty six of polyphenols were characterised from the ethanolic extract of *Ananas comosus* L. Leaves by HPLC-DAD-MS. The study which employed mobile phase consist of a gradient of 0.1% formic acid in water and 0.1% formic acid in acetonitrile resulted with detection of up to 26 different phenolic compounds. Structures of each phenolic compound were proposed based on the fragmentation patterns observed in the MS. *Ananas comosus* L. leaves extract was known for its antioxidant property. Therefore, results have clearly showed that plant phenols were account for the antioxidative effects of the extract and HPLC-DAD-MS is an appropriate tool for detecting and characterising structures of polyphenols in the extract [106].

Comparison of both HLPC and LCMS chromatographic profiles would provide information on the varieties of polyphenols presents in an extract. Related plants produce similar type of flavonoids which are useful in the plant classification and can be used to resolve taxonomic problem. Variation in the flavonoid profile was able to determine the morphology and ecology difference of two populations *Agauria salicifolia*. HPLC/UV-DAD spectra indicated the presence of phenolic compound and the λ_{max} of peak indicated the family of phenolic compound. For compound without commercially available reference, a full scan mode analysis followed by MS/MS experiments in negative ionisation was appropriate. LC/MS chromatograms of both populations revealed similar

flavonoid profile but the difference between the populations was found in the concentration of the compounds [107].

Reversed HPLC method was developed to determine a content of gallic acid, corilagin and ellagic tannin from 70% methanol extract of peel,pulp and seed tissue of longan fruits (*Euphoria longana* Lam.) obtained from different cultivars. Cultivar with the highest content of polyphenols will be selected for breeding and cultivation program [108]. It can be concluded that both HPLC and LCMS provided reliable data on composition of polyphenols in plant extract. Nevertheless, further information on the position of the substituent groups and the positive identification of the compounds would require the use of NMR. However NMR would require extensive sample purification and more quantity of samples needed for LCMS. Therefore, the employment of both HPLC and LCMS is rapid and applicable to extract and compound even at low concentration.

Mimosaceous plants

The destruction of tropical forest in the developing countries leaves researchers with limited time to investigate and possibilities of loosing opportunities for a successful therapeutic development. The comprehensive data on the plants with health impact encourages the investigation to better comprehend the human-plants relations. The overlapping use of plants as food and medicine is the example of traditional knowledge and management of natural resources which have been culturally constructed.

In addition to the advancing analytical research, systematics approach has been an important tool in the pharmacognostical practise and contributes to the incremental growth of collection data. Related families often contain similar types of compounds and understanding of the systematic position of a medicinal plant species allows some deductions to be made about the biologically active secondary natural products from the species. In addition, such information offers a strong foundation to advance in the field of ethnopharmacology [22].

Mimosaceae members have been well known as food and medicinal plants. It is one of 3 families from the Order Fabales. The family is consisted of 40 genera of trees and shrubs but rarely herbs, which are often prickly. Although the consumption of Mimosaceous plant

fruits causes pungent smell in breath and urine, the plants are well known delicacy in the countries of South East Asia region, either being eaten raw or cooked. The characteristic of the families are presented in Table 6. Order Fabales belonging several medicinal plants, countless agricultural products and poisonous plants. Several species which are already investigated scientifically and those which have entered the pharmacopeia have already given impact to the health care.

Table 6 The characteristic of families from the order of Fabales [109]

Characteristic	**Family**		
	Mimosaceae	**Caesalpiniaceae**	**Fabaceae**
1. Flower : comprises of a corolla of 5 petals	Actinomorphic, hermaphrodite, small, spicate, racemose or capitates.	Zygomorphic, conspicuous, racemose	Zygomorphic
Petals	Valvate, small, free or connate and hypogynous	Imbricate with the adaxial one overlapped by a pair of lateral petal	Imbricate with the adaxial petal overlapping the lateral petals
Calyx	Tubular,valvate, and 5 lobbed	Consist of 5 sepals which are imbricate and free	
Anthers	Small, 2-celled, open lengthwise and marked with a deciduous gland at the apex	The andrecium consists of 10 stamens, the anthers of which are tetrasporangiate, dithecal and open lengthwise or by a terminal or base pore	The andrecium comprises of 10 stamens

Gynecium	Consist of a single ovaries attached to marginal placentas	Curved and consists of a single carpel, forming a unilocular ovary containing two to several ovules attached on a marginal placenta	Consist of a single carpel forming a single-locular ovary
2. Fruits	pods	Pods	pods
3. Seeds	Starchy and oily, containing galactomannans, toxic non-protein amino acids, steroids and lectins (glycoprotein).		
4. Leaves	Bipinate and stipule	Pinnate, bipinnate or less frequently simple and stipulate	Simple or compound and stipulate
5. Phytochemicals	Tannins, mucilages, gums and an usual series of amino acids	Anthraquinone glycosides and tannin	Tannins, mucilages, anthraquinones, isoflavonoids, triterpenoid saponins, cyanogens glycosides, quinolizidine, indole and simple tetrahydroisoquinoline alkaloids.

Systematic study of plants consisted of chemotaxonomy study provides good correlation between the taxonomy and occurrence of plant secondary compounds. It may be possible to discover related phytochemical in related species, genera or families. Different degrees of activity in the different species may have some taxonomic predictive values. Chemotaxonomic approach has been revealed significant biological activity of many members of families. As previously reported, medicinal plants, food, beverages and industrial by product has been at an interest of present researchers. The random selection approach applies the collection and selection of plant material regardless of the existing

studies on their chemistry or biological activity. Although this approach is purely serendipitous it stands a chance that random plant selection will give extract with good activity.

Due to the reputations as traditional food, the biological activities of Mimosaceous plant have been previously evaluated along with other food plants. A study on *Parkia speciosa*; the most consumed Mimosaceous plants showed that the *P. speciosa* water extract had weak response in ferric reducing, cupric ion chelating and DPPH radical assay although the extract had high total phenolic content [25], nevertheless the consumption of pod of *P. speciosa* showed a hypoglycaemic effect [110]. Whereas, during the assessment of 26 Thai indigenous plants, total phenolic, total flavonoid and DPPH scavenging activity of seed and bud of *Leucaena glauca* along with *Acacia catechu* bark prepared by 95% ethanolic were determined. Among the Mimosaceous plants, extract of *A. catechu* exhibited highest content of phenolic, flavonoids and the strongest scavenging activity, followed by bud and seed of *L. glauca* [111].

A study was carried out to evaluate the safety and anticarcinogenic of edible Mimosaceous seeds. The presence of formaldehyde in edible seeds of Mimosaceous plant; *Parkia speciosa*, *Archidendron jiringa* and *Leucaena leucocephala* were determined. Natural occurring formaldehyde in food has been a concern of food safety. Results showed a substantial content of formaldehyde were found in both raw and boiled seeds. Nevertheless, the content was lower in the boiled seeds, suggesting that all both raw and boiled seeds were safe to be consumed [112].

Djengkol bean or *Archidendron jiringa* is another common delicacy. The fruits are consumed raw, roasted or fried. Djenkolic acid; a sulphur containing amino acid is the principle compound in *A. jiringa* (Figure 5). However, toxic effect to the renal has been reported upon high consumption of Djenkolic acid [113].

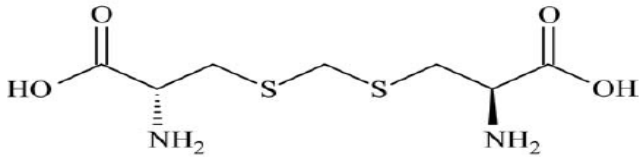


Figure 5 Chemical structure of Djenkolic acid [113]

Oral administration of saponin isolated from bark of *Albizzia lebbeck* on the reproductive system of male albino rats was determined. Three main saponins namely Albizia saponin A, B and C were isolated from methanolic extract of the bark. Sixty days of oral administration brought significant lost in testis weight, and reduced in sperm cell count, thus concluded that saponins of *Albizia lebbeck* bark showed antifertility activity [114]. The n-butanol fraction and isolated flavonoids from *Acacia pennata* were studied for the anti-inflammatory property from their ability to inhibit both cyclooxygenase enzymes (COX 1 and COX 2). It was found that n-butanol fraction of *A. pennata* exhibited good COX 1 inhibiting activity but weak COX 2 inhibition. The potent COX 1 inhibitor was contributed by glycosylation on the structure of flavonoids, thus clarify the anti-inflammation activity mechanism of action both n-butanol fraction and isolated flavonoids of *A. pennata* was by inhibiting inflammation mediated by COX 1 enzyme [115].

Table 7 Application of Mimosaceous plants in Thai traditional medicine [26].

Species	**Thai name**	**Part used**	**Preparation**	**Application**
Acacia catechu	Sisiat	Branch and twig	Make a tea of dried powdered extract	Antiemetic
			Simmer extract in water to make thick paste	Anti-diarrhea
		Wood	Topical	Astringent

			application	
Adenanthera pavonina	Maklam taa chang	Wood Essential oil	paste Soap, shampoo	Cooling effect, antipyretic Dermatitis, inflammation on skin
Mimosa pudica	Maiyarap	Whole plant	Decoction	Analgesic, diuretic
Neptunia oleracea	Phak kachet	Young stem and leaves	Eaten raw	Antipyretic,detoxifier
Xylia xylocarpa	Daeng	Flower Stem, bark	Decoction Decoction	Cardiac tonic, antipyretic Antipyretic,antidiarrheal, laxative

Members of Mimosaseae have been studied for the claimed medical, nutrient and chemical properties [25-30]. Characterized by an impressive phytochemical diversity, polyphenols especially flavonoids and tannin are the common ones [114, 115]. Numbers of phytochemicals have been isolated from Mimosaceous plants and the biological properties were evaluated. Nevertheless, it was found that most of studies were carried on the seeds, because of the utilization of seed as food. However, there are a number of Mimosaceous plants of Thailand which have not yet been investigated.

Previously, twenty species of Thai plants in Mimosaceae family were exhaustively extracted with petroleum ether, dichloromethane and ethanol respectively. The plant extracts were studied for their α-glucosidase inhibition activity by spectrophotometry using 1-deoxynojirimycin as a positive control. The result showed that most percentage yields of the plants were in ethanol extract [116]. In accordance with previous studies, it was concluded that it was encouraging to continuously evaluate the ethanolic extracts of Mimosaceous plants especially those of species and plant part which have not yet been reported.

CHAPTER III

METHODOLOGY

Plant Materials

Plant materials from 20 species of Mimosaceae consisted of bark, stem, leaves, seed coat, pericarp, stem bark, branch, twig, cotyledon, seed and branch bark studied were as the following. Table 8 showed the selected species, Thai names and plant parts. Samples are collected from botanical garden or buy in the local market around Ratchaburi, Rayong, Pathumthani, Nakhonpathom and Bangkok. Samples are deposited at the Faculty of Pharmaceutical Sciences Herbarium, Chulalongkorn University. The identification and authentication of each species was carried out by Associate Prof. Nijsiri Ruangrungsi. All samples were air dried before grounded and were stored at room temperature.

Table 8 Selected species in the study with Thai names and plants parts

Species	**Thai name**	**Plant Part**
Acacia catechu (L.f) Willd.	สีเสียด (Sri siad)	leaves
Acacia farnesiana (L.) Willd.	กระถินหอม (Kra thin hom)	twig
Acacia rugata Merr.	ส้มป่อย (Som poi)	leaves
Acacia rugata Merr.		pericarp
Acacia pennata (L.) Willd.	ชะอม (Cha om)	twig
Adenanthera microsperma Teijsm.	มะกล่ำตาไก่ (Ma kram taa klai)	branch
Adenanthera pavonina L.	มะกล่ำตาช้าง	pericarp
Adenanthera pavonina L.	(Ma kram taa chang)	branch
Adenanthera pavonina L.		leaves
Adenanthera pavonina L.		Seed coat
Albizia lebbeck (L.) Benth.	จามจุรีสีทอง (Jam ju ree sri thong)	leaves
Albizia lebbeckoides (DC.) Benth.	ข่าง (klang)	leaves
Albizia procera (Roxb.) Benth.	ทิ้งถ่อน (Thig thon)	stem bark
Albizzia myriophylla Benth.	ชะอมไทย (Cha om thai)	leaves

Archidendron jiringa (Jack) I.C Nielsen	ลูกเหนียง	pericarp
Archidendron jiringa (Jack) I.C Nielsen	(Lung neung)	seed
Cathormion umbellatum (Vahl) Kosterm.	ระกำป่า	leaves
Cathormion umbellatum (Vahl) Kosterm.	(Rha kham pa)	bark
Cathormion umbellatum (Vahl) Kosterm.		branch
Entada rheedii Spreng	สะบ้ามอญ	seed coat
	(Sa ba mon)	
Entada rheedii Spreng		cotyledon
Leucaena glauca Benth.	กระถิน (Kra-thin)	pericarp
Leucaena glauca Benth.		twig
Mimosa pudica L.	ไมยราบ (Mai ya lap)	twig
Neptunia oleracea Lour.	ผักกระเฉด (Pak kra ched)	twig
Parkia speciosa Hassk.	สาโท (Sato)	pericarp
Parkia speciosa Hassk.		seed
Pithecellobium dulce Benth.	มะขามเทศ	stem bark
Pithecellobium dulce Benth.	(Ma Kham Thad)	leaves
Pithecellobium dulce Benth.		pericarp
Samanea saman (Jacq.) Merr	ก้ามปู (Klam pu)	leaves
Samanea saman (Jacq.) Merr		branch
Xylia xylocarpa (Roxb.) Taub	ไม้แดง (Mai Daeng)	bark
Xylia xylocarpa (Roxb.) Taub		stem
Xylia xylocarpa (Roxb.) Taub		leaves

Extraction

The samples weighed 10 to 30 g were extracted by Soxhlet apparatus for 8 hours with 500 ml solvent at temperature 60˚C. Petroleum ether, dichloromethane and ethanol were used as solvent for extraction in an ascending polarity consecutively. The solvents in the extract were removed with a rotary evaporator and water bath respectively. The extracts yield were weighed, recorded and stored at 4˚C until it was analyzed.

Brine shrimp Lethality assay

Fresh eggs of *A. salina* were purchased from the local pet shop at Chatuchak market, Bangkok. Artificial sea water was prepared by dissolving 3.8 g sea salt per litre of

distilled water and was aerated for 24 hours. Into a two portioned aquarium, the eggs were hatched in the darkened area of the aquarium covered by aluminium foil whereas the other part of the aquarium was put under a light. With the help from a light source, the larvae (nauplii) were attracted to the side of the aquarium and were easily collected from the non hatched eggs. One day old nauplius was transferred into a vial containing 5 ml of artificial sea water (30 per vial) and was allowed to stand for another 24 hours under illumination. Extracts prepared in DMSO at the concentrations of 10, 100 and 1000 µg/ml was added into each vial. Five vials were prepared for each concentration. The controls were prepared in the same manner except that pure DMSO was used instead of the extract. Three replicates were prepared for each concentration. After 24 hours, the numbers of survivors were counted. Next, the percentage of death and LD_{50} was calculated [117].

Disk diffusion method for antibacterial activity

Bacterial strains: *Staphylococcus aureus* (ATCC 6538P), *Bacilus subtillis* (ATCC 6633) and *Escherichia coli* (ATCC 25922), were obtained from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. The bacterial strains were maintained on Muller-Hinton agar. The subcultures were prepared 24 hours before use. Two to three colonies of the microbial culture from 24 hour old plate were suspended in normal saline and the turbidity of the culture was adjusted to match the 0.5 Mc Farland standard.

The assay was modified from [118]. Ten µl of a suspension was pipette on the agar plate. A lawn culture was prepared on Muller-Hilton agar using sterile cotton swab. Sterile 6 mm filter paper discs were placed on the culture and impregnated with 10 µl of extract (100mg/ml prepared in DMSO). Then, the plates were incubated at 37˚C for 24 hours. Disc impregnated with 10 µl DMSO was made as control. After incubation, the diameter of clear zone around each disc were measured and recorded in millimeter (mm). The absence of a zone inhibition was interpreted as the absence of activity. Each extract was tested in triplicate and was repeated twice.

Broth microdilution assay for antimicrobial evaluation

Bacterial strains: *Staphylococcus aureus* (ATCC 6538P), *Bacilus subtillis* (ATCC 6633) and *Escherichia coli* (ATCC 25922), fungals: *Saccharomyces cerevisiae*

(ATCC 9763) and *Candida albicans* (ATCC 10230). They were obtained from the Department of Microbiology, Faculty of Pharmaceutical Sciences, Chulalongkorn University. Both bacterial and fungal strains were maintained on Muller-Hinton and Sabouraud agar respectively. The subcultures were prepared 24 hours before use. Two to three colonies of the microbial culture from 24 hour old plate were suspended in normal saline and the turbidity of the culture was adjusted to match the 0.5 Mc Farland standard.

The assay was carried out according to [119]. For broth microdilution, a microbial suspension in broth was prepared by adding 10 µl of normal saline microbial suspensions to 1ml Muller-Hinton or Sabouraud broth. The assay was carried out according to [46] with modifications. Into a sterile 96-well microplate, 50 µl of microbial suspended in broth was added to the wells containing 50 µl of plant extract (final concentration: 0.2-1.0 mg/ml) or control. Control was prepared by diluting 50 µl DMSO with broth to obtain final volume of 1ml. After 20 hours incubation at 37 °C, 20 µl of *P*-Iodonitrotetrazolium (INT) reagent dissolved in water (1 mg/ml) was added into each well. The lowest concentration of Mimosaceous plant extract inhibiting the growth of test microorganisms indicated by INT was defined as the MIC of an extract. Next, the content of the known MIC wells were streaked onto fresh nutrient agar plates. The plates were further incubated at 37˚ C for 24 hours. The least concentration of extract with no microbial growth observed on the plate was considered as the MBC (Minimum bactericidal concentration) or MFC (Minimum fungicidal concentration) value.

Determination of free radical scavenging activity

Free radical scavenging activity was evaluated by ability to scavenge DPPH; a stable synthetic free radical. The activity was determined according to [120]. Aliquot of 100 µl of DPPH in methanol (126µM) was pipette into 96 well microplate, followed by 100µl of Mimosaceous extract prepared in ethanol. The reaction was allowed to incubate in 30 minutes at room temperature. Next, the absorbance was measured at 517 nm using microplate reader. All extract were analysed in triplicate. The control was reaction mixture with ethanol substituting the extract. The DPPH radical scavenging activity was calculated by the following equation: Radical scavenging activity (%)= (Absorbance $_{control}$ –Absorbance $_{sample}$)/ Absorbance $_{control}$ x 100. The scavenging activity of Mimosaceous

extracts was expressed as the concentration necessary to scavenge free radical by 50% (IC_{50}).

Reducing power assay

The assay was carried out according to [121]. Extract of Mimosaceous plants in DMSO (20 mg/ml) was diluted with 0.1M potassium phosphate buffer (NaH_2PO-K_2HPO_4) at pH 6.6 to obtain different concentrations in a range of 0.02 to 1.0 mg/ml. A 148µl of each concentration was pipette into a 96-well microplate, followed by 50µl of 1% potassium ferricyanide (w/v). The mixture was incubated at 50 °C for 20 min. Next, 50µl of 10% tricloroacetic acid (w/v) and 2µl of 1% of ferric chloride (w/v) was added into each well. The mixture was mixed until homogenized before being measured spectrophotometrically at 700 nm. The assay was carried out in triplicate. The graph of absorbance at 700 nm against the correspondent extract concentration was plotted. The EC_{50} was arbitrarily defined as the concentration of extract that exhibited absorbance of 0.5 at 700nm obtained from a line of best fit from the plotted graph.

Nitric oxide scavenging assay

Extracts of Mimosaceous plants in DMSO (20 mg/ml) was diluted with ultrapure water to obtain different concentrations in a range of 0.05-5 mg/ml. The assay was carried out according to [122] with modifications. Into a 96 microwell plate, 50µl of extract or control (ultrapure water) was added into 50 µl of aqueous sodium nitroperusside (5 mM). The mixture was incubated for 2 hours. Next 100 µl of Griess reagent (0.5% sulphanilamide in ultrapure water, 0.16% naphtylethylenediamine dihydrochloride in 20% acetic acid, 1:1) was added and was immediately read at 570nm. The nitric oxide scavenging activity was calculated as below:

Scavenging activity (%)=(Absorbance $_{control}$ –Absorbance $_{sample}$)/ Absorbance $_{control}$ x 100.

The concentration of extracts necessary to scavenge 50% of the produced nitric oxide (EC_{50}) was obtained from a graph of scavenging activity (%) against a plant extract concentrations.

Screening of chelating activity

Ferrozine has been used to quantify the free Fe2+. Presence of chelating agent will disrupt the formation of Fe^{2+}-Ferrozine and eventually fading the magenta colour of the

complex. The method has been used to evaluate the metal chelating activity of extract. The assay was carried out according to [123]. Extract was prepared in ethanol (5 mg/ml). In the 96 well microplate, 15µl of $FeCl_2$ in ultrapure water (2 mM) was added into 110 µl of extract in various concentrations in ethanol. The reaction started by addition of 75 µl of aqueous Ferrozine (5 mM). The mixture was left for 10 minutes, before the absorbance of reaction was measured at 562 nm. EDTA was used as positive control (0.10, 0.13, 0.16 mM). The control was reaction mixture with ethanol substituting the extract. Fe^{2+} chelating activity of test compound was calculated as:

Chelating activity (%)=(Absorbance $_{control}$ –Absorbance $_{sample}$)/ Absorbance $_{control}$ x 100.

Screening of monophenolase and diphenolase inhibition activities

Mimosaceous extracts were first diluted in dimethyl sulfoxide (DMSO) and were further diluted with working buffer. For the experiment, the working buffer was 0.1M potassium phosphate buffer (NaH_2PO-K_2HPO_4) at pH 6.8. All of the samples tested were prepared in the buffer. Tyrosinase enzyme was prepared in buffer (5370 U/ml) and stored at -20˚C in 1 ml aliquots prior to use. The assay was performed in 96 well microplate as previously described [124] with some modification. Both L-Tyrosine and L-DOPA were used as substrates to evaluate the inhibition activity of Mimosaceous extract against monophenolase and diphenolase activity respectively.

Into the 96 well microplate, 100µl of each extracts (500 µg/ml) were pipetted. Next, 50 µl of L-tyrosine solution or L-DOPA solution (4 mM in buffer) was added and followed by 50 µl of 26.86 U/ml mushroom tyrosinase (EC 1.1418.1) in the same buffer. The reaction was monitored incubated for 20 before measuring the absorbance at 475 nm using microplate reader. Control was the mixture of all reagents with 2.5% DMSO in buffer substituting the extract. All determination was carried out in triplicate. The percentage inhibition of enzyme by extract was calculated as; Inhibition (%)= (A $_{control}$- A $_{sample}$)/A $_{control}$ x 100%.

Determination of total phenolic content

Quantification of total phenolic content of extract was determined using Folin-Ciocalteu's phenol reagent modified from [125]. Phenolic compounds in the extract will form a blue color complex with Folin Ciocalteu reagent after adjusted with alkali. Briefly, 80µl of extract in methanol was pipette into 96 well microplate, followed by 100µl of 15% Folin

Ciocalteau. Distilled water was added to adjust the volume to 200µl. The mixture was left for 5 minutes before addition of 100 µl sodium carbonate (Na_2CO_3) aqueous (0.105 g/ml). The absorbance of extract was measured at 756nm after incubation at 30˚C for 60 minutes. All determinations were performed in triplicate. Different concentrations of gallic acid (0.03, 0.06, 0.12, 0.25, 0.5 and 1 mg/ml) were used to prepare a standard curve. The concentration of total phenolic compounds in all extract was expressed as mg of gallic acid equivalents per g dry weight of extract using a linear equation.

Detection and characterization of polyphenols from extracts by HPLC-PDA and LC/MS

Detection of polyphenols of the Mimosaceous ethanolic extract was carried out by HPLC-PDA and the constituents of extracts which showed greatest activity in the selected assays were analyzed by LC/MS. One mg/ml sample was filtered through the Ultrafree-MC membrane centrifuge-filtration unit (hydrophilic PTFE, 0.20 µm, Milipore), and 5µl of the filtrate was loaded into a TSK-gel Super ODS column (2.0 µm, 2.0 x 100 mm, TOSOH, Tokyo, Japan) at 40˚C. The mobile phase consisted of water (A) and acetonitrile (B) both containing 0.1% formic acid with the flow rate of 200µl/min. The HPLC system was consisted of a pair of LC 10AD VP pumps, a DGU 12A degasser, a CTO 6A column oven, an SPD 10A VP photodiode array detector (PDA), and a SCL 10A VP system controller (Shimadzu, Kyoto, Japan).

The gradient condition for HPLC-PDA was 0%-55% for 35 minutes. The LC separation was performed by an Agilent 1100 HPLC system (Agilent Technologies, Palo Alto, CA) with gradient condition of 5%-60%B for 35 minutes. The entire eluent was directed into the mass spectrometer which it was diverted to waste at 2.5 min after injection to avoid any introduction of salts into the ion source.

The MS analysis was performed using a Finnigan LCQ Deca XP plus Ion Trap MS instrument (Thermo Fischer Scientific, San Jose, CA) equipped with electrospray ionization (ESI) interface. The ESI conditions of negative mode were as follows: capillary temperature 300˚C; sheath gas flow rate, 35 (arbitrary units); ESI source voltage, 5000V; capillary voltage, 43 V; and tube lens offset 15 V. Scan range 150 to 1000 was applied. MS/MS was applied to ions that acquired further structural analysis.

CHAPTER IV

RESULTS AND DISCUSSIONS

Brine Shrimp Lethality Test

This assay was carried out to evaluate the cytotoxic effect of each Mimosaceous plants ethanolic extract. In the previously reported brine shrimp assay, the studied extract was pipetted on the paper disc, and was allowed to set in the vial of brine shrimp for 24 hours [40]. However, it was observed that such method was not suitable to study the cytotoxicity of Mimosaceous plants ethanolic extract. Preliminary, the extract of *Xylia xylocarpa* bark (10µg/ml in DMSO) was pipetted on the paper disc, whereas the same concentration was directly pipetted into the vials containing brine shrimp. The brine shrimp was found not affected by the extract on paper disc, but was found lethal when the extract was directly pipetted into the vial. The brine shrimp exposed directly to DMSO also was not affected which ensured the safety of directly pipetted DMSO into the brine shrimp vial. Therefore, in this study all extracts were directly exposed to the brine shrimp in the vial.

Ethanolic extract of 20 species Mimosaceous plants evaluated for their cytotoxicity activity by brine shrimp with *Artemia salina* are shown in table 9. Extracts of Mimosaceous plants at concentrations ranging from 1000, 100, 50, 10, 5 and 1 µg/ml were exposed to the 48 hours old of *A. salina* for 24 hours. From the 35 extracts tested, 13 extracts showed cytotoxicity effect at LC_{50} 3.45 to 48.12µg/ml calculated from the lethality percentage. The extracts tested with brine shrimp lethality test have been classified as toxic with LC_{50} value < 1000 µg/ml and non-toxic LC_{50} value > 1000 µg/ml [40]. Therefore, the remaining extracts which caused no lethality to brine shrimp at concentration 1000 µg/ml were regarded as non toxic (NT).

Table 9 Cytotoxicity activity of Mimosaceous ethanolic extract to brine shrimp.

Species	**Plant part**	**LC_{50} (µg/ml)**
Pithecellobium dulce Benth	stem bark	3.45
Archidendron jiringa (Jack) I.C Nielsen	pericarp	3.64
Albizia procera (Roxb.) Benth	stem bark	3.91
Samanea saman (Jacq.) Merr.	branch	4.56
Samanea saman (Jacq.) Merr.	leaves	4.84
Cathormion umbellatum (Vahl) Kosterm.	bark	7.64
Entada rheedii Spreng	seed coat	8.96
Parkia speciosa Hassk.	pericarp	18.49
Acacia rugata Merr.	pericarp	37.59
Adenanthera microsperma Teijsm.	leaves	37.69
Xylia xylocarpa (Roxb.) Taub	leaves	46.23
Xylia xylocarpa (Roxb.) Taub	bark	46.98
Xylia xylocarpa (Roxb.) Taub	stem	48.12
Acacia catechu (L.f) Willd.	leaves	NT
Acacia farnesiana (L.) Willd.	twig	NT
Acacia rugata Merr.	leaves	NT
Acacia pennata (L.) Willd.	twig	NT
Adenanthera pavonina L.	seed coat	NT
Adenanthera pavonina L.	leaves	NT
Adenanthera pavonina L.	branch	NT
Adenanthera pavonina L.	pericarp	NT
Albizia lebbeck (L.) Benth.	leaves	NT
Albizia lebbeckoides (DC.) Benth.	leaves	NT
Albizzia myriophylla Benth.	leaves	NT
Archidendron jiringa (Jack) I.C Nielsen	seed	NT
Cathormion umbellatum (Vahl) Kosterm.	leaves	NT
Cathormion umbellatum (Vahl) Kosterm.	branch	NT
Entada rheedii Spreng	cotyledon	NT

Leucaena glauca Benth	twig	NT
Leucaena glauca Benth	pericarp	NT
Mimosa pudica L.	twig	NT
Neptunia oleracea Lour	twig	NT
Parkia speciosa Hassk.	seed	NT
Pithecellobium dulce Benth	leaves	NT
Pithecellobium dulce Benth	pericarp	NT

The highest cytotoxicity effect of Mimosaceous plants was exhibited by the extract of *Pithecellobium dulce* stem bark with LC_{50} of 3.45µg/ml. Figure 6 showed the HPLC-PDA chromatogram of *P.dulce* bark extract.

Figure 6 HPLC-PDA chromatogram of *P. dulce* bark extract

The peaks at retention time of 13.31, 14.31 and 16.36 in *P. dulce* bark (figure 6) extract exhibited λ_{max} at 280nm was indicated as tannin. Analysis by ESI-MS of peak at min 14.31 showed the presences of ion at *m/z* 833 which has reported as trimer of proanthocyanidin, consisted of catechin monomer [126].

From the table, some of species known as food, for instance the extracts from twig of *Acacia pennata*, *Leucaena glauca*, *Neptunia oleracea* and seed of *Parkia speciosa* were

found non toxic to the brine shrimp, except for the pericarp of *Parkia speciosa* that showed cytotoxic effect at 18.49µg/ml. The pericarp of *P. speciosa* is known been eaten along with the seed in Thailand and countries of South East Asia. Other than the taste, the pericarp of *P. speciosa* has been believed to have antidiabetic activity. Previously study found that the hypoglycaemic effect of the pericarp chloroform extract was contributed by compound stigmast-4-en-3-one; a plant sterol [110]. Despite its beneficial effect of *P. speciosa* pericarp which was contributed by the non polar compound, the ethanolic extract of *P. speciosa* pericarp exhibited a cytotoxicity effect although the value could be considered as moderate in comparison with the other Mimosaceous extracts.

Spectrometric quantification by Folin reagent showed total phenolic content of *P. speciosa* pericarp ethanolic extract at 343.18 ± 8.24 mg gallic acid equivalent / g extract. The constituents of the *P. speciosa* ethanolic extract were investigated by HPLC-PDA (figure 7).The chromatogram at 280 nm of the extracts showed unresolved hump eluting between 10 to 20 min. Such hump in the 280nm chromatographic profile could be attributed to polymeric polyphenols as observed in the cider apple skin and pulp [126]. Peak (a) which has retention time at 11:68 min showed a single absorption band ($\lambda_{max.}$) at 281nm which were quite similar to that of standard epicatechin, thus suggesting the presence of the compound in the extract.

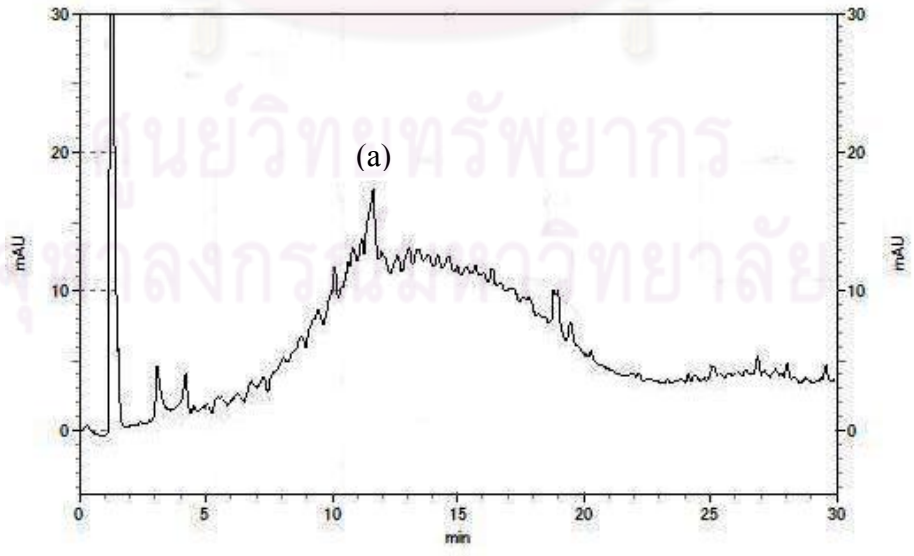


Figure 7 HPLC-PDA chromatogram of *P. speciosa* pericarp extract

The cytotoxicity of some Mimosaceous plants was studied previously. For instance, the cytotoxicity of triterpenoid saponins isolated from the seed kernels of *Entada rheedii* was evaluated from the cell viability assay. The extract showed moderate cytotoxic potency against tumor cell line [127]. The cytotoxic property of the seed kernel was consistent with the cytotoxic activity of *E. rheedi* seed coat in this study (IC_{50}=8.96 µg/ml), however the cotyledon was not toxic to brine shrimp.

The absence of cytotoxicity activity from *Acacia catechu* leaves ethanolic extract which contained 270.23 ± 9.81 mg gallic acid equivalent per g extract was observed in this study. This result was supported with previously study that showed 90 days oral administration of *A. catechu* extract rich in (+) catechin combined with other extract produced no evidence in toxicity in rats [128]. *Acacia catechu* plant is known for high content of catechin compound therefore, the standardized extract has been a control extract to compare the catechin content of other extract [128].

Condensed tannin or proanthocyanidin made from the monomer of flavan-3-ol as catechin which abundant in the fruits and green tea has been toxicologically investigated [129,130]. Polyphenol extract from apple was reported safe and no toxic at average dietary level from the results of an acute oral- toxicity test, and a 90-day subchronic-toxicity test showed no significant hematological, clinical,chemical, histopathological, or urinary effects at a dose of 2000 mg/kg [131]. Response from study conducted on 30 healthy volunteers along with results of mutagenicity test, also indicated that catechin-type monomer and low oligomers of proanthocyanidin were safe for human consumption [132].

Saponins and tannins have been reported from the alcoholic extract. HPLC-PDA chromatogram of both *P. speciosa* and *P. dulce* ethanolic extracts exhibited presence of tannin which was consistent with previously study. However, saponin was not detected from the condition of the HPLC-PDA employed. It was reported that the main problem in the HPLC analysis of saponins was the detection. Only a few saponins exhibited absorption maxima in UV range for instance at 254 nm, whereas majority of saponins do not possess chromophores necessary for UV detection [133]. Nevertheless, the presence

of triterpene saponins of *P. dulce* defatted seeds [134] and triterpenoid saponins with *N*-acetyl sugar from methanolic extract of *Albizia procera* bark [135] have been reported.

Therefore, based on the previously studies [127,128] and the observed cytotoxic activity of *A. procera* stem bark ethanolic extract (IC_{50}=3.91 µg/ml), the presence of saponins were unambiguously contributed to the cytotoxicity of Mimosaceous ethnolic extracts to brine shrimp.

The brine shrimp lethality test has been the elementary assay used to warrant the safety of medicinal plants usage. Results from this study reported for the first the cytotoxcity activity of *P. dulce* stem bark and *A. jiringa* pericarp. The saponins from both *E. rheedii* and *A. procera* were reported exhibiting cytotoxicity against cancer cell lines [127, 135]. It could be suggested that the extracts in this study as *S. saman* and *C. umbellatum* which shown cytotoxicity activity may also have potential of anticancer activity. In addition, despite of the beneficial consumption of *P. speciosa* pericarp [110] and the traditional utilization of *X. xylocarpa* bark decoction as antipyretic and antidiarrheal [26], the toxicological effects as reported in this study should not be neglected.

Antibacterial activity

The antibacterial activity of the ethanolic extracts (final concentration 1mg/ml) was screened by both disc diffusion and broth microdilution methods. From the total of 35 extracts evaluated by paper disc method, only 6 extracts were found exhibiting the antibacterial activity (table 10), whereas no inhibition to *B. subtilis* was observed.

Table 10 Antibacterial activity of extracts determined by disc diffusion method. The final concentration of the extract on the disc was 1 mg/ml. Diameter of the inhibition was including 6mm of the paper disc diameter and expressed as mean ± SD.

Bacteria Strain	**Extract**		**Diameter of inhibition zone (mm ± SD)**
	Species	Plant part	
E. coli (gram negative)	*Samanea saman* (Jacq.) Merr.	Branch	16.67 ± 1.15
	Samanea saman (Jacq.) Merr.	Leaves	20.67 ± 0.55
	Acacia catechu (L.f) Willd.	Leaves	9.67 ± 0.55
S. aureus (gram positive)	*Archidendron jiringa* (Jack) I.C Nielsen	pericarp	13.33 ± 0.45
	Pithecellobium dulce Benth.	Stem bark	8.33±0.45
	Parkia speciosa Hassk.	Pericarp	7.67 ± 0.58
	Xylia xylocarpa (Roxb.) Taub.	Leaves	12.33 ± 0.58
B. subtilis	No inhibition of growth was found		

In the broth microdilution method, the ability of each extract at final concentration 1 mg/ml to inhibit the growth of *S. aureus*, *B. subtilis* and *E. coli* in the 96 well microplate was indicated by the absence of reddish-pink colour after the addition of INT (2-4-iodophenyl-3-4-dinitrophenyl-5-phenyltetrazolium chloride); a tetrazolium salt. From table 10, a total of 12 extracts showed antibacterial activity to *S. aureus*, whereas a total of 13 extracts and 4 extracts inhibited the growth of *B. subtilis* and *E. coli*, respectively. Results from both paper disc and broth microdilution methods were

compared. It was found that, in the broth microdilution method, 13 Mimosaceous extracts showed antibacterial activity to *B. subtilis* compared to none in the paper disc method.

In the paper disc assay, the antibacterial activity was quantitatively determined by the presence or absence of clear zone around the disc impregnated with the extract. However, previous study has reported that the size of the inhibition zone could be affected by the extract rate of diffusion from paper disc to agar medium, thus affected the results [136]. The low ability of Mimosaceous plantes ethanolic extract to diffuse from the paper disc into a medium was observed previously in the brine shrimp cytotoxicity assay. Therefore, in this study the broth microdilution method was more appropriate for a rapid quantitative determination of the antimicrobial activity of Mimosaeous plants ethanolic extracts due to the direct contact of the extract to the bacteria. Furthermore, the 96-well microplate maximized the number of extract to be evaluated in a time compare to paper disc assay. Comparison of results from paper disc and broth microdilution resulted that the absence of an inhibition zone was not necessarily meant the extract was not active.

The MIC of Mimosaceous plant extracts were determined by broth microdilution method. Thirteen extracts obtained from the broth microdilution screening were diluted to determine their minimum inhibition concentration (MIC). Different extract concentrations of extract were prepared in broth to give final concentration of 0.1, 0.2, 0.4, 0.6, 0.8, 1 and 1.2 mg/ml in the microwell.

The determination of bacterial growth in broth dilution method to find the MIC could be done for instance by microscopic evaluation [118]. However, in this study the MIC was determined by calorimetric approach using tetrazolium salt as an indicator of bacterial growth which was reported more convenient. Bacteria and fungi are able to convert the tetrazolium salts into colour formazan derivatives which can be visualized. In the well where microorganism growth occurred, the addition of INT was resulted in the change of colour from yellow to purple and remaining yellow indicated the absence of growth [53]. Other known tetrazolium salt is the MTT (3-4,5-dimethylthiazol-2-yl-2,5-diphenyltetrazolium bromide) which has been used widely in the study of cell lines to

determine the cell lines viability. No difference in sensitivity between both INT and MTT in colouring the microorganism. However in reaction with microorganism, MTT produced insoluble purple formazan which has to be dissolved while no dissolution was necessary with INT [137].

The lowest concentration of extract inhibiting growth of test microorganisms indicated by INT salt was defined as the minimum inhibition concentration (MIC) of a Mimosaceous extract. Extract with MIC < 1mg/ml was exhibited good activity [138]. Apparently, the MBC determinations are undertaken less frequently as a research tool compared to MIC, however despite the advantages provided by tetrazolium salt, the vagueness of visible growth arises due the colour of Mimosaceous plant extracts. The content of each well which showed no reddish-pink colour after the addition of INT were streaked onto fresh nutrient agar plates. The plates were further incubated at 37˚ C for 24 hours. The least concentration of extract with no microbial growth observed on the plate was considered as the MBC (Minimum bactericidal concentration) value (table 11). The MIC and MBC of extracts which previously did not showed antibacterial activity to the particular strains during the screening and the extracts which may have MBC more that 1 mg/ml were not determined (ND).

Table 11 The MIC and MBC values of Mimosaceous plants ethanolic extract. The MIC and MBC values were final concentration of extract in the well and were presented as mg/ml (ND: not determined).

Species	Plant material	Bacteria strains					
		S. aureus		*B. subtilis*		*E. coli*	
		MIC	MBC	MIC	MBC	MIC	MBC
Acacia catechu (L.f.) Willd	leaves	1	1	0.8	0.8	1	>1
Acacia farnesana (Linn) Welld.	twig	ND	ND	0.8	1	ND	ND
Albizia procera (Roxb)	stem bark	1	1	0.4	0.4	ND	ND
Archidendron jiringa I.C Nielsen	pericarp	0.8	1	0.4	0.4	1	>1

Cathormion umbellatum (Vahl)	leaves	0.8	1	0.8	1	0.8	1
Cathormion umbellatum (Vahl)	bark	0.6	1	0.4	0.4	ND	ND
Entada rheedii Spreng	seed coat	0.2	0.4	0.1	0.1	ND	ND
Parkia speciosa Hassk.	pericarp	0.4	0.8	0.1	0.1	ND	ND
Pithecellobium dulce Benth	stem bark	0.4	0.8	0.2	0.2	ND	ND
Samanea saman (Jacq.) Merr.	leaves	1	1	0.8	1	ND	ND
Xylia xylocarpa (Roxb.) Taub	stem	0.4	0.6	0.2	0.2	ND	ND
Xylia xylocarpa (Roxb.) Taub	bark	0.4	0.6	0.2	0.2	ND	ND
Xylia xylocarpa (Roxb.) Taub	**leaves**	**0.4**	**0.6**	**0.1**	**0.1**	**0.8**	**1**

From 35 extracts tested, only 13 extracts were active as antibacterial agent. The antibacterial activity of *A. catechu* leaves in the study was supported by the previous reports. Both aqueous and ethanolic extracts of *Acacia catechu* bark exhibited high activity against all enterohaemorrhagic *Escherichia coli* strains. The antibacterial activity of both extracts indicated the presence of metabolic toxin or broad spectrum antibiotic compound in *A. catechu* [139]. It was also found that a reduction of 10-15% in bacterial growth was seen on a wool sample dyed with a commercially optimized natural dye powder of *Acacia catechu* [140].

A number of extracts have been traditionally used to treat microbial related diseases; nevertheless, the absence of the activity exhibited was reported in the antimicrobial assay. For instance, both extracts *A. lebbeck* pod and *L. glauce* seed known by the indigenous people in India to treat eye infection and diarrhoea, respectively exhibited the absence of antimicrobial activity at concentration of 1mg/ml by agar dilution method [141]. In another study, the 95% ethanol extract of *A. lebbeck* leaves was reported showing showed no activity against *S. aureus*, *E. coli* and *C. albicans* at the concentration ≤ 3 mg/ml, although from the qualitative analysis, although the extract was positive with flavonoids and tannin [142]. Accordingly, the absence of the antibacterial activity of *A. lebbeck* leaves and *L. glauca* twig was also observed in the antibacterial assays of this study.

Based on the MIC values (table 11), each extracts showed different response to each bacterium. For instance, *A. farnesiana* twig was found active to *B. subtilis* only, whereas 4 extracts; *A. catechu* leaves, *A. jiringa* pericarp, *C. umbellatum* leaves and *X. xylocarpa* leaves showed inhibition against all bacteria. Ten extracts exhibited equal MIC and MBC values indicated the high effectiveness of observed Mimosaceous plants ethanolic extracts in inhibiting the *B. subtilis* growth.

The application of INT salt was convenient to determine the MIC for *S. aureus* and *B. subtilis*. Although the formazan production was increased with time, the well was remained distinguishable compared to the control well. From the results, a total of 13 extracts showed antibacterial activity evaluated by broth microdilution method. From this study the INT salt was found not suitable for *E. coli* as the red formazan produced was too high. This was in accordance with the previously report [137] which also caused difficulty to visibly determine the MIC for most of the extracts against *E. coli*. However, in most reported study, *E. coli* was found resistant to plant extract [138]. As observed in this study, *E. coli* was also found resistant to most extracts evaluated both in the disc and broth microdilution assays. Nevertheless, the broth microdilution method was inexpensive, less laborious and economical. The method appears to have the potential to become a tool, which may improve the approach to evaluate potential antibacterial agent by providing the results in a simple way [143].

The susceptibility of bacteria gram positive to plant extracts has been reported frequently [46]. Both *S. aureus* and *B. subtilis* are the gram positive bacteria, which as observed in this study showed the susceptibility to Mimosaceous plants extracts compared to *E. coli* which is the gram negative bacteria. Bacteria are prokaryotic cells. Bacterial wall is varied considerably between species which has been classified according to their reaction in Gram staining. The gram negative bacteria are known for its complex bacterial wall consisted of lipopolysaccharide cell membrane while gram positive is consisted of peptidoglycan which observed as able to remain its purple colour during the gram staining. Bacteria also can be categorized according to the shapes as spherical (coci), rods (bacilli) or helical (spirilla). The selection of bacteria in this study was based on their characteristic as gram-reaction and cell morphology (Table 12).

Table 12 The basic characteristic of bacteria studied

Species	Gram reaction	Cell morphology
Staphylococcus aureus	positive	Non-spore forming cocci
Bacillus subtilis	positive	Endospore-forming bacilli
Escherichia coli	negative	Non-spore forming bacilli

The resistance observed in *E. coli* was contributed by the outer membrane surrounding the cell wall in gram negative bacteria. Higher resistance of gram negative bacteria to external agents has been attributed to the presence of lipopolysaccharides in their outer membranes, which hindered the access and resist the lytic action of most extracts exhibiting activity [138]. Therefore, in order to inhibit microbial growth, the agent must able to penetrate the permeability barriers of the cell. Although the mechanism was unknown, Mimosaceous plants ethanolic extracts were active in inhibiting the growth of gram positive bacteria particularly the endospore-forming bacilli. The influences of chemical composition of extract with the antibacterial activity have been previously reported. It was concluded that antimicrobial properties were closely related to the structure, especially the presence of functional groups [139].

This study reported for the first time the antibacterial activity of *A. jiringa* pericarp, *C. umbellatum* leaves and *X. xylocarpa* leaves. The HPLC-PDA chromatogram of *X. xylocarpa* (figure 8) and analysis by ESI-MS was carried out to investigate the responsible compound for observed the antibacterial activity of the extract.

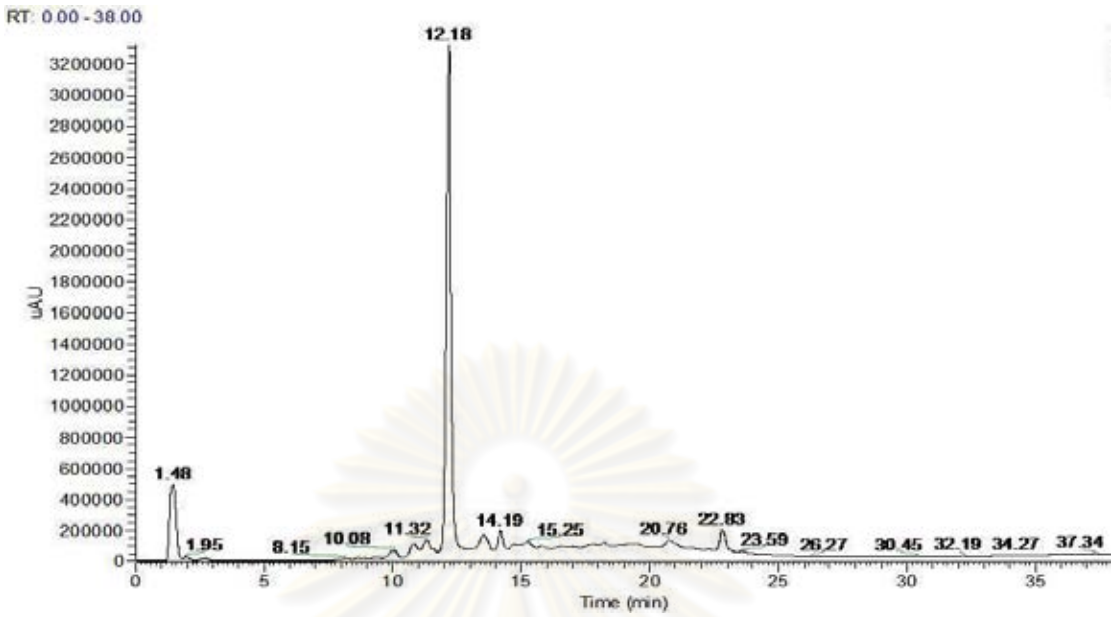


Figure 8 HPLC-PDA chromatogram of *X. xylocarpa* leaves

From figure 8, the prominent peak at min 12.18 exhibited λ_{max} at 257 and 348 which indicated as flavonoid glycoside. Analysis by ESI-MS revealed ion at *m/z* 463. Ion at *m/z* 463 fragmented to produce ions which have similar fragmentation to myricetin whereas loss of 146 was corresponded to the neutral loss of hexoside [144]. Thus according to literature, $[M-H]^-$ at *m/z* 463, was tentatively identified as myricetin-3-*O*-hexoside. Presence of ion at *m/z* 561 and 545 corresponded as proathocyanidin dimer was detected by ESI-MS. Fragmentation of each ion respectively to ion 305 indicated presence of epigallocatechin. Thus, both ions at *m/z* 561 and 545 were the epigallocatechin derivatives.

Phenolics are known to act as antimicrobial by damaging cell walls and membranes and by precipitating essential cell proteins. The extract of *X. xylocarpa* leaves consisted of epicatechin derivatives as observed from the ESI-MS results, thus in accordance with the bactericidal capacity of catechin which has been related to the ability in perturbing the membrane structure from the bacterial-like model membrane [145]. The presence of free hydroxyl groups appears to be important for the flavonoids antimicrobial activity [146], nevertheless, the aglycone, type of substitute group and position of substitute groups also attributed to the effectiveness of flavonoids as antimicrobial agent [147] as observed from the antibacterial activity contributed by myricetin-3-*O*-hexoside.

Antifungal activity

Due to the advantages of disc paper assay observed during the antibacterial evaluation of Mimosaceous ethanolic extract, the antifungal activity was evaluated by broth microdilution assay in 96 well microplate with INT salt as fungal growth indicator. A loop of content in each well was streaked into fresh agar plate to observe the growth after 24 hours to determine the minimum fungicidal concentration (MFC). Different extract concentrations of extract were prepared in broth to give final concentration of 0.1, 0.25, 0.5, 1, 2.5 and 5 mg/ml in the microwell. The final concentration of extracts was increased to 5 mg/ml because most of the extracts showed no inhibitory effect at final concentration lesser than 1.0 mg/ml. From 35 extracts, 17 extracts showed antifungal activity (table 13). At final concentration of 5 mg/ml, extracts which showed fungal growth as indicated by INT was considered as had no antifungal activity (NA).

Table 13 The MIC and MFC values of Mimosaceous plants ethanolic extract. The MIC and MFC values were final concentration of extract in the well and were presented as mg/ml

Sample	**Plant part**	***C. albicans***		***S. cerevisiae***	
		MIC	MFC	MIC	MFC
Acacia catechu (L.f.) Willd	leaves	NA	ND	0.5	1
Acacia farnesana (L.) Welld.	twig	NA	ND	2.5	2.5
Adenanthera microsperma Teijsm.	leaves	2.5	>5	NA	ND
Albizia lebbeck (L.) Benth.	leaves	2.5	2.5	NA	ND
Albizia lebbeckoides (D.C)	leaves	2.5	5	NA	ND
Albizzia myriophylla Benth	leaves	2.5	>5	NA	ND
Archidendron jiringa I.C Nielsen	pericarp	NA	ND	0.25	0.5
Cathormion umbellatum (Vahl)	leaves	NA	ND	0.5	1
Entada rheedii Spreng	seed coat	NA	ND	1	2.5
Leucaena glauca Benth	twig	NA	ND	2.5	2.5
Leucaena glauca Benth	pericarp	2.5	>5	2.5	>5

Pithecellobium dulce Benth	leaves	NA	ND	0.5	1
Samanea saman (Jacq.) Merr.	branch	NA	ND	0.25	0.5
Samanea saman (Jacq.) Merr.	leaves	2.5	5	0.25	0.25
Xylia xylocarpa (Roxb.) Taub	stem	NA	ND	0.5	1
Xylia xylocarpa (Roxb.) Taub	bark	NA	ND	0.5	1
Xylia xylocarpa (Roxb.) Taub	leaves	NA	ND	2.5	>5

*NA: No Activity ND: Not Determined

From the results, the MIC value of antifungal was found higher than observed in the antibacterial activity. Fungi are recognized as a sister group to animals and are more distantly related to plants. Two selected fungal in this study are the yeast. Both *S. cerevisiae* and *C. albicans* are yeasts which essentially is a single celled fungi which although has a simple morphology are a highly specialized group. Yeast is a fungal which known by the basic reproduction is by budding. Almost all organic material is subjected to attack or spoilage by fungi, thus fungicides are of great importance as preservatives. Antifungal also has been used to fight fungal diseases which affected plants. Fungi is unlike bacteria are eukaryotic cells which difficult the development of antifungal compounds that do not harm either animal or plant hosts since all three eukaryotic groups have much in common biochemically. However, one of the defining characteristics of fungi is the structure and composition of the cell wall. The development of compounds that target this cellular component may offer a high degree of specificity in the targeting of fungal pathogens [148].

It was observed that fungal was less susceptible to Mimosaceous plants ethanolic extracts compared to bacteria and among the two studied fungals, 13 of Mimosaceous plant extracts inhibited the growth of *S. cerevisiae* compared to 6 extracts against *C. albicans*. The highest inhibition activity against *S. cerevisiae* exhibited by *A. jiringa* bark, both *S. saman* branch and leaves extracts with MIC at 0.25 mg/ml, along with the MIC for *C. albicans* at 2.5 mg/ml was observed. Previously, the constituents of *S. saman* bark extract as sterol and flavonoids were reported [134] which could be contributed to the observed antifungal activity.

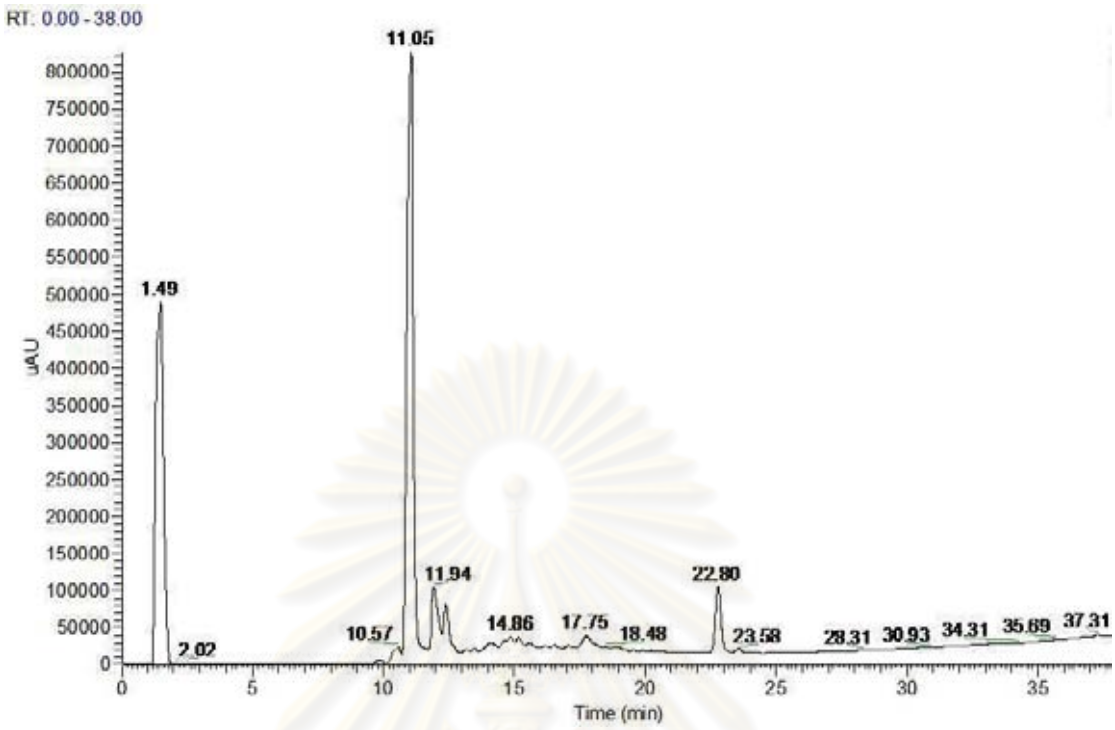


Figure 9 HPLC-PDA chromatogram of *S. saman* leaves

The HPLC-PDA of *S. saman* leaves extract exhibited the prominent peak at min 11.05 exhibited λ_{max} at 253, 267 (shoulder) and 352 nm, indicating flavonoid glycosides. Analysis by ESI-MS detected ion of flavonoid glycoside at m/z 755 [149] which fragmented to ion at m/z 609, and 300 indicating, rutin and quercetin, respectively in MS/MS. Neutral loss of 146 amu signifying presence of deoxyhexose. Therefore, the peak was tentatively identified as rutin-deoxyhexoside. However, sterol was not detected in the ethanolic extract of *S. saman* leaves in this study as previously described in the bark extract [134].

As with bacteria, the cell wall is therefore a major target for antifungal agent. However, the outer wall of the fungal cell is a complex multilayered structure where amorphous, granular and fibrillar structures interact with one another to give the cell a rigid shape and to confer osmotic stability. Another activity of antifungal agents is inhibiting the respiration in fungi by disturbing the enzyme systems that transfer hydrogen from the dehydrogenases to molecular oxygen [148].

Antioxidant activity

The antioxidant activity has been attributed to various mechanisms which have been contributing directly or indirectly in the prevention of pathogenesis and deterioration of food [150]. A number of assays both *in vitro* and *in vivo* have been proposed to evaluate the antioxidant activity of an extract or a chemical compound. The antioxidant activity *in vitro* is demonstrated by changes in the absorbance which can be observed by the changes in colours during a specified time and was measured spectrophotometrically at a defined wavelength. Assays to evaluate scavenging activity of antioxidant to radicals found *in vivo* have also been developed. The non radical as nitric oxide which is implicated in the oxidative stress can be easily generated and the ability of potential antioxidant to scavenge nitric oxide can be assessed by 96 well microplate and microplate reader. The antioxidant assays employed in the microplate have been well accepted due to its simplicity and reproducibility.

In this study, the antioxidant activities of Mimosaceous plants extracts in this study were described from the abilities to scavenge DPPH radical, scavenge nitric oxide, chelate iron, and reducing power. The radical scavenging, reducing capacity and metal chelating properties of antioxidants are known to eliminate and prevent the generation of free radical.

The concentration range of extracts evaluated with this study was prepared according to the previously studies on plant extracts. For instance, final concentration prepared for both metal chelating and nitric oxide scavenging assays was 5 mg/ml. It was found higher concentration of the extracts interfering with the sensitivity and accuracy of the assay. High absorbance of the reaction was produced due to the intense colour of the extracts compared to control. Therefore, selected concentration of extract that caused no discoloration to the reaction mixtures was regarded as showing no activity.

Due to the preparation of extract, each Mimosaceous extract was expected to be rich in phenolic content. Therefore, quercetin which is a flavonoid was selected to be a positive control to compare the antioxidant activity of each extract.

DPPH radical scavenging activity

In this study the ethanolic extracts from plant parts of Mimosaceae with good free radical scavenging activity were evaluated from their ability to inhibit or scavenge the DPPH radical. The concentration of extract to inhibit 50% DPPH radical (IC_{50}) is shown in table. Extract of *Xylia xylocarpa* leaves demonstrated highest DPPH scavenging activity (IC_{50}=0.01 ± 0.0001 mg/ml) which was comparable with positive control Quercetin (IC_{50}=0.01 ± 0.0014 mg/ml). From 35 extracts studied, a good DPPH scavenging activity was observed in 12 extracts with IC_{50} values were and less than 0.1mg/ml (Figure). From the highest IC_{50} values, the *S. saman* leaves exhibited the lowest DPPH scavenging activity.

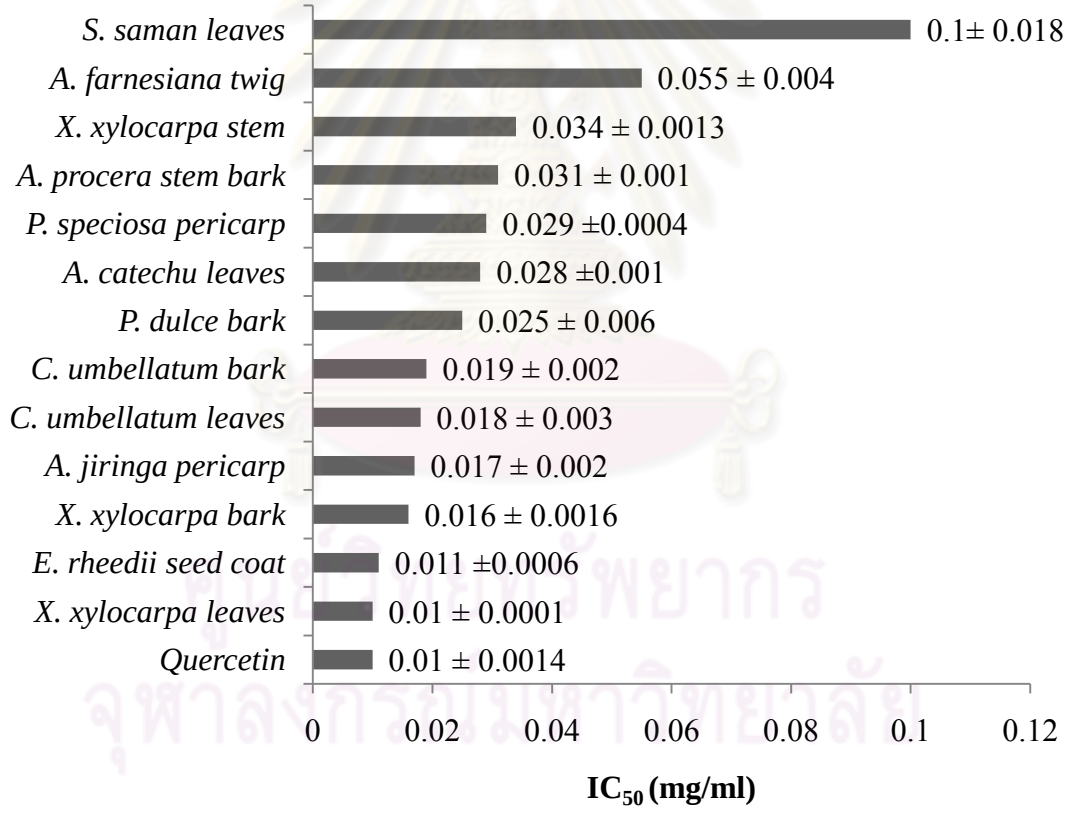


Figure 10 DPPH radical scavenging activity of Mimosaceous extract. IC_{50} values ≤0.1mg/ml

The antioxidant activity of crude extract has been closely related with the constituents of the extract. For instance, it was reported that the bark extract of *Acacia catechu* gave the highest scavenging activity (IC_{50}= 0.05µg/µg DPPH), due to its high phenolic content (178mg Gallic acid equivalent/g dry weight of plant extract) and flavonoid content (42mg Rutin equivalent/g dry weight of plant extract) [25]. The total phenolic content of *X. xylocarpa* leaves extract was 605.52 ± 5.32 mg gallic acid equivalent per gram extract, which also was the highest among all Mimosaceceous extract in this study. Therefore, it was suggested that the DPPH radical scavenging observed in this study was contributed by its phenolic content.

The mechanism of antioxidant has mainly been described as able to scavenge free radical which reducing is an ability to donate electron or hydrogen. DPPH is a stable free radical and accepts an electron to become a stable molecule. Because of the odd electron, DPPH radical shows a strong absorption at 517 nm in visible spectroscopy (deep violet color). As this electron becomes paired off in the presence of a free radical scavenger the absorption vanished and the resulting decolorization is with respect to the number of electron taken up. The availability, reproducibility and convenience of this reaction have made the DPPH radical a widely used method to screen the potential of plant extract as antioxidants. As a result, it has become a routine assay in studying antioxidant [151].

Reducing power

In assessing the antioxidant property of an extract, it is important that the extract able to maintain its stability after being reduced, which not turn into free radical itself. Reducing capacity of compound has been served as a significant indicator of potential antioxidant activity. It was suggested that there is a direct correlation between the antioxidant activity and reducing power of components of some plants. Therefore, other than ability to scavenge DPPH free radical, the reducing ability of 35 Mimosaceous plants ethanolic was evaluated by ability to reduce the ion Fe^{3+} to Fe^{2+}.

In this assay, the yellow color of the test solution changes to various shades of green and blue depending on the reducing power of each extract. The presence of the antioxidant

known for its reducing ability causes the conversion of the Fe^{3+}/ ferricyanide complex in this method to the ferrous form (Fe^{2+}). By measuring the formation of blue color at 700nm, the Fe^{2+} concentration can be monitored. The higher absorbance indicates the higher reducing power [152].

The highest reducing capacity was exhibited by the extracts of *Cathormion umbellatum* bark followed by *Entada rheedii* seed coat. From 35 extracts studied, the reducing activity of 12 extracts were compared with positive control; Quercetin which showed reducing capacity at mean 3.85 ± 0.14 µg/ml (figure 11).

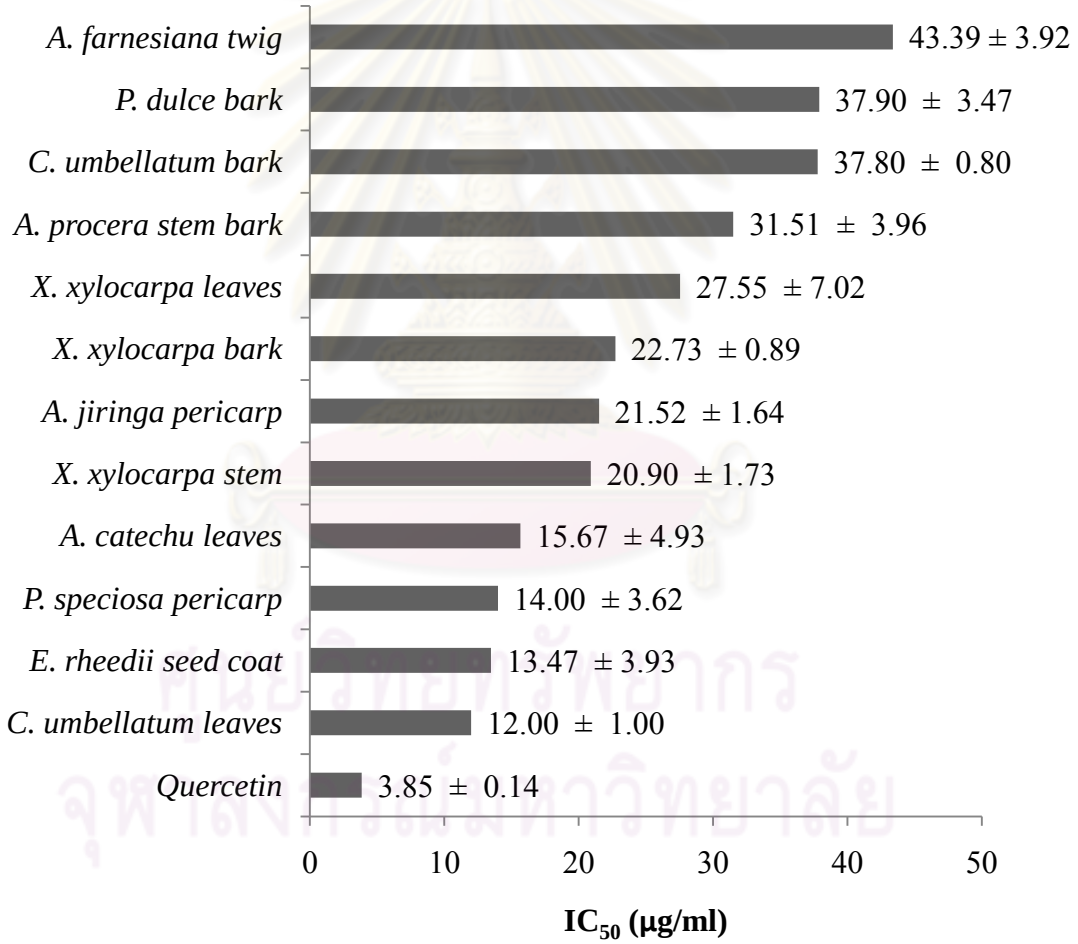


Figure 11 Reducing power of Mimosaceous extracts. IC_{50} values <50 µg/ml

The results suggested that Mimosaceous plants extracts have reducing potency which important to donate electron to free radical, nevertheless the reducing ability of Mimosaceous extract was found lower compared to Quercetin. Although the DPPH radical scavenging and reducing power assay has been closely related, as both assays evaluate the reducing ability of extracts, it showed from the results that *X. xylocarpa* leaves extract which the most active against DPPH with the IC_{50} comparable with positive control Quercetin was not the extract with the best reducing ability.

In both DPPH radical scavenging and reducing assays, positive control Quercetin showed the highest activity in DPPH radical scavenging and reducing power compared to Mimosaceous extracts. However, it was concluded that there may not always extract with highest radical scavenging exhibiting highest reducing power [153]. Structure antioxidant activity of Quercetin such as free OH group at 3'4' OH (catechol) group, 3-OH at ring C, OH group at A ring as in position 5 and 7, along with 2-3 double bond conjugated with a 4-oxo function which contributed to the reducing or donating properties of Quercetin has been reported [154]. Therefore, the different results could be contributed by different reagent used in the assay and also the chemical constituent of the Mimosaceous plants ethanolic extracts.

Nitric oxide scavenging activity

Nitric oxide (NO) is a non radical gaseous molecule which has been one of the simplest compounds found to be continuously produced in humans and animals. It is derived from L-arginine through the enzyme nitric oxide synthase (NOS) and by NO donors, such as sodium nitroprusside (SNP). NO has been showing to play an unprecedented range of roles in biological systems; acting as a universal intracellular and transcellular signaling molecule and the regulator of vascular tone, cell proliferation and apoptosis [155].

However, overproduction of nitric oxide (NO) has been associated with chronic inflammation, cell death, and onset of atherosclerosis [156]. *In vitro*, sodium nitroprusside (SNP) decomposes in an aqueous solution at physiological pH to produce NO. Under

aerobic conditions, NO reacts with oxygen to produce stable nitrite product which can be quantify from the reaction with Griess reagent. Antioxidants were reported able to prevent the cytotoxity and lipid peroxidation activities of SNP derived by NO [157]. Therefore, NO scavenger may be of therapeutic benefit in various types of inflammation.

From the 35 extracts tested, only 8 extracts exhibiting NO scavenging activity and the activity of positive control quercetin; a known antioxidant phenolic exhibited IC_{50} at 150.8 ± 11.32 µg/ml was more pronounced than the NO scavenging activity of Mimosaceous extracts at concentration of 0.05-5 mg/ml. The highest NO scavenging activity was showed by the extracts of *A. lebbeckoides* leaves and *P. dulce* bark (figure 12).

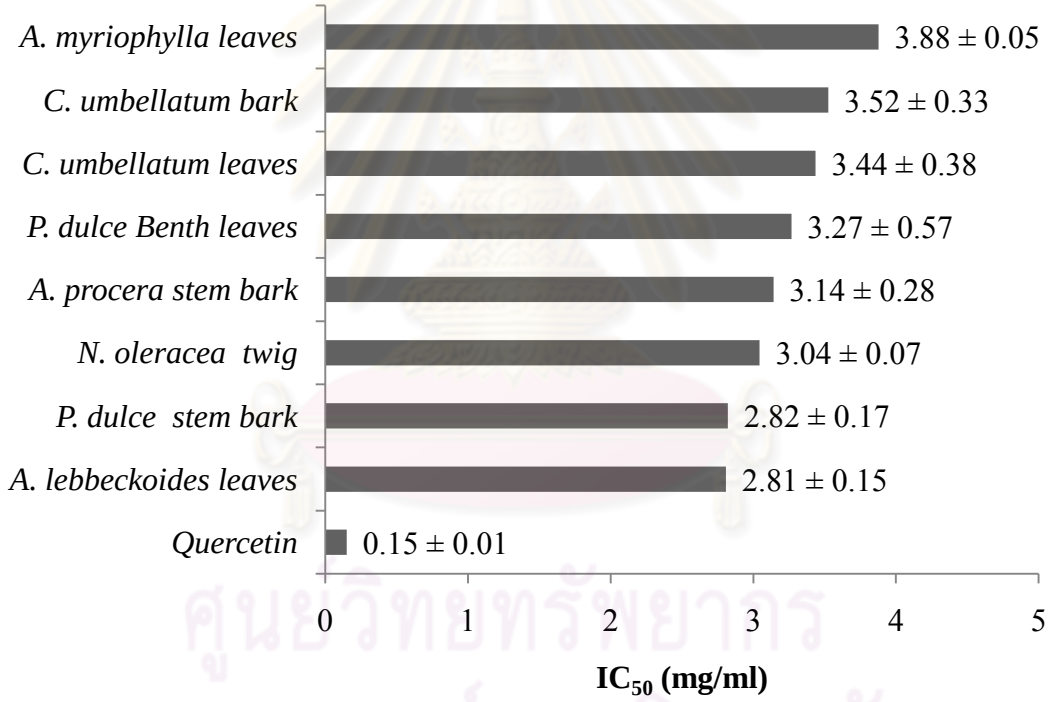


Figure 12 Mimosaceous extract with NO scavenging property. (Data are presented as mean ± STD)

Previously, the structural requirement of quercetin and other 72 flavonoids to inhibit NO production was reported. Thus, the excellent scavenging activity of quercetin observed in this study was in accordance with the previous study. Quercetin showed pronounced NO scavenging activity due to its chemical structures which possessed free

OH groups and absence of glycoside group [158]. In a more complex assay, NO was generated in cultured animal cells by irritating the cells with inflammatory agent such as lipopolysaccharide (LPS), interleukin or tumor necrosis factor. The accumulation of nitrite produced due to the response to inflammatory agent in the culture medium will be measured using Griess reagent.

However, in the assay employed, NO was directly generated from SNP. The generation of NO from SNP provided simple but reliable alternative and, the assay was sufficient to evaluate the ability of extract to scavenge the generated NO. For instance, when a solution of 5 mM SNP in PBS was incubated at 25°C for 2 hours, a time-dependent nitrite was generated, which was decreased by the presence of extracts in a dose-dependent manner [159]. In this study, the mean of nitrite produced during the 2 hours incubation of SNP was 59.56 ± 2.26 µM. The nitrite produced was referred from the absorbance of known sodium nitrite concentrations treated with Griess reagent (figure 13).

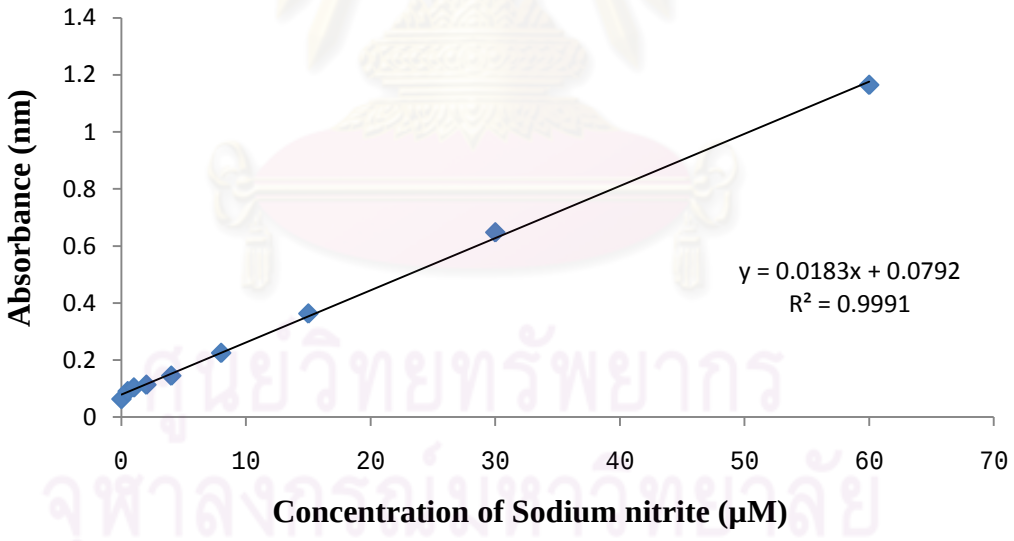


Figure 13 Standard graph used to determine the concentration of NO

The search for natural NO production inhibitors have been gaining attention. It was reported that NO scavenging ability has been contributed to the DNA damage inhibition in SNP-mediated macrophages of *Uraria crinita* root extract which known for anti-inflammatory activities [159]. Thus, the extract may have the property to counteract

the effect of NO formation and in turn may be of considerable interest in preventing the ill effects of excessive NO generation in the human body. The scavenging activity may also help to arrest the chain reactions initiated by excess generation of NO that are detrimental to human health [160]. It was suggested that it might be beneficial to human health to consume foods which could scavenge free radicals [159], therefore this result may encourage the consumption of *N. oleraceae* twig which is commonly found in Thai dishes that has been known as a detoxicant [111].

Metal chelating activity

Transition metals have a major role in the generation of oxygen free radical in the living organism. Iron is among essential element for living organism. Iron exists as ferrous (Fe^{2+}) and ferric (Fe^{3+}) ion. The Fe^{3+} is biologically inactive however after being reduced to Fe^{2+}, it involves in the production of hydroxyl radical ($OH^{\bullet}$) through Fenton reaction in the presence of cuprous or ferrous ion. Therefore, transition metal as iron (Fe^{2+}) has been described participating in free radical generation, stimulating lipid peroxidation and inhibiting the function of membrane protein [161]. Metal chelating capacity of antioxidant was significant in reducing the concentration of transition metal participated in lipid peroxidation [162].

From 35 extracts, only 6 extracts exhibited metal ion chelating property. Analysis of metal ion-chelating property showed that *Leucaena glauca* twig extract displayed the lowest IC_{50} values which indicated high metal chelating activity (figure 14). However, the activity of both extracts were low compared to the EDTA which showed very good activity as Fe^{2+} chelator against Ferrozine (IC_{50}=0.119 ± 0.001mM).

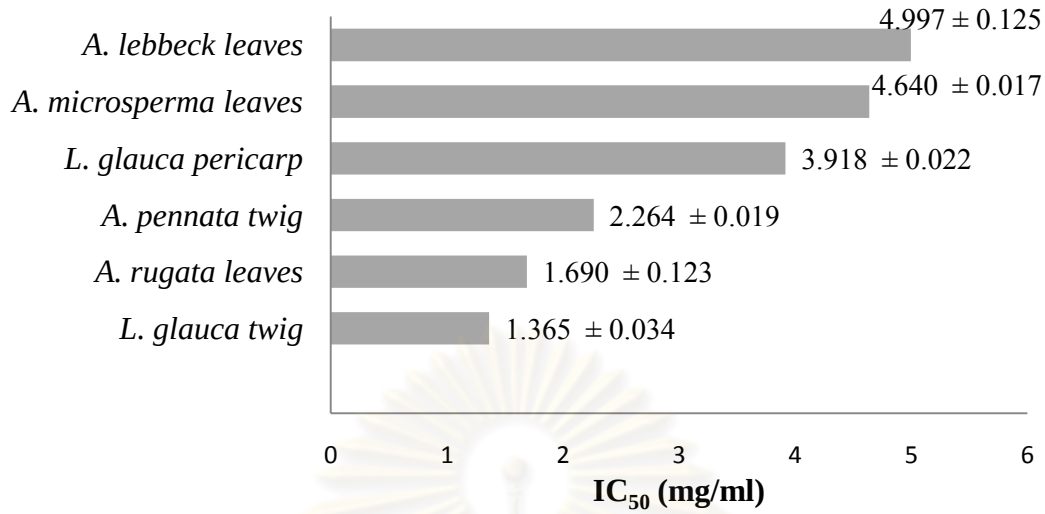


Figure 14 Mimosaceous extract with metal ion chelating property. (Data are presented as mean ± STD)

In studying the metal chelating effect of a substance, ferrozine has been used to quantify the free iron (Fe^{2+}). The presence of chelating agent will disrupt the formation of Fe^{2+}-Ferrozine and eventually fading the magenta color of the complex. The method has been used to evaluate the direct metal chelating activity of extract [123]. The metal chelating was first carried out at concentration 5 mg/ml to screen the extract with good response. Therefore, extract that caused no discoloration to the reaction mixtures could be regarded as showing no inhibition actions.

From the results, two common vegetable in Thailand, namely *L. glauca* and *A. pennata* exhibited iron chelating property. The ethnopharmacological properties of both vegetables reported were being carminative, relieve flatulence for *A. pennata* and relieve diarrhea for *L. glauca* [111]. Although the IC_{50} values showed by both extracts were far lower compared to EDTA, binding iron property of food is important to suppress the accessibility of the iron to oxygen molecules thus partly explain the health protective effect. However, further studies are required to investigate the iron binding property of both vegetables, due to the adverse effect of iron chelator such as reducing iron absorption that beneficial to health [163].

Tyrosinase enzyme inhibition activity

Tyrosinase is the enzyme known to initiate melanogenesis. The enzyme catalyses the hydroxylation of L-Tyrosine to 3,4-diydroxyphenylalanine (DOPA) in a activity known as monophenolase and the oxidation of DOPA to Dopaquinone, known as diphenolase. Search for natural tyrosinase activity inhibitor either by inhibiting the hydroxylation of L-tyrosinase or oxidation of L-DOPA has been of considerable interest both in the food and cosmetic industry primarily related to pigmentation. Natural tyrosinase inhibitors from the edible plants can be a candidate for tyrosinase inhibitory material [164].

In the *in vitro* tyrosinase inhibition assay, both diphenolase and monophenolase inhibition activity of Mimosaceous plant extracts were evaluated by employing L-DOPA and L-Tyrosinase as substrates for the tyrosinase enzyme commercially extracted from mushroom, respectively. From the results, 7 Mimosaceous extracts exhibited inhibition to both tyrosinase enzyme activities. It was also observed that at 4 mM of substrates, percentage inhibition of monophenolase showed by the extract concentration of 500μg/ml was higher than the diphenolase (figure 15).

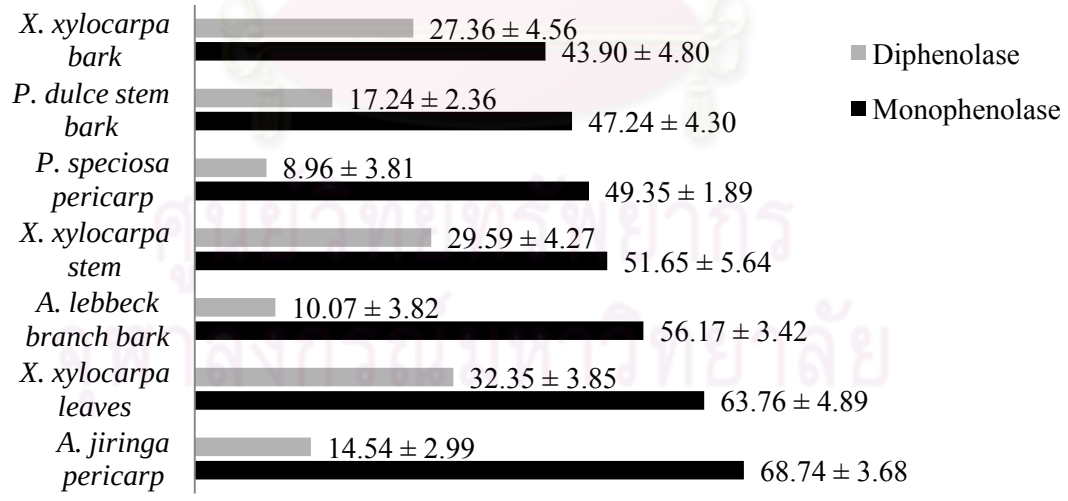


Figure 15 Mimosaceous extract with Tyrosinase enzyme inhibition property. (Data are presented as mean ± STD)

As depicted by the extract of *A. jiringa* bark, the extract showed the highest inhibition of monophenolase (68%) but only 14% of diphenolase inhibition at 500µg/ml. A similar response was also observed in the extract of *X. xylocarpa* leaves. Previously, the IC_{50} of crude extracts in monophenolase inhibition was found lower compared to the diphenolase inhibition activity [165]. This observation was supported by the study on the tyrosinase inhibition from the extract of *Sideroxylon inerme* bark. The extract exhibited better IC_{50} values in monophenolase compared to diphenolase inhibition activity. Presence of epigallocatechin gallate and procyanidin B1 in the extract was reported responsible for the tyrosinase inhibition. Both compounds were also exhibited good DPPH scavenging activity and melanin production inhibition. The results supported the traditional usage of *S. inerme* bark as a skin lightener in South Africa and likely find the phytochemical responsible for the whitening and antioxidant effects [166].

Monophenolase activity of tyrosinase is generally defined as the first step in the melanization pathway and consists of the hydroxylation of monophenols to diphenol. Thus, the activity of monophenolse observed could be related with the ability of the Mimosaceous extracts to donate hydrogen, which inhibited the hydroxylation by tyrosinase enzyme to occur. However, it was reported that the effect of isolated flavonoids from licorice as potent monophenolase inhibitor was contributed by its structural similarity with L-tyrosine substrate thus acted as a competitive substrate to react with tyrosinase enzyme. It was also observed that the diphenolase inhibitory effect of compounds which potent as monophenolase inhibitor were lower. Even at the high concentration, no significant effects were observed on diphenolase activity with DOPA as substrate [90] which was in accordance with this study.

It was suggested that diphenolase activity of tyrosinase was inhibited by chelating the copper in the tyrosinase enzyme, as exhibited by flavonoid kaempferol isolated from saffron flower and kojic acid; a known diphenolase inhibitor [89]. Nevertheless, different inhibitory effects observed between the monophenolase and diphenolase activities of Mimosaceous plants extracts could be due to the involvement of different substrate molecules in those two reactions [167].

In a comparison of arbutin; a naturally cosmetic vehicle and whitening agent with selected polyphenols, results indicated ellagic acid, gallic acid and tannic acid showed better diphenolase inhibition activity than Arbutin, except for coumarin which exhibited the lowest activity among all [168]. It was reported that polyphenols inhibit the tyrosinae enzyme by being a competitor to the substrates of tyrosinase enzymes; L-DOPA or L-Tyrosine. Most polyphenols competitors have ortho-dihydroxyphenols; a structure that resembles the substrate L-DOPA, to compete with L-DOPA to bind with the enzyme at the same site [169]. While for the inhibition of monophenolase, the polyphenols with structure of 4-hydroxychalcone that similar with L-Tyrosine resulted being a competitor to L-Tyrosine [90]. However, in this study, the samples employed are of crude extract which was a mixture of polar compounds that could have different effect on the enzyme. Probably as crude extracts, were not capable to compete for the substrate which resulted in the absence of tyrosinase inhibition activity from the other Mimosaceous plants extracts.

Total phenolic content of Mimosaceous plants ethanolic extract

Phenolic compounds are known for vast variety of chemical structures and previously studies have demonstrated the effects of phenolic structures on their biological properties. A study proposed that the structure and content of the phenolic compounds that responsible for the good activity of an extract can be optimized during the drying process and solvent extraction condition. The quantification of polyphenols by Folin ciocalteu has been a routine assay despite its questioned sensitivity related to its ability to react with other constituents as protein. Therefore, ethanolic extract in this study was prepared by sequential extraction with different polarity solvents to eliminate other constituents that may interfere with Folin ciocalteu reaction.

The extraction procedure can also largely affect the determination of the phenolic content. The use of sequential solvents with different polarity allows a separation of compounds according to their solubility in the solvents, thus simplify and reduce the chemical complexity when taken into the extract, whereas it has been suggested that the heat might disrupted the cell wall and liberate the polyphenol compounds which in turn increase the pool of accessible compounds [144]. Furthermore phenolic contents from plant extracts were closely dependent on the solvent polarity [170].

From the results (table 14), *Xylia xylocarpa* leaves showed the highest content of phenolic content equivalent to 605.52 mg gallic acid per each gram extract, followed by *Entada rheedii* seed coat and pericarp of *Parkia speciosa* extract. The lowest amount of phenolic compound was exhibited by extract of *C. umbellatum* branch with 7.26 mg gallic acid per gram extract, which was the lowest compared to the other Mimosaceous plants branch extracts. Nevertheless, the phenolic content of seeds as *P. speciosa* and *A. jiringa* were not likely to reveal high phenolic content since both seeds are largely constituted of compounds such as lectin [171, 172].

Table 14 Total phenolic content of Mimosaceous plants ethanolic extract

Species	**Plant material**	**Total Phenolic content (mg galic acid /g extract)**
Xylia xylocarpa (Roxb.) Taub.	leaves	605.52 ± 5.32
Entada rheedii Spreng	seed coat	429.14 ± 8.46
Parkia speciosa Hassk.	pericarp	343.18 ± 8.24
Xylia xylocarpa (Roxb.) Taub.	stem	333.56 ± 8.34
Xylia xylocarpa (Roxb.) Taub.	bark	326.48 ± 0.33
Pithecellobium dulce Benth.	bark	323.83 ± 4.43
Archidendron jiringa (Jack) I.C Nielsen	pericarp	320.94 ± 1.71
Acacia catechu (L.f) Willd.	leaves	270.23 ± 9.81
Cathormion umbellatum (Vahl) Kosterm.	bark	269.93 ± 4.76
Albizia procera (Roxb.) Benth.	stem bark	249.00 ± 6.49
Leucaena glauca Benth.	twig	249.91 ± 4.71
Acacia farnesiana (L.) Willd.	twig	209.78 ± 3.21
Cathormion umbellatum (Vahl) Kosterm.	leaves	199.25 ± 2.01
Samanea saman (Jacq.) Merr.	leaves	180.85 ± 2.72
Acacia pennata (L.) Willd.	twig	177.29 ± 1.15
Adenanthera pavonina L.	seed coat	168.47 ± 1.27
Pithecellobium dulce Benth.	leaves	159.97 ± 7.06
Acacia rugata Merr.	leaves	137.93 ± 4.02
Entada rheedii Spreng	cotyledon	118.78 ± 1.80
Albizzia myriophylla Benth.	leaves	104.97 ± 2.18

Albizia lebbeckoides (DC.) Benth.	leaves	102.47 ± 1.04
Leucaena glauca Benth.	pericarp	81.06 ± 3.02
Adenanthera pavonina L.	branch	77.38 ± 1.96
Mimosa pudica L.	twig	74.90 ± 2.51
Neptunia oleracea Lour.	twig	61.62 ± 2.05
Adenanthera pavonina L.	pericarp	54.38 ± 2.76
Albizia lebbeck (L.) Benth.	leaves	55.65 ± 1.36
Pithecellobium dulce Benth.	pericarp	43.43 ± 2.40
Acacia rugata Merr.	pericarp	42.94 ± 1.28
Samanea saman (Jacq.) Merr.	branch	29.28 ± 1.37
Archidendron jiringa (Jack) I.C Nielsen	seed	26.99 ± 0.62
Adenanthera pavonina L.	leaves	22.51 ± 0.35
Adenanthera microsperma Teijsm.	leaves	19.34 ± 0.61
Parkia speciosa Hassk.	seed	15.48 ± 0.61
Cathormion umbellatum (Vahl) Kosterm.	branch	7.26 ± 1.14

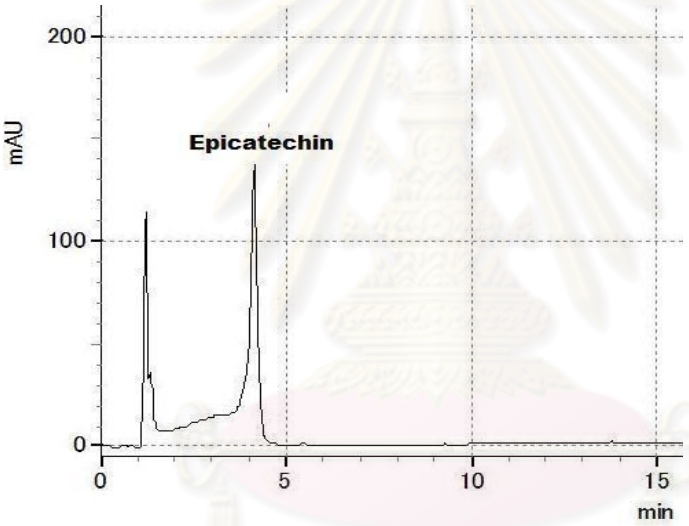
It was observed that among the same species, different plant material showed different total phenolic content. For instance, total phenolic content of Mimosaceous plants leaves was different from their bark. This could be resulted by the transportation and distribution of polyphenols during the development of plant [173]. Furthermore, the tissue system of stem, bark, leaves and pericarp also are structurally different from each other which influence the accumulation of chemical compound. Nevertheless, the values given by extract reaction with Folin-Ciocalteu reagent were only an approximate estimation of the total phenolic content [174].Total phenolic compound does not give a full picture of the quantity or quality of the phenolic constituents in the extracts. Therefore, in many studies, the characterizations of the extract have been frequently suggested.

Analysis of Mimosaceous plants ethanolic extract

Optimization of HPLC mobile phase

Rapid and efficient HPLC-DAD-MS/MS method provides fast screening of polyphenols without further clean-up procedure due to the method of extracts preparation in this study. Reversed phase HPLC using an acidic water-methanol gradient elution system is very common for the separation of flavonoids and tannins, however better chromatographic separation was achieved using a gradient of acidified water and acetonitrile mobile phase (figure 16).

a) acidic water-methanol gradient elution system



b) acidified water and acetonitrile gradient elution system

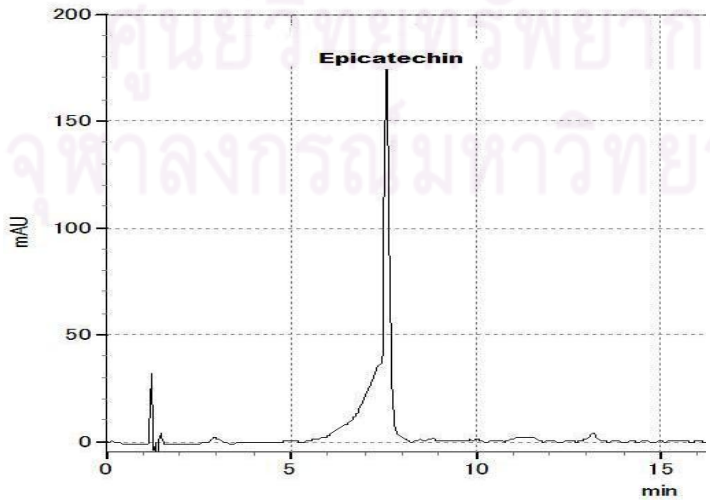


Figure 16 The resolution of standard epicatechin in the HPLC mobile phase. (a) is Methanol- acidified water and (b) is acetonitrile-acidified water. It was observed that acetonitrile-acidified water (b) showed better resolution. Thus, the gradient of 0-60% acetonitrile was employed for the separation of Mimosaceous plant ethanolic extracts in HPLC-PDA.

HPLC profile of Mimosaceous plants ethanolic extract

A preliminary study of the UV spectra of the peaks gave a first indication of the family of phenolic compounds. Based on the UV absorption of prominent peaks in the extracts, the peaks were arranged according to their identical retention time and UV absorption (table 15 and 16). The peaks that showed λ_{max} near 280nm was regarded as flavan-3-ol (including dimers), whereas the peaks exhibited two major absorption bands in UV spectra: around λ_{max} 260 nm and 350 nm, thus were putative flavonoids.

Table 15 The retention time (RT) and λ_{max} of putative flavan-3-ol compounds and derivatives from Mimosaceous plants ethanolic extracts.

RT (min)	λ max (nm)	Species	Plant part
2.79	268	Gallic acid	Standard
7.69	278	Epicatechin	Standard
3.91	283	*Parkia speciosa* Hassk.	seed
3.91	283	*Acacia rugata* Merr.	pericarp
3.91	263	*Albizia lebbeck* (L.) Benth.	branch
4.05	256, 291	*Albizia procera* (Roxb) Benth.	stem bark
4.13	257	*Pithecellobium dulce* Benth.	stem bark
4.9	273	*Entada rheedii* Spreng	cotyledon
4.96	273	*Entada rheedii* Spreng	pericarp
5.53	287	*Acada pennata* (L.) Willd.	twig
6.15	278	*Cathormion umbellatum* (Vahl) Kosterm.	bark

6.29	272	*Albizia lebbeck* (L.) Benth.	branch bark
6.78	276	*Acacia catechu* (L.f.) Willd.	leaves
6.78	282	*Adenanthera pavonina* L.	seed coat
7.48	296	*Parkia speciosa* Hassk.	seed
8.25	280	*Xylia xylocarpa* (Roxb.) Taub	bark
8.81	268	*Cathormion umbellatum* (Vahl) Kosterm.	leaves
8.81	272	*Cathormion umbellatum* (Vahl) Kosterm.	bark
8.81	269	*Acacia catechu* (L.f.) Willd.	branch
9.36	261	*Acacia catechu* (L.f.) Willd.	leaves
10.01	272	*Archidendron jiringa* I.C Nielsen	pericarp
10.01	266	*Acacia catechu* (L.f.) Willd.	leaves
10.2	267	*Adenanthera pavonina* L.	leaves
10.56	268	*Neptunia oleracea* Lour	twig
10.84	280	*Pithecellobium dulce* Benth.	stem bark
11.05	264 sh	*Acacia catechu* (L.f.) Willd	leaves
11.33	283sh	*Adenanthera pavonina* L.	branch
11.39	264sh	*Acacia catechu* (L.f.) Willd	leaves
11.39	280	*Xylia xylocarpa* (Roxb.) Taub	bark
11.6	280	*Pithecellobium dulce* Benth.	stem bark
11.68	281	*Parkia speciosa* Hassk.	pericarp
11.82	266	*Adenanthera pavonina* L.	leaves
12.3	266	*Cathormion umbellatum* (Vahl) Kosterm.	leaves
12.58	263	*Pithecellobium dulce* Benth.	pericarp
13.14	277	*Albizia lebbeck* (L.) Benth.	branch bark
13.43	269	*Acacia catechu* (L.f.) Willd.	leaves
13.8	278	*Pithecellobium dulce* Benth.	stem bark

Table 16 The retention time (RT) and λ_{max} of putative flavonoids compound from Mimosaceous plants ethanolic extracts.

RT(min)	λ max (nm)	Species	Plant part
8.11	269,331	*Albizia lebbeck* (L.) Benth.	leaves
8.26	269,334	*Acacia rugata* Merr.	leaves
8.95	253,267sh,352	*Samanea saman* (Jacq.) Merr.	leaves
9.09	268,338	*Acacia rugata* Merr.	leaves
9.09	271,334	*Acada pennata* (L.) Willd.	twig
9.09	271, 348	*Mimosa pudica* (L.)	twig
9.86	263sh,350	*Cathormion umbellatum* (Vahl) Kosterm.	leaves
9.86	257, 348	*Xylia xylocarpa* (Roxb.) Taub	leaves
9.92	267sh, 351	*Albizia lebbeck* (L.) Benth.	leaves
9.93	263sh,347	*Leucaena glauca* Benth.	twig
10.01	268,338	*Acacia farnesiana* (L.) Willd.	twig
10.01	268,336	*Albizzia myriophylla* Benth.	leaves
10.01	267sh,342	*Mimosa pudica* L.	twig
10.07	268, 337	*Adenanthera pavonina* L.	pericarp
10.14	268,336	*Acacia rugata* Merr.	leaves
10.28	267 sh, 347	*Albizia lebbeckoides* (D.C) Benth.	leaves
10.98	267, 338	*Neptunia oleracea* Lour	twig
11.32	254, 263sh, 347	*Pithecellobium dulce* Benth.	pericarp
11.33	256, 344	*Pithecellobium dulce* Benth.	leaves
11.4	264, 346	*Acacia farnesiana* (L.) Willd.	twig
11.46	253,264sh,348	*Leucaena glauca* Benth.	twig
11.67	222,267,331sh	*Albizia lebbeck* (L.) Benth.	leaves
11.96	269sh,342	*Acacia farnesiana* (L.) Willd.	twig
11.96	269sh,342	*Albizzia myriophylla* Benth.	leaves
12.1	267,335	*Albizia lebbeck* (L.) Benth.	leaves

12.16	268,348	*Mimosa pudica* L.	twig
12.17	263sh,343	*Neptunia oleracea* Lour	twig
12.58	263,342	*Pithecellobium dulce* Benth.	leaves
12.94	222, 329	*Adenanthera microsperma* Teijsm.	branch

Flavonoids occur very commonly in plants and widely distributed in foods. It has the basic 15-carbon atoms in their basic nucleus and is arranging in a C_6-C_3-C_6 configuration that is two aromatic rings linked by a three carbon (C_3) which form a third ring. The rings are labelled as A, B and C (figure 17).

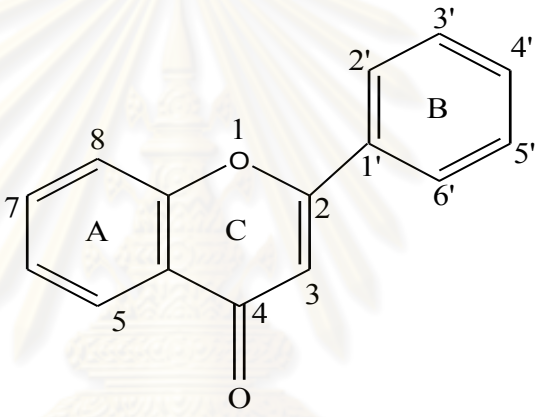


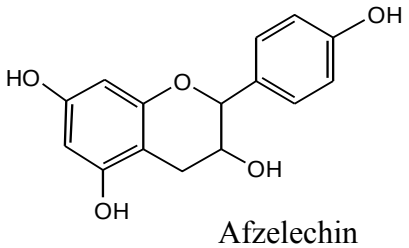
Figure 17 Basic structure of flavonoids [175]

Flavonoids are classified into flavonol, flavone, flavanol, flavanone based on the variation in the number and the arrangement of the hydroxyl group. The hydroxyl group or presence of substituted group at the C_3 of ring C in the flavonoids distinguishes the classes and also conferring the biological activity of particular flavonoids. Further modification of flavonoids resulted to the hydroxylation, methylation and glycosylation of hydroxyl group of the flavonoid nucleus [176].

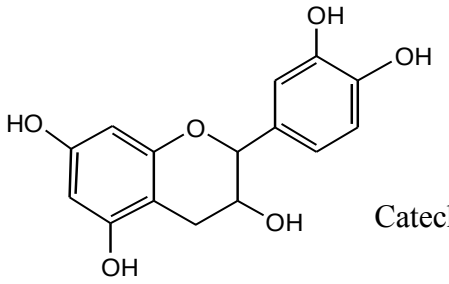
Flavonoids occur as aglycones; which are the flavonoids with no sugars attached, flavonoid *O*-glycoside, flavonoid *C*-glycoside, and methylated flavonoids. Commonly flavonoids present as flavonoid *O*-glycoside; where hydroxyl groups of the flavonoids are substituted with sugars. The formation of glycosidic bond occurs between the phenolic hydroxyl of the phenolic compound and an OH group of the sugar molecule. The

glycosidic link formed at certain favored positions at position 3, 5 and 7. Glucose is the most common sugar found but other sugars as galactose, rhamnose, xylose, and arabinose are also encountered [175].

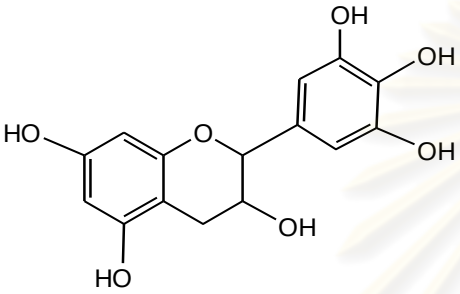
Tannins are natural polyphenol which have been gaining attention due to their astringency caused by their ability to bind protein and metal. Furthermore the beneficial health effects of plants rich tannin has been ascribed frequently. Correspondingly, various methods have been developed to detect the compounds from food, beverages and plant extracts. Tannins can be divided into condensed (flavan-3-ol as monomer) or proanthocyanidin and hydrolyzed (gallic acid or ellagic acid as the building block). Two asymmetric carbon atoms (C_2 and C_3) of flavan-3-ol give 4 optical isomers. For instance, catechin can exist as; (+) catechin, (-)catechin, (+) epicatechin, and (-)epicatechin. The flavan-3-ol can also be linked to gallic acid (catechingallate or gallocatechingallate). The monomers when a linkage is existed at C_4-C_8 or C_4-C_6 it is called B-type and if additional ether linkage is present between C_2 and C_7 it is called A-type (figure 18).



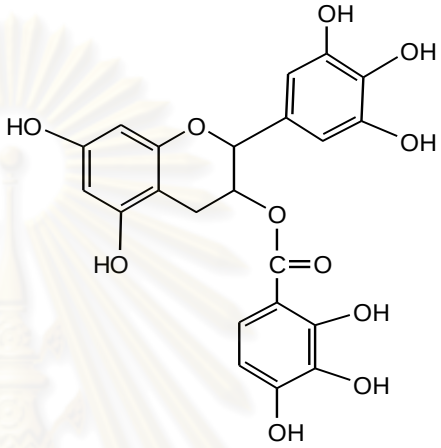
Afzelechin



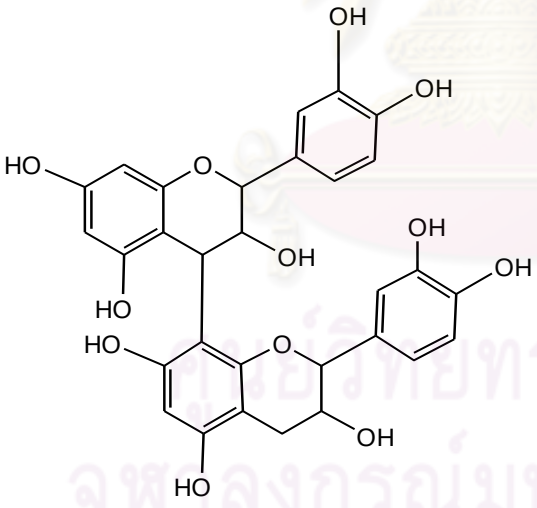
Catechin



Gallocatechin



Gallocatechin-gallate



Catechin-catechin linked via C_4-C_8

Figure 18 Examples of the monomers and proanthocyanidin compound

Comparing with retention time and maximum absorption of standard gallic acid and epicatechin, none of the peaks obtained from Mimosaceous plants extracts consisted of gallic acid or epicatcehin. However, based on the maximum absorption, the peaks obtained could be the derivatives of epicatechin, which due to bigger size of molecule; the peaks appeared later than the standard epicatechin which also could be the condensed tannin. The absence of hydrolysable tannins may be due to the woody material studied [172].

HPLC coupled to an absorbance monitor as photodiode array detector (PDA) have been a very useful tool to characterize an extract. However, distinguishing between compounds with similar absorbance spectra was not affordable. HPLC linked to a mass spectrometer has been used to distinguish the compounds on the basis of their mass spectra.

Identification of polyphenols by ESI-MS

Electrospray ionization (ESI) is a soft ionization technique that gives molecular ion peaks, making it useful for molecular weight determination. In the negative ESI-MS, the identification of the aglycone moiety is done by the formation of radical aglycone $[Y_0\text{-}H]^{-\bullet}$ and aglycone fragment Y_0^-. Several pathways of fragmentation have been described. Frequently, in negative mode ionization the fragmentation is initiated by water loss following retro-diels-alder (RDA) fission, heterocyclic ring fission (HRF), benzofuran-forming fission (BFF) and quinine methide (QM) fission.

The tentative identification of selected prominent peaks from HPLC-PDA by ESI-MS was based on the chromatographic behaviour, retention time (RT), deprotonated ion $[M\text{-}H]^-$, MS fragments and neutral loss, whereas where necessary, analysis by MS/MS was carried out. The type of attached glycosides was identified according to the lossess of hexose, rhamnose, glucuronide and pentose moieties (162, 146, 176, 132 amu from the spectrum, respectively). Compounds showing the same molecular ion and MS fragment but eluting (earlier or later) were assumed that the sugar group was linked to a different position but the actual position could not be determined [177].

The characterizations were also aided by the following elution order (figure 19)

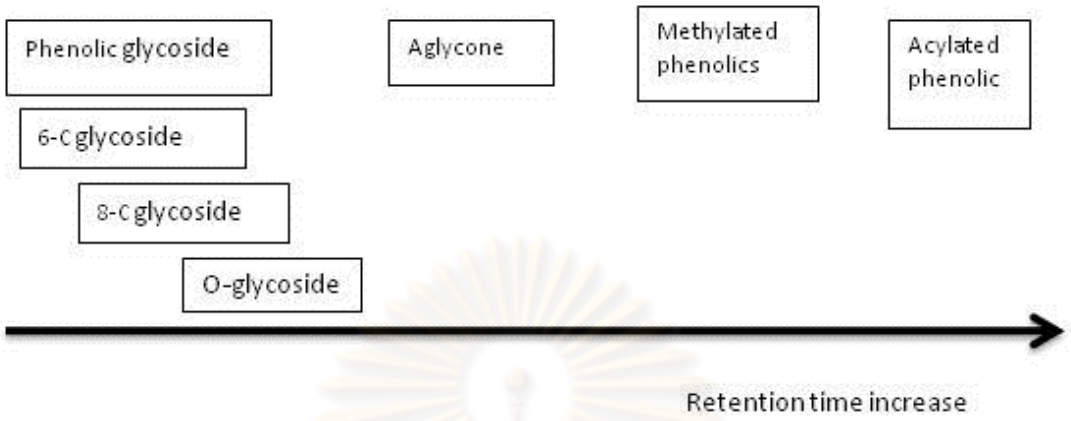


Figure 19 Elution order of phenolic compounds in HPLC

From the peaks obtained in the HPLC, flavonoids and tannin were putatively presence in the ethnolic extracts of Mimosaceous plants. However, with a scanning range between *m/z* 150-1000, only a total of 21 compounds consisted of flavonoids glycosides and proanthocyanidins were tentatively identified from the selected extracts (table 17), whereas the remaining peaks were unable to be characterized.

Table 17 Flavonoids and proanthocyanidins from selected Mimosaceous plants ethanolic extract.

No	Tentatively identified Compound	RT (min)	Species	Plant part	$[M\ H]^-$
1	Quercetin-3-*O*-hexoside [178]	12.95	*Pithecellobium dulce* Benth	pericarp	463
2	Quercetin-3-*O*-pentoside [178]	13.92	*Acacia farnesiana* (L.) Willd.	twig	433
		13.94	*Leucaena glauca* Benth	twig	433
		14.04	*Mimosa pudica* (L.)	twig	433
3	Quercetin-3-*O*-rhamnoside [178]	14.34	*Acacia catechu* (L.f.) Willd	leaves	447
		14.35	*Acacia farnesiana* (L.) Willd.	twig	447
		14.34	*Albizia lebbeckoides* (DC.) Benth.	leaves	447
		14.28	*Leucaena glauca* Benth.	twig	447
		14.38	*Neptunia oleracea* Lour	twig	447
		14.4	*Pithecellobium dulce* Benth.	leaves	447
		14.4	*Pithecellobium dulce* Benth.	pericarp	447
		14.38	*Xylia xylocarpa* (Roxb.) Taub	leaves	447
4	Quercetin-3-*O*-hexose-gallate [144]	11.73	*Acacia catechu* (L.f.) Willd	leaves	615
		11.77	*Acacia farnesiana* (L.) Willd.	twig	615
5	Rutin [179]	12.71	*Albizia lebbeck* (L.)	branch	609

			Benth	bark	
		12.7	*Albizia lebbeck* (L.) Benth.	leaves	609
		12.7	*Mimosa pudica* (L.)	twig	609
		12.65	*Neptunia oleracea* Lour	twig	609
6	Kaempferol 7-*O* rutinoside [144]	12.86	*Adenanthera pavonina* L.	leaves	593
		12.94	*Albizia lebbeckoides* (DC.) Benth.	leaves	593
7	Myricetin-3-*O*-hexoside [144]	10.91	*Cathormion umbellatum* (Vahl) Kosterm.	leaves	479
8	Myricetin-3-*O*-rhamnoside [144]	12.31	*Cathormion umbellatum* (Vahl) Kosterm.	leaves	463
		12.32	*Leucaena glauca* Benth.	twig	463
		12.37	*Xylia xylocarpa* (Roxb.) Taub	leaves	463
9	Myricetin-3-*O*-deoxyhexose-gallate [178]	15.58	*Cathormion umbellatum* (Vahl) Kosterm.	leaves	615
10	Luteolin-7-*O*-glucoside [180]	14.85	*Albizia lebbeck* (L.) Benth.	leaves	447
11	Luteolin methylated glycoside [178]	15.99	*Pithecellobium dulce* Benth.	leaves	477
		16.04	*Pithecellobium dulce* Benth.	pericarp	477
12	6-C-pentosyl-8-C-hexosyl apigenin [181]	11.45	*Acacia rugata* Merr	leaves	563
		11.46	*Acacia pennata* (L.)	twig	563

			Willd.		
		11.27	*Adenanthera pavonina* L.	pericarp	563
		11.31	*Neptunia oleracea* Lour	twig	563
13	Vitexin [182]	12.62	*Adenanthera pavonina* L.	pericarp	431
14	Vitexin-5-*O*-pentoside [182]	12.73	*Acacia pennata* (L.) Willd.	twig	563
15	Vitexin-5-*O*-rhamnoside [182]	12.8	*Acacia rugata* Merr.	leaves	577
		12.61	*Albizzia myriophylla* Benth	leaves	577
16	2"-*O*-rhamnosyl-isoorientin [181]	10.13	*Acacia pennata* (L.) Willd.	twig	593
		10.22	*Acacia rugata* Merr	leaves	593
		10.06	*Adenanthera pavonina* L.	pericarp	593
		10.21	*Albizia lebbeck* (L.) Benth.	leaves	593
		10.18	*Neptunia oleracea* Lour	twig	593
17	gallocatechin derivatives [183, 126]	13.83	*Xylia xylocarpa* (Roxb.) Taub	leaves	545
18	dimer epigallocatechin derivatives [183, 126]	11.8	*Xylia xylocarpa* (Roxb.) Taub	leaves	561
19	trimer epigallocatechin derivatives [183, 126]	13.7	*Xylia xylocarpa* (Roxb.) Taub	leaves	833
20	dimer afzelechin - epicatechin [183, 126]	10.07	*Xylia xylocarpa* (Roxb.) Taub	stem	561
		10.01	*Samanea saman* (Jacq.) Merr.	leaves	561

21	trimer catechin derivatives [126]	14.16	*Pithecellobium dulce* Benth	stem bark	833
		14.41	*Xylia xylocarpa* (Roxb.) Taub	stem	833
		14.4	*Samanea saman* (Jacq.) Merr.	leaves	833

Characterization of flavonoid-glycosides

Initially, flavonoid compounds of both flavonol and flavone glycoside were detected from the λ_{max} values obtained by the HPLC-PDA. From the data obtained by ESI/MS, the glysosylated flavonol were consisted of aglycones quercetin, kaempferol and myricetin, which were identified from the fragmentation of the deprotonated ion, $[M-H]^-$ to ion at *m/z* 300, 285 and 316, respectively. The $[M-H]^-$ ion and fragmented ions of apigenin and luteolin derivatives were characterized based on the literatures.

The deprotonated ion, $[M-H]^-$ at *m/z* 463, 433 and 447 fragmented to produce ion at m/z 300 that corresponded to standard quercetin. The enlargement of the signals at *m/z* 867 and 895 (figure 20 and 21) showed that the distance of isotopic peaks was one mass unit demonstrating that those ions were $[2M-H]^-$, which frequently detected in the negative ion electrospray ionization. The neutral loss of 162,132 and 146 from each $[M-H]^-$ ion, respectively, were demonstrating a presence of hexose, pentoside and deoyhexoside. Successively, the $[M-H]^-$ ions were tentatively identified as the deprotonated ion of compounds quercetin-3-*O*-hexoside, quercetin-3-*O*-pentoside, and quercetin-3-*O*-rhamnoside, respectively, according to the literatures. Rutin in the extracts was identified according to the retention time and fragmented ion that matched with the standard rutin. Ion at *m/z* 615 was fragmented to ion at *m/z* 463 and 300 indicated neutral loss of galloyl group and hexoside, respectively (figure 22). The aglycone was identified as quercetin from ion at *m/z* 300 produced. Therefore, ion at *m/z* 615 was tentatively identified as quercetin-3-*O*-galloylglucoside and apparently the compounds of this type are quite sporadic in the flora [144].

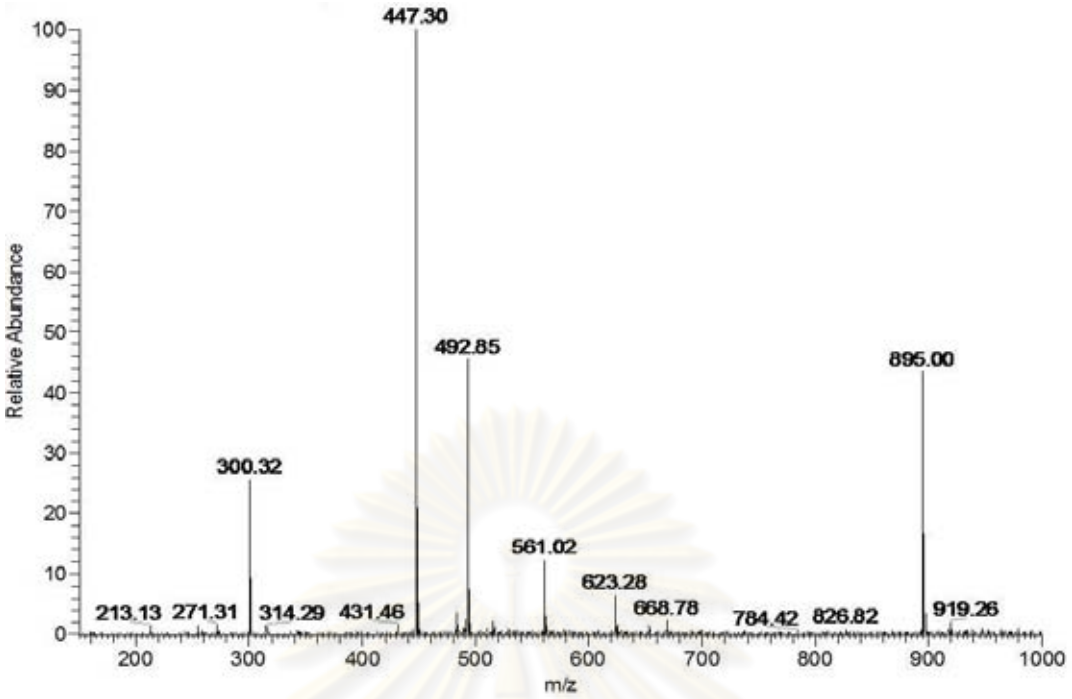


Figure 20 MS spectra of quercetin-3-*O*-rhamnoside from extract of *Albizia lebbeckoides* leaves

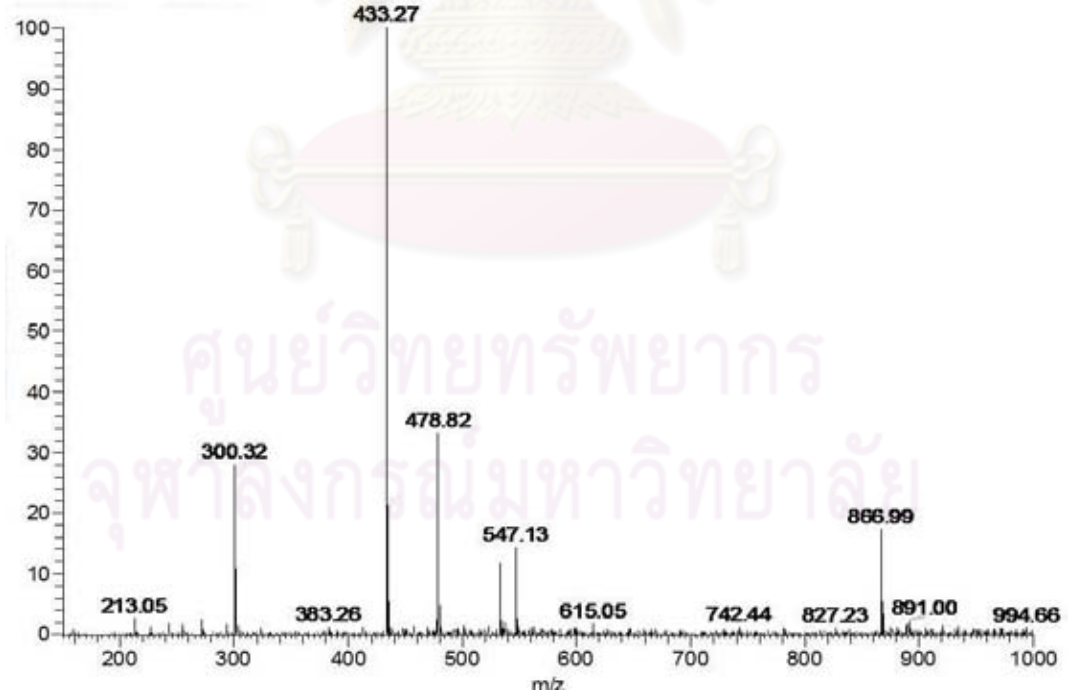


Figure 21 MS spectra of quercetin-3-*O*-pentoside from extract of *Leucaena glauca* twig

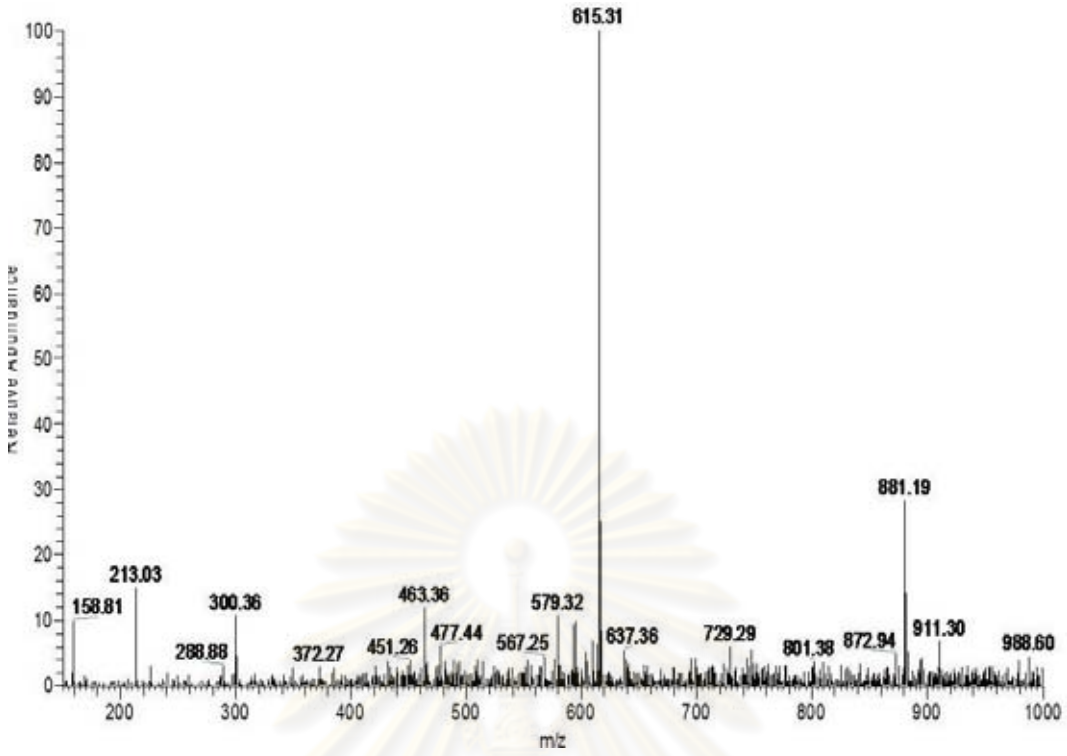


Figure 22 MS spectra of quercetin-3-*O*-galloylglucoside from extract of *Acacia farnesiana* twig

The deprotonated ion at m/z 593 was characterized as kaempferol-rutinoside based on the fragmented of the $[M-H]^-$ ion to ion at m/z 285 which indicated kaempferol, whereas the loss of in 308 corresponded to loss of rutinoside [144]. Thus, it was tentatively identified as kaempferol-7-*O*-rutinoside according to the literature [144].

Ion at *m/z* 447 fragmented to ion at 285 which could be the deprotonated luteolin or kaempferol (MW 286) along with the loss of 162 Da indicated the loss of hexose. However, ion at *m/z* 447 was tentatively identified as luteolin 7-*O*-glucoside based on the absence of ion at *m/z* 327 (figure 23). Supposedly, the ion at *m/z* 327 is common in all of *O*-7 glucoside. However, in the luteolin 7-O-glucoside, the absence of the ion was appeared to be caused by hydroxylation at the 3'-position of B ring [180].

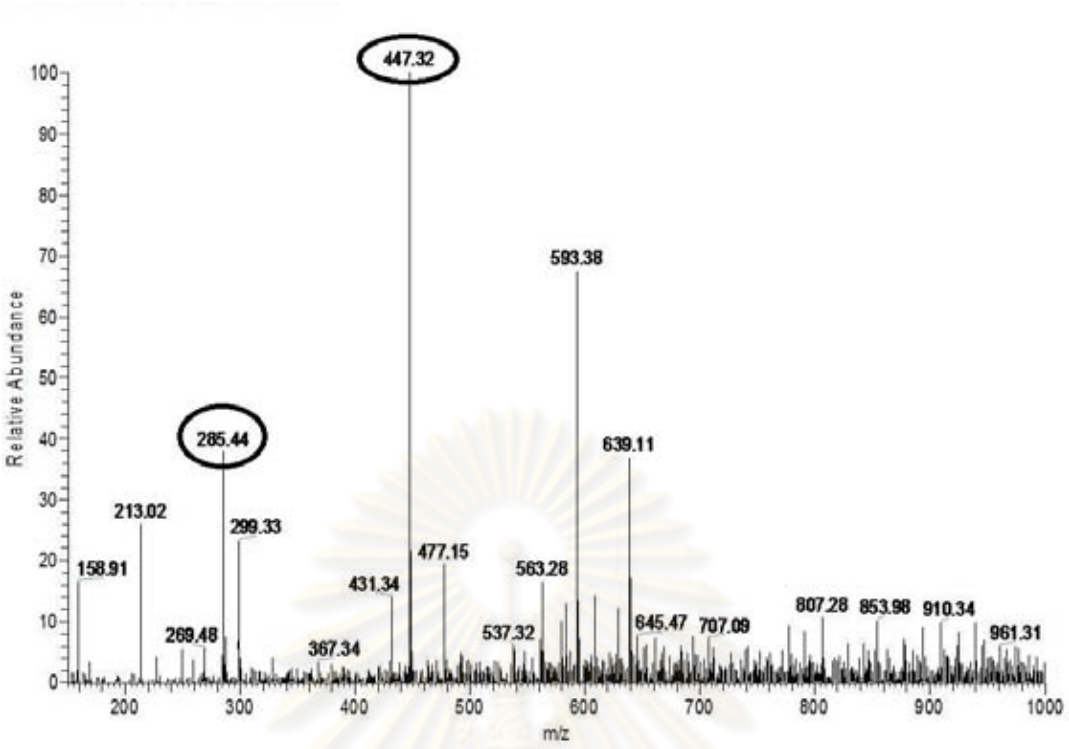


Figure 23 MS spectra of luteolin-7-*O*-glucoside from *Albizzia lebbeck* leaves extract

Characterization of tannins

Since mass spectrometry cannot distinguish between stereoisomers like (+)/(-) catechin/epicatechin or (+)/(-) gallocatechin the names catechin and gallocatechin for all isomers are used. Ion characteristic for catechin (trans) or epicatechin (cis) can be found at m/z 289 and 291 which could represent a mixture of isomers and could not be distinguished by ESI-MS spectra, thus their identities were confirmed by comparing the RT with standards catechin and epicatechin.

From the analysis by ESI-MS, ions at *m/z* 833 and 849 detected in both *X. xylocarpa* bark and leaves extracts were initially recognized as proanthocyanidin trimer. The ion at *m/z* 833 of *X. xylocarpa* bark fragmented to ion 561 and 289 which were similar with previous study [183, 126]. Thus, the ion at *m/z* 833 (figure 24) was characterized as proanthocyanidin with two epiafzelechin and one epicatechin subunits.

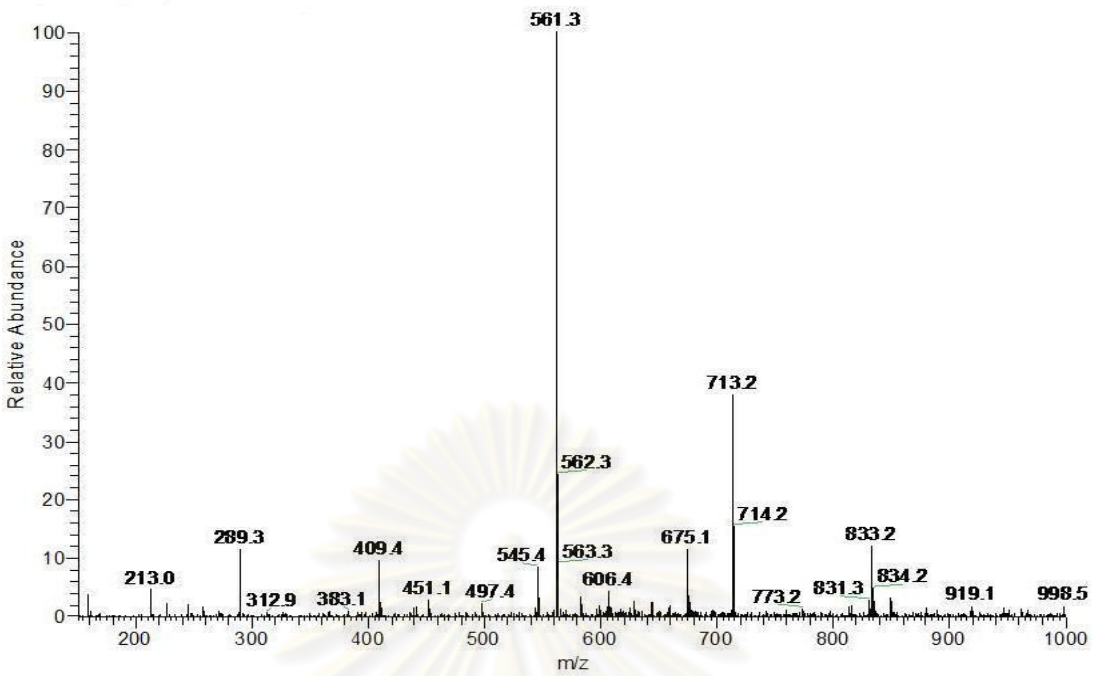


Figure 24 MS spectra of proanthocyanidin trimer from extract of *X. xylocarpa* bark

Ions at *m/z* 833 and 849 of *X. xylocarpa* leaves fragmented to ion at *m/z* 305, thus both ions (figure 25) were characterized as the derivatives of epigallocatechin; a trihydroxylated unit of condensed tannin [183, 126]. The ions at *m/z* 289 and 305 observed in both extracts were consistent with the standard epicatechin and epigallocatechin respectively.

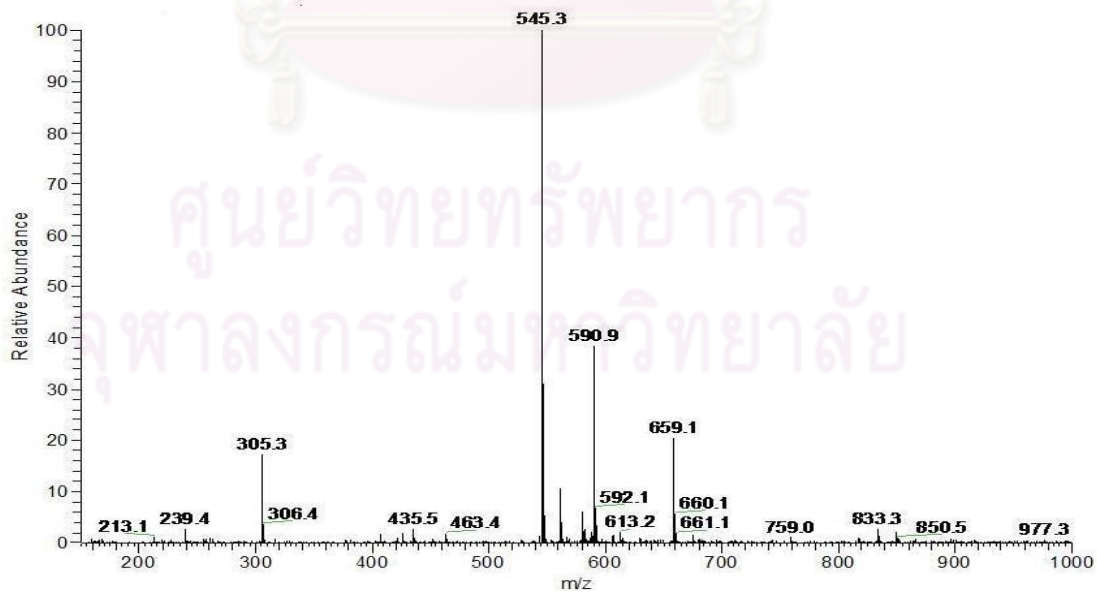


Figure 25 MS spectra of epigallocatechin derivatives from extract of *X. xylocarpa* leaves

Influences of phytochemical content on the biological activity of Mimosaceous plants ethanolic extracts

It was reported that the chemical structure of gallic acid derivatives and flavan-3-ol family as procyanidins type B exhibited good electron or hydrogen donating property in DPPH assay [184]. An increase in the number of hydroxyl group led to higher DPPH scavenging activity, specially three hydroxyl groups at B ring. The loss of one hydroxyl group decreased activity slightly but the loss of two hydroxyl groups significantly decrease the activity, whereas glycosylation resulted in the decrease in the DPPH radical scavenging activity [185]. The previous studies were in accordance with the highest DPPH activity of *X. xylocarpa* leaves that could be contributed by the presence of aglycone epicatechin and epigallocatechin derivatives in the extracts as detected by ESI-MS.

Polyphenols are also known for contributing to the electron donating property that reflected in the reducing capacity of an extract [186]. However, it was corroborated that an increase in the number of free hydroxyl group of phenolic compound led to higher activity whereas glycosylation resulting decrease in donating property of phenolic compounds [187]. Therefore, although the *X. xylocarpa* leaves exhibited the highest total phenolic content, the presence of glycosylated myricetin in the extract may have hindered the reducing capacity of the extract.

Analyses of the *L. glauca* twig extract that exhibited highest iron chelating activity resulted presence of quercetin-3-*O*-rhamnoside, quercetin-3-*O*-pentoside and myricetin-3-*O*-rhamnoside in the extract. Previously, flavonoids exhibited low chelating property due to the presence of 2-3 double bond and 4-keto group [188]. However, from the analysis of extract, quercetin glycosides were deduced contributed to the metal ion chelating property of *L. glauca* leaves extract. The results were supported by previous study which reported, both quercetin and rutin were highly effective chelators of transition metals [189].

The highest NO scavenging activity was showed by the extracts of *A. lebbeckoides* leaves and *P. dulce* bark. The extract of *P. dulce* bark was selected to be characterized from ESI-MS due to its higher total phenolic content. Proanthocyanidin trimer consisted of epicatechin and epiafzelechin units were detected in the *P. dulce*

bark extract. The contribution of such compounds to the NO scavenging property of *P. dulce* bark extract was previously observed in the high direct scavenging activity against NO generated from SNP of catechin and catechin derivatives exhibited by green tea tannins [190].

The tyrosinase inhibition property of flavonoids has been studied. Previously study on diphenolase inhibition of flavonols; kaempferol and quercetin showed that free hydroxyl group at the C_3 position was important in eliciting their tyrosinase inhibition activity. The glycosylation at C_3 did not show any inhibitory activity in L-DOPA oxidation. The ability to chelate the copper metal in tyrosinase enzyme has been suggested as another mechanism of tyrosinase inhibition. The tyrosinase inhibition of kojic acid was contributed by its ability to chelate copper in the enzyme, therefore structural similarity of flavonols and kojic acid suggested that flavonols also able to chelate copper in the tyrosinase enzyme as long as their 3-OH group was free [89]. Data of ESI-MS showed that myricetin-3-*O*-rhamnoside was characterized from the *Xylia xylocarpa* leaves extract, therefore the high tyrosinase inhibition activity of *X. xylocarpa* leaves extract could be contributed by epigallocatechin derivatives that were also presented in the extract.

Phenolics are known to act as antimicrobial by damaging cell walls and membranes and by precipitating essential cell proteins. The extract of *X. xylocarpa* leaves consisted of epicatechin derivatives as observed from the ESI-MS results, thus in accordance with the bactericidal capacity of catechin which has been related to the ability in perturbing the membrane structure from the bacterial-like model membrane [191]. The presence of free hydroxyl groups appears to be important for the flavonoids antimicrobial activity [192]. However, the extract of *Cathormion umbellatum* leaves that consisted of myricetin-3-*O*-hexoside and myricetin-3-*O*-rhamnoside was also exhibited good antibacterial activity. Therefore, the aglycone, type of substitute group and position of substitute groups also attributed to the effectiveness of flavonoids as antimicrobial agent [193].

Despite of being rich in flavonoids and tannins, other phytochemicals also contributed to the antimicrobial activity of extract [194]. The extract of *E. rheedii* seed coat exhibited good reducing activity but no prominent peaks of polyphenols were detected by HPLC-PDA and HPLC-ESI-MS, especially *E. rheedii* is known for its

saponin and has been used as detergent. The contribution of other compounds to the observed antioxidant and antimicrobial activity of Mimosaceous plants may also possible. Mimosaceous plants extracts were suspected to contain saponins but the selected chromatographic condition, type of ionization and MS scan range in this study were not suitable for the characterization of such compounds.

For instance, it was reported that ESI-MS spectra of $[M-H]^-$ ions of saponin provide structural information on the sequence of sugar chains with lower sensitivity than that in positive ion. This was because the negative mode involve deprotonated ion, which have less internal energy to produce fragments. Comparing to positive mode, saponin molecules combined with an alkali metal ion to generate new bond in an exothermic reaction. The energy released may excite more bond cleavage to fragment which is useful for the identification. There was also no cross ring ion detected at all which makes a tremendous difference between the negative and positive mode spectra. Positive mode provide type of fragments that gives the information on the primary sequence and branching in terms of classess of monosaccharide in the saponin chain, while the cross ring types of ion providing the information of linkages between sugar residues [195].

CONCLUSION AND RECOMMENDATION

The overwhelming previous studies on the *in vitro* biological activities of plant ethanolic extracts due to their phenolic compounds prompted this study. Despite the other studies carried out previously on the extract of Mimosaceous plants and phenolic compounds, this study provided information on bioactivities of the ethanolic extract evaluated from selected bioassays but also reports the phytochemical composition of the Mimosaceous ethanolic extract exclusively prepared by successive extraction using sohxlet apparatus. The ethanolic extracts prepared by successive extraction in soxhlet apparatus, was later found simplifying the analysis of extract by both HPLC-PDA and HPLC-ESI-MS when the clean up procedure was not necessary. Quercetin 3-*O*-rhamnoside was the most frequent compound detected. The compound was found in 8 of Mimosaceous plants ethanolic extracts. The extract of *X. xylocarpa* leaves was found to be rich with proanthocyanidin of epigallocatechin derivatives. The extract from *X. xylocarpa* leaves exhibited good DPPH scavenging, antimicrobial and tyrosinase inhibition activity which was likely to be contributed by the proanthocyanidins as reported by previously reported studies. Currently, *X. xylocarpa* is among dominant species in natural mixed deciduous forests of Thailand. The species has been most valued as part of future restoration strategies of native forest in northern Thailand [196]. The nutrient and chemical evaluation of raw seeds of *X. xylocarpa* as the cheapest and potential alternative protein source to the low income population was reported [197]. Although the decoction of flower and bark have been used in Thai traditional medicine as antipyretic [26], this study reported for the first time the *in vitro* biological activities of the leaves and bark extract of the plant. Therefore, the activities reported may add value to the *X. xylocarpa* and provide guidance for a further research on this species. Twenty species of the Mimosaceous plants selected in this study were consisted of not only those which have been known well in Thailand as both edible and medicinal plants but also those which have not been studied previously. The ethanolic extracts of *Pithecellobium dulce* stem bark, *Archidendron jiringa* pericarp, *Entada rheedii* seed coat, *Leucaena glauca* twig, leaves of *Samanea saman*, *Cathormion umbellatum*, *Albizzia lebbeck* and *Albizzia lebbeckoides* were the extracts with potential biological activities observed in this study. The selected assays may not able to reflect the effects of the extracts *in vivo* because all the reactions were generated in microplate. Nevertheless, the assays

selected from the renowned reported studies were appropriate and have been carried out frequently as preliminary or complementary assays. In addition, all determinations were carried out in 96 well microplate which provided rapid bioactivities determination of the extracts. The plant materials were collected from botanical garden or bought from local markets. Although the variation in locality and geographical have influenced to the phytochemical production of plants, but it was reported that plants produce similar type of constituents regardless of their localities. For example, two populations *Agauria salicifolia* revealed similar flavonoid profile but the difference between the populations was showed by the concentration of each flavonoid compound [77]. Generally, the results of this study are hoped able to raise the appreciation to the species of the Mimosoideae subfamily which currently undervalued for their unknown biological properties, supporting their reputation as Thai traditional medicine plants, thus encouraging further exploration. However, to truly investigate the ethanolic Mimosaceous plant extracts, some details in this study should be approved. It was found that most of the chromatograms presented were not separated at the baseline. Although the extracts have been simplified by sequence of extraction solvents, the extracts were still consisted of the mixture of compounds with similar polarity that was likely to co-elute. Optimization of mobile phase is necessary for a separation of compounds in the HPLC that consequently allow more accurate characterization of the compounds present in the extract. HPLC-ESI-MS also was not able to provide precise molecular structures of the phenolic compound in the extracts especially without standard compounds. Lack of details on the C_4-C_8 or C_4-C_6 position of the interflavan linkage and position of glycosidic group were unresolved since the experimental conditions did not allow the elucidation of such features. ESI-MS cannot distinguish the isomer, for example catechin from epicatechin because of the same retention time and fragmentation ions. Other than the λ_{max} values**,** fragmentation ions and neutral loss, tentative characterization was based on the previously reported literatures on the compounds that are common. Regardless of the absence details, results from this study were sufficient to report the *in vitro* bioactivities and characterization of prominent constituents available in the ethanolic extracts of Mimosaceous plants.

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APPENDIX

APPENDIX

Results of DPPH radical scavenging

Species	**Plant material**	**DPPH IC_{50} (mg/ml)**
Xylia xylocarpa (Roxb.) Taub	leaves	0.01 ± 0.0001
Entada rheedii Spreng	seed coat	0.011 ± 0.0006
Xylia xylocarpa (Roxb.) Taub	bark	0.016 ± 0.0016
Archidendron jiringa I.C Nielsen	pericarp	0.017± 0.002
Cathormion umbellatum (Vahl)	leaves	0.018± 0.003
Cathormion umbellatum (Vahl)	bark	0.019± 0.002
Pithecellobium dulce Benth	bark	0.025± 0.006
Acacia catechu (L.f.) Willd	leaves	0.028 ± 0.001
Parkia speciosa Hassk.	pericarp	0.029± 0.0004
Albizia procera (Roxb)	stem bark	0.031 ± 0.001
Xylia xylocarpa (Roxb.) Taub	stem	0.034± 0.0013
Acacia farnesiana (Linn) Welld.	twig	0.055 ± 0.004
Samanea saman (Jacq.) Merr.	leaves	0.100±0.0018
Albizia lebbeckoides (D.C)	leaves	0.103 ± 0.001
Leucaena glauca Benth	twig	0.114 ± 0.002
Pithecellobium dulce Benth	leaves	0.114 ± 0.006
Adenanthera pavonina L.	branch	0.132± 0.004
Adenanthera pavonina	seed coat	0.136 ± 0.004
Mimosa pudica (Linn)	twig	0.146± 0.004
Albizzia myriophylla Benth	leaves	0.161 ± 0.014
Leucaena glauca Benth	pericarp	0.221± 0.007
Samanea saman (Jacq.) Merr.	branch	0.343 ± 0.031

Pithecellobium dulce Benth	pericarp	0.345 ± 0.035
Neptunia oleracea Lour	twig	0.345± 0.01
Entada rheedii Spreng	cotyledon	0.367 ± 0.015
Adenanthera pavonina Linn.	pericarp	0.387 ± 0.011
Archidendron jiringa I.C Nielsen	seed	0.449 ± 0.013
Acacia pennata (L.) Willd.	leaves	0.812 ± 0.053
Albizia lebbeck (L.) Benth.	leaves	0.834 ± 0.01
Cathormion umbellatum (Vahl)	branch	1.056 ± 0.021
Adenanthera pavonina L.	leaves	1.556± 0.034
Acacia rugata Merr.	twig	3.036± 0.010
Adenanthera microsperma Teijsm.	leaves	3.820 ± 0.625
Acacia rugata Merr	pericarp	3.850 ± 0.516
Parkia speciosa Hassk.	seed	5.153 ± 0.560

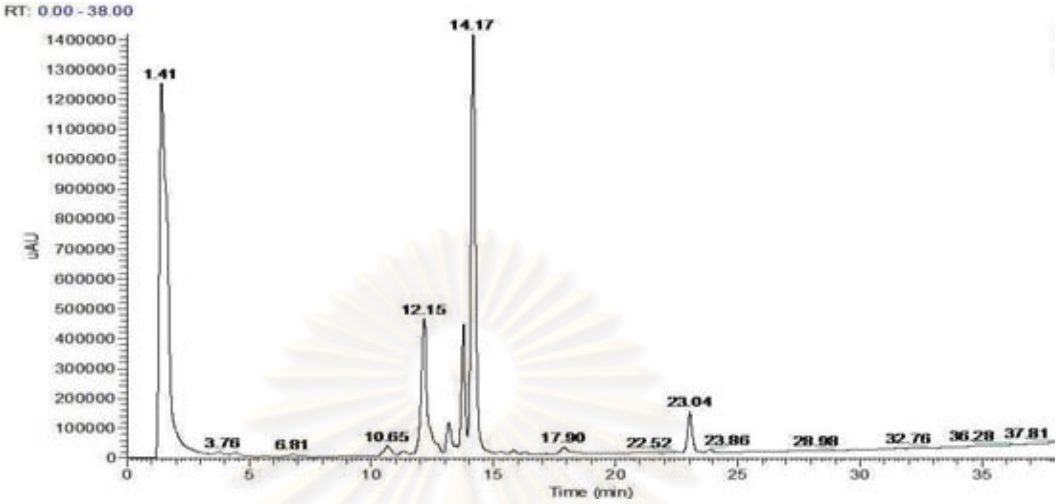
Results of reducing power assay

Species	**Plant material**	**Reducing power (IC_{50} ug/ml)**
Cathormion umbellatum (Vahl)	leaves	12.00±1.00
Entada rheedii Spreng	seed coat	13.47±3.93
Parkia speciosa Hassk.	pericarp	14.00± 3.62
Acacia catechu (L.f.) Willd	leaves	15.67± 4.93
Xylia xylocarpa (Roxb.) Taub	stem	20.9 ±1.73
Archidendron jiringa I.C Nielsen	pericarp	21.52±1.64
Xylia xylocarpa (Roxb.) Taub	bark	22.73±0.89
Xylia xylocarpa (Roxb.) Taub	leaves	27.55±7.02
Albizia procera (Roxb)	stem bark	31.51±3.96
Cathormion umbellatum (Vahl)	bark	37.80±0.80
Pithecellobium dulce Benth	bark	37.90±3.47
Acacia farnesana (Linn) Welld.	twig	43.39 ± 3.92
Samanea saman (Jacq.) Merr.	leaves	59.85±2.61
Leucaena glauca Benth	pericarp	57.68 ± 5.96
Pithecellobium dulce Benth	pericarp	61.88±5.44
Leucaena glauca Benth	twig	64.94± 2.60
Pithecellobium dulce Benth	leaves	65.98± 4.64
Albizia lebbeckoides (D.C)	leaves	82.00± 19.08
Mimosa pudica (Linn)	twig	104.17±10.82
Adenanthera pavonina Linn.	pericarp	104.26± 11.86
Adenanthera pavonina L.	branch	120.34± 3.17
Adenanthera pavonina	seed coat	134.80± 14.74
Albizzia myriophylla Benth	leaves	135.94±14.13

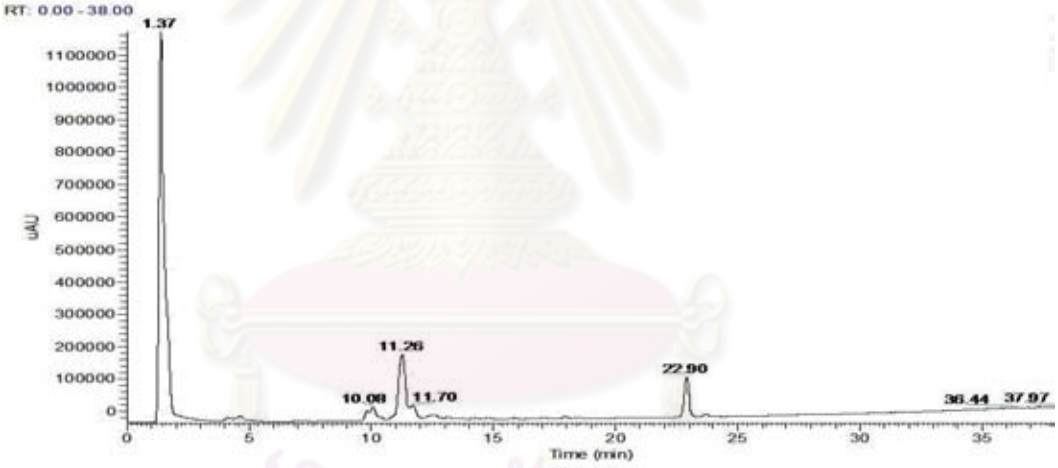
Samanea saman (Jacq.) Merr.	branch	158.53± 14.82
Acacia pennata (L.) Willd.	leaves	190.34 ± 11.98
Neptunia oleracea Lour	twig	196.30± 10.86
Entada rheedii Spreng	cotyledon	207.38±8.94
Adenanthera pavonina L.	leaves	303.38± 4.03
Archidendron jiringa I.C Nielsen	seed	376.79± 4.49
Albizia lebbeck (L.) Benth.	leaves	384.54± 37.99
Cathormion umbellatum (Vahl)	branch	460.43± 73.89
Parkia speciosa Hassk.	seed	529.97± 38.12
Acacia rugata Merr	pericarp	621.13 ± 5.95
Adenanthera microsperma Teijsm.	leaves	854.35± 60.13
Acacia rugata Merr.	twig	944.94± 84.69

HPLC-PDA chromatogram of selected Mimosaceous plants ethanolic extract. (Values on the chromatogram indicated the retention time (min) of each peak)

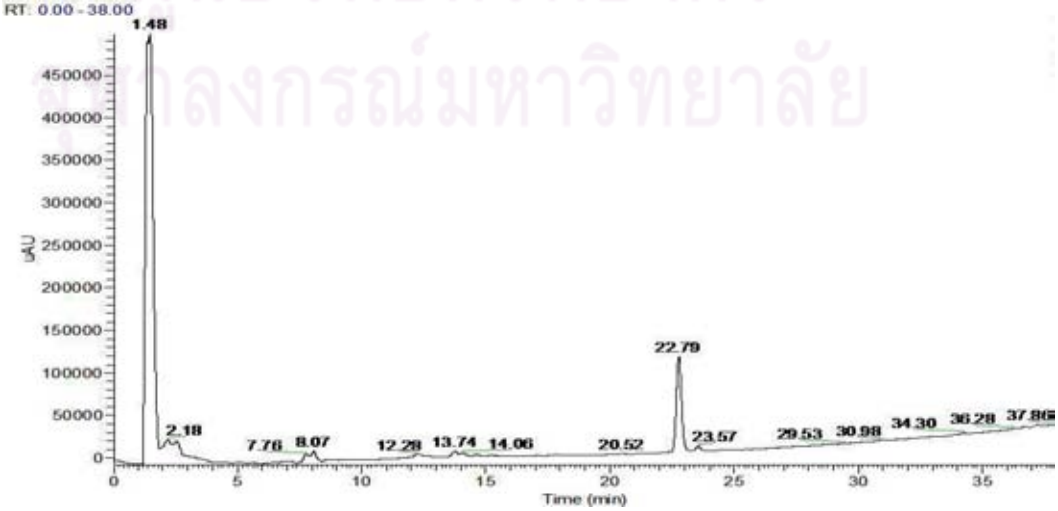
1. *Leucaena glauca* twig



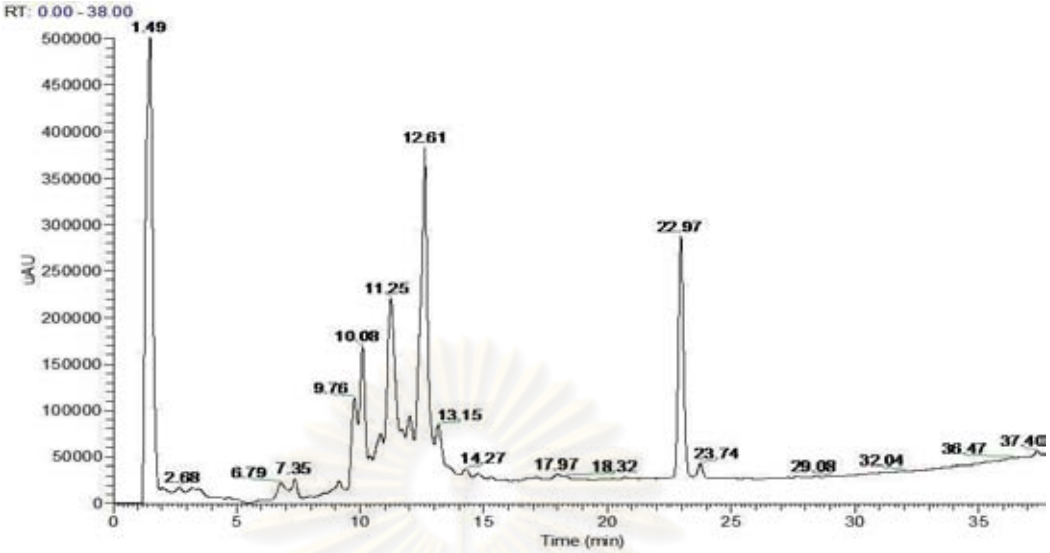
2. *Acacia pennata* twig



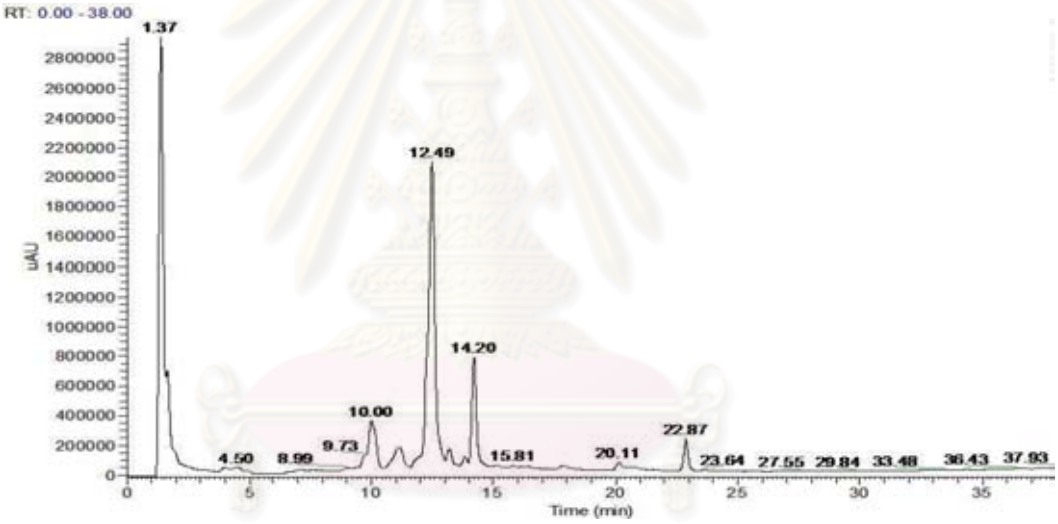
3. *Parkia speciosa* seed



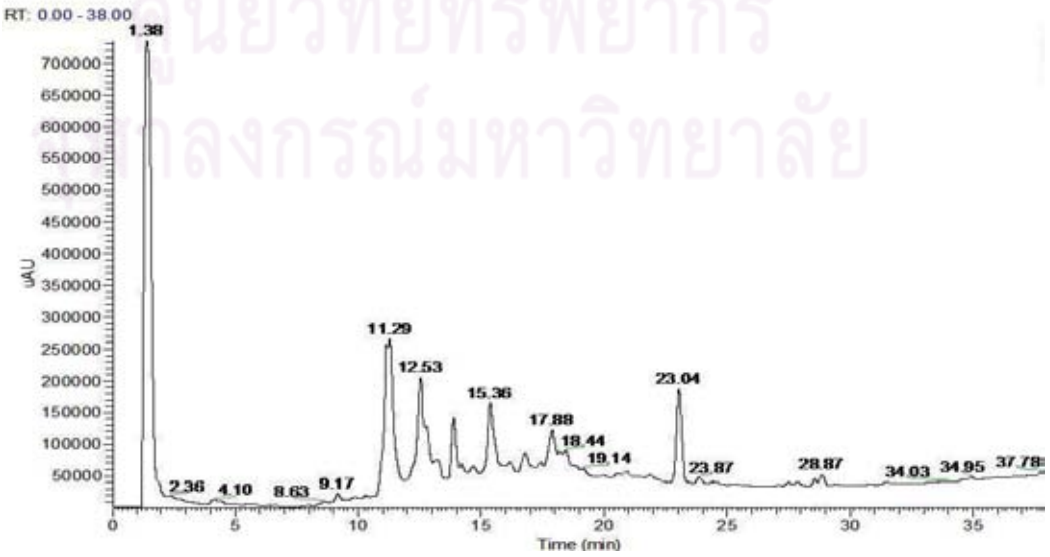
4. *Acacia rugata* leaves



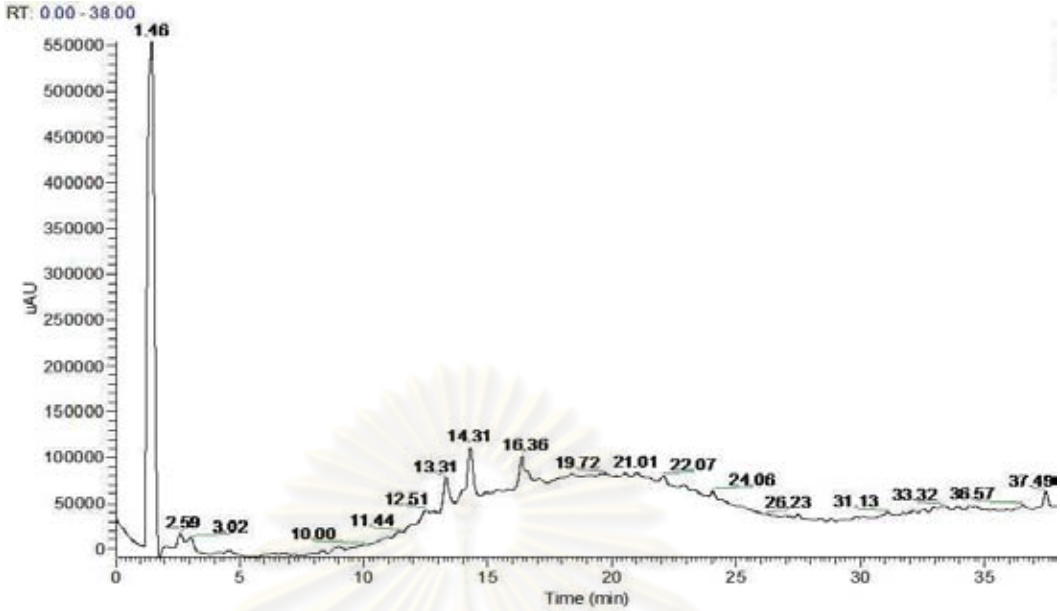
5. *Neptunia oleracea* twig



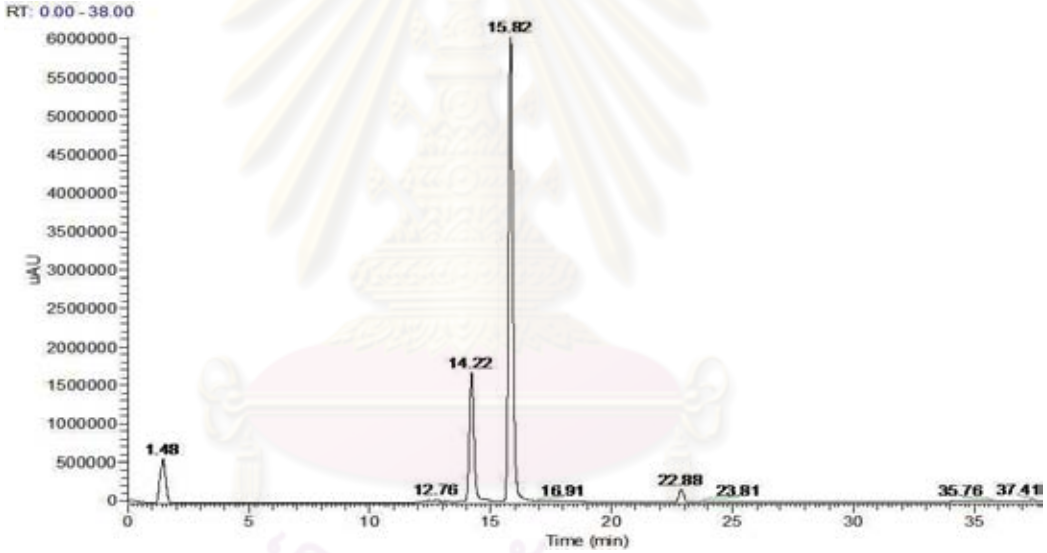
6. *Mimosa pudica* twig



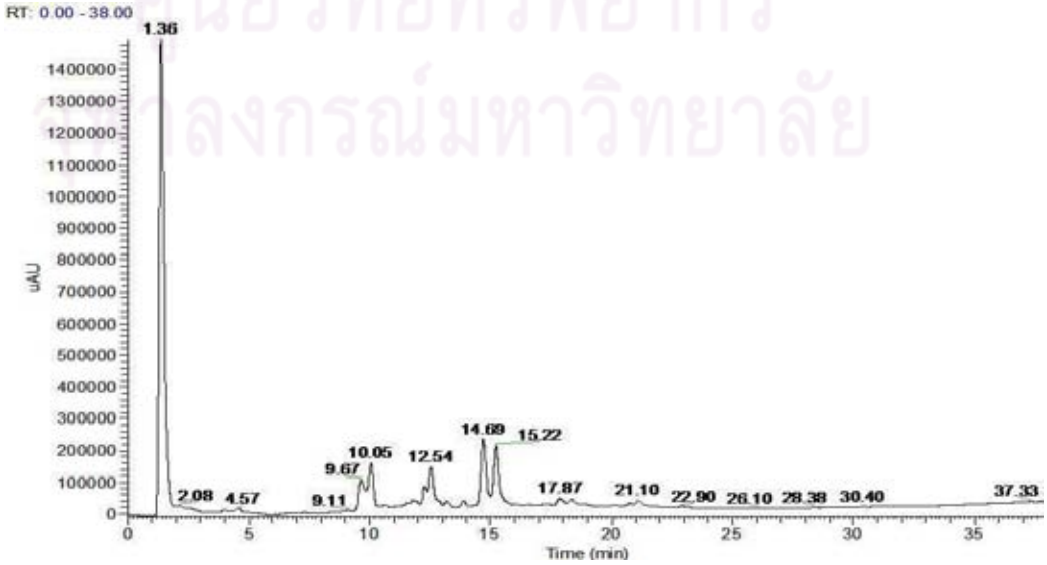
7. *Pithecellobium dulce* stem bark



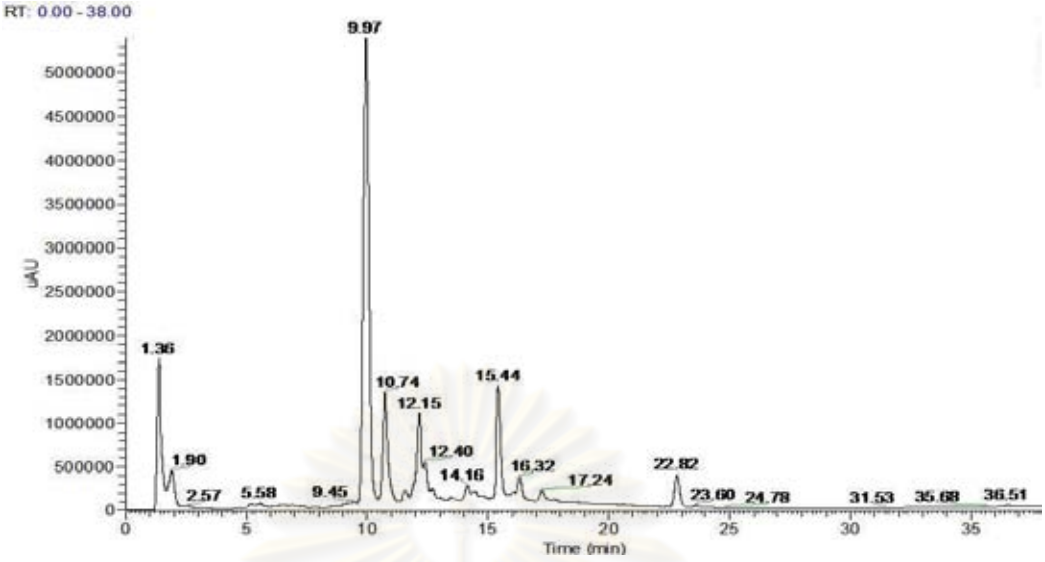
8. *Pithecellobium dulce* leaves



9. *Albizia lebbeck* leaves

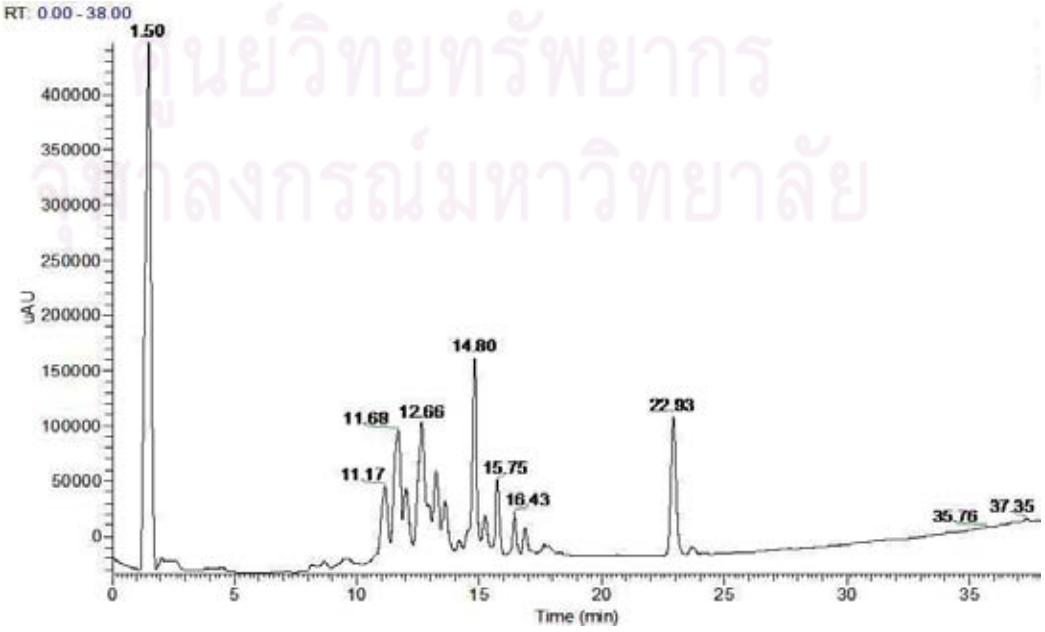


10. *Cathormion umbellatum* leaves

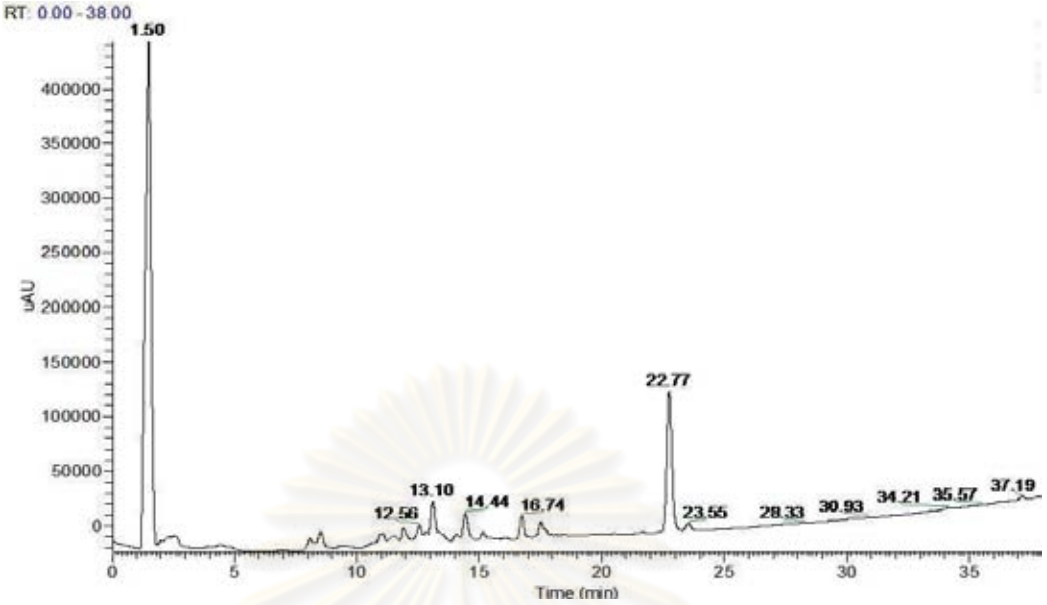


11. *Cathormion umbellatum* bark

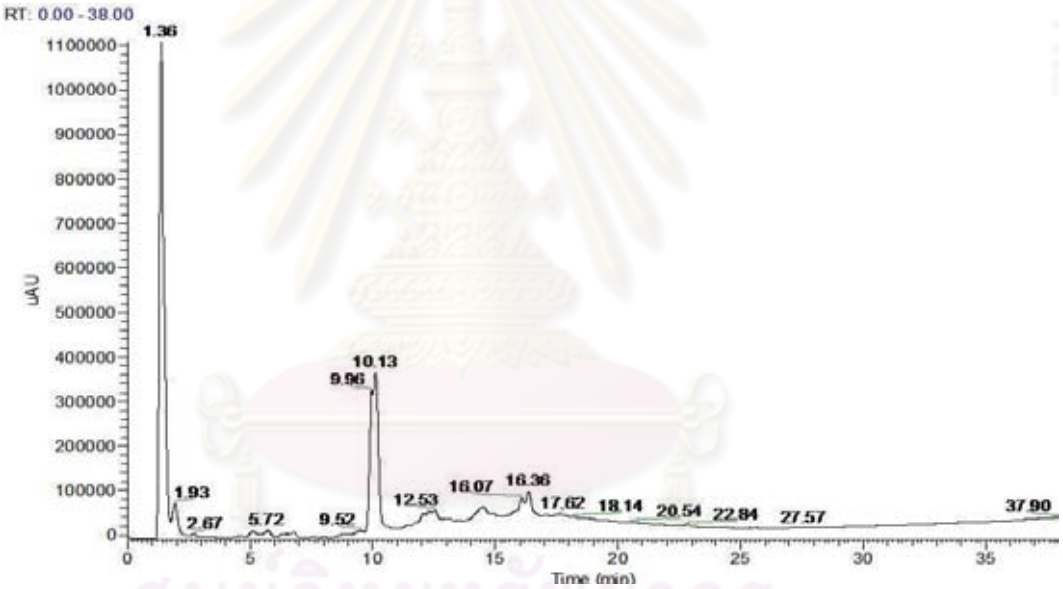
12. *Adenanthera pavonina* leaves



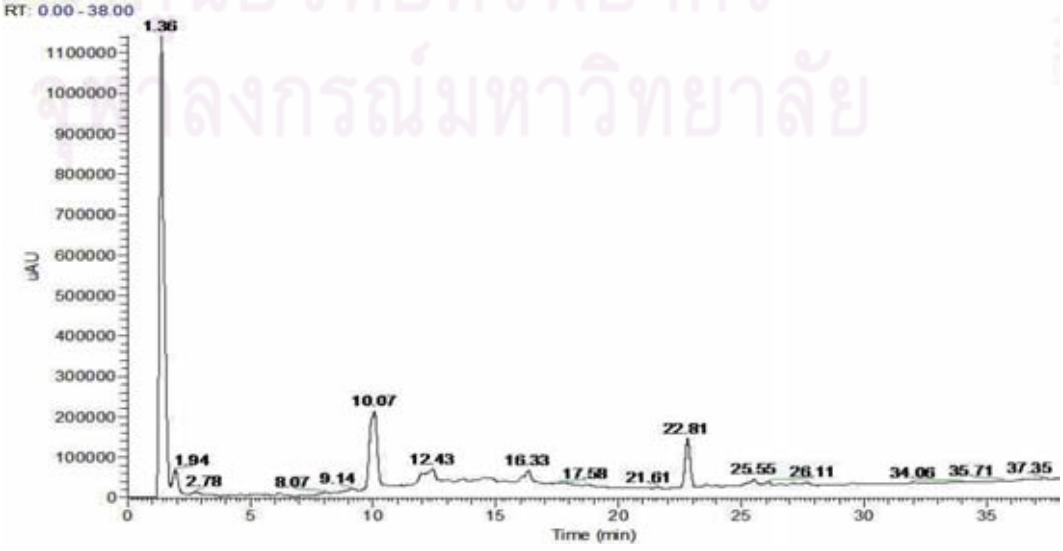
13. *Adenanthera microsperma* leaves



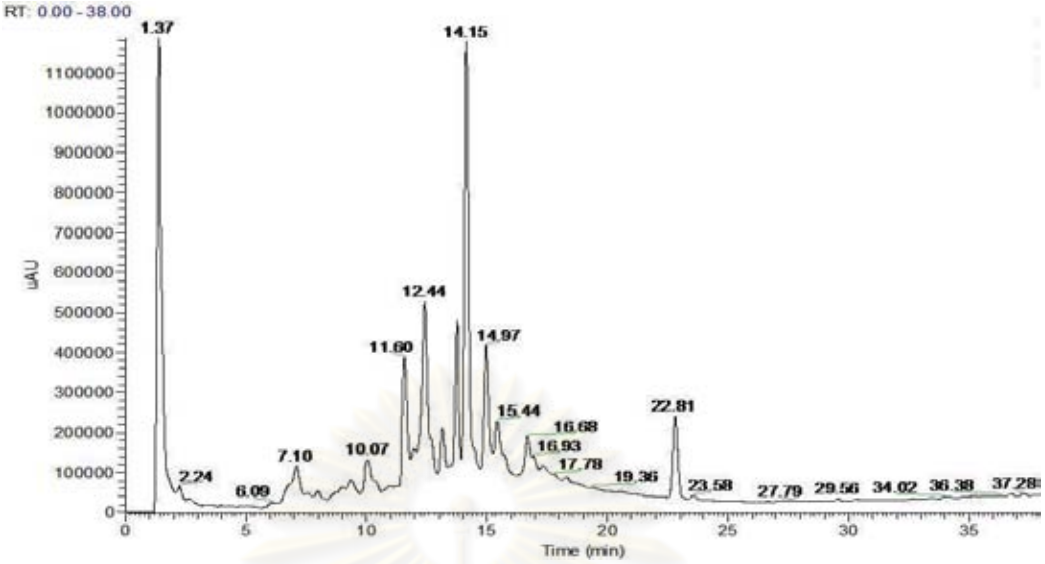
14. *Albizia lebbeck* branch bark



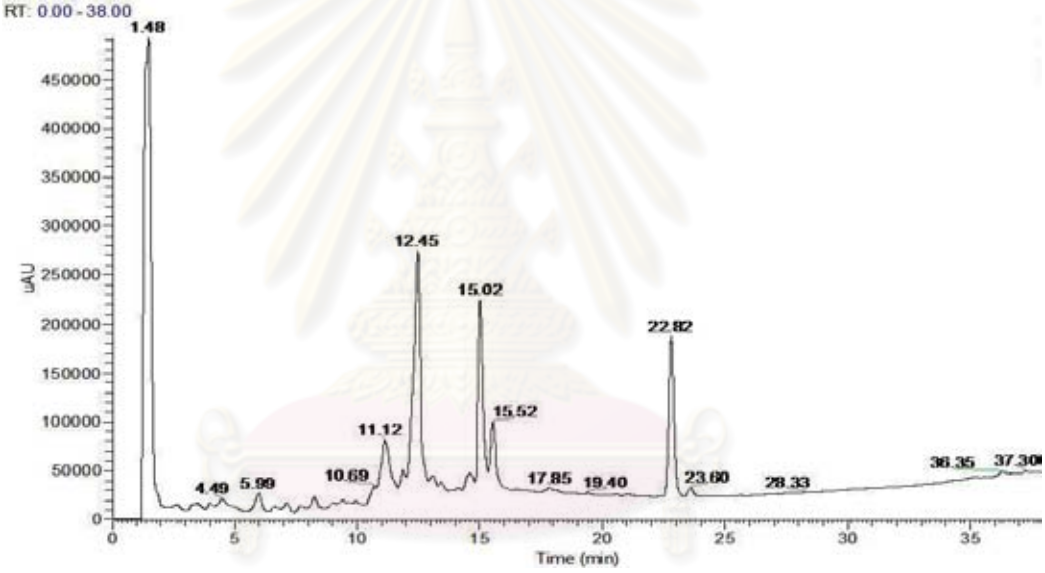
15. *Archidendron jiringa* pericarp



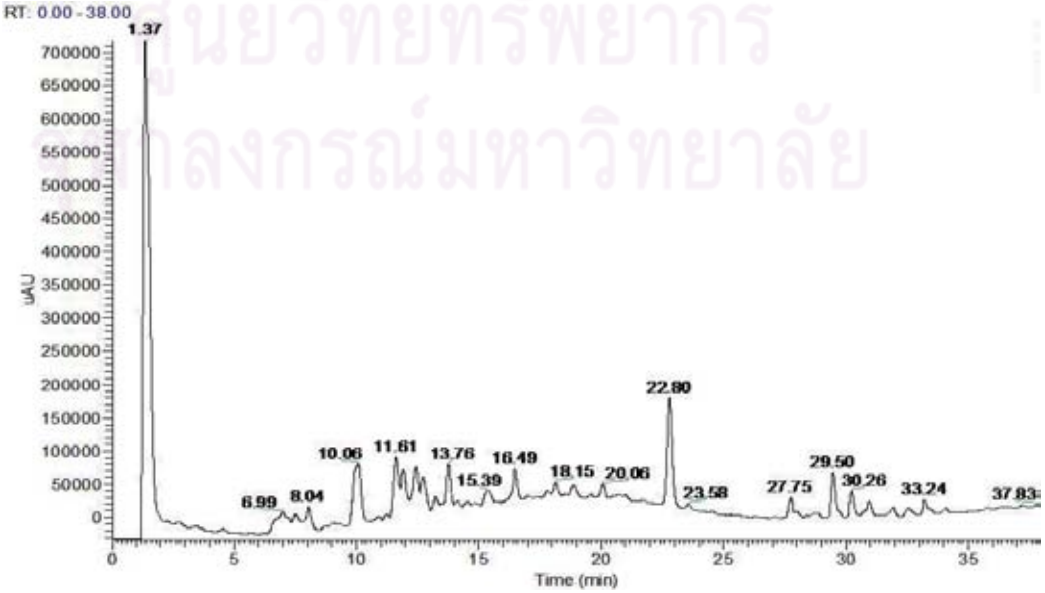
16. *Acacia farnesiana* twig



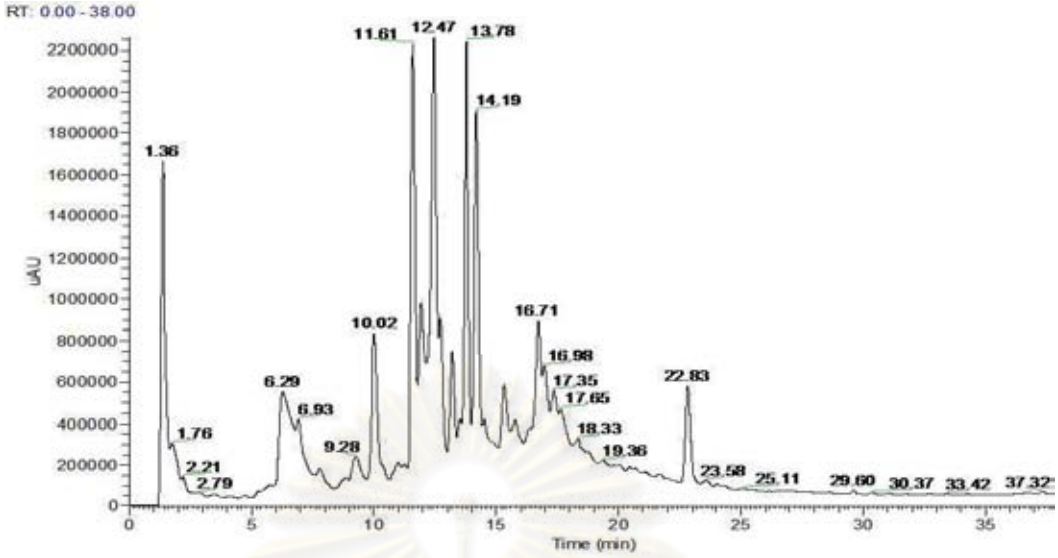
17. *Albizia myriophylla* leaves



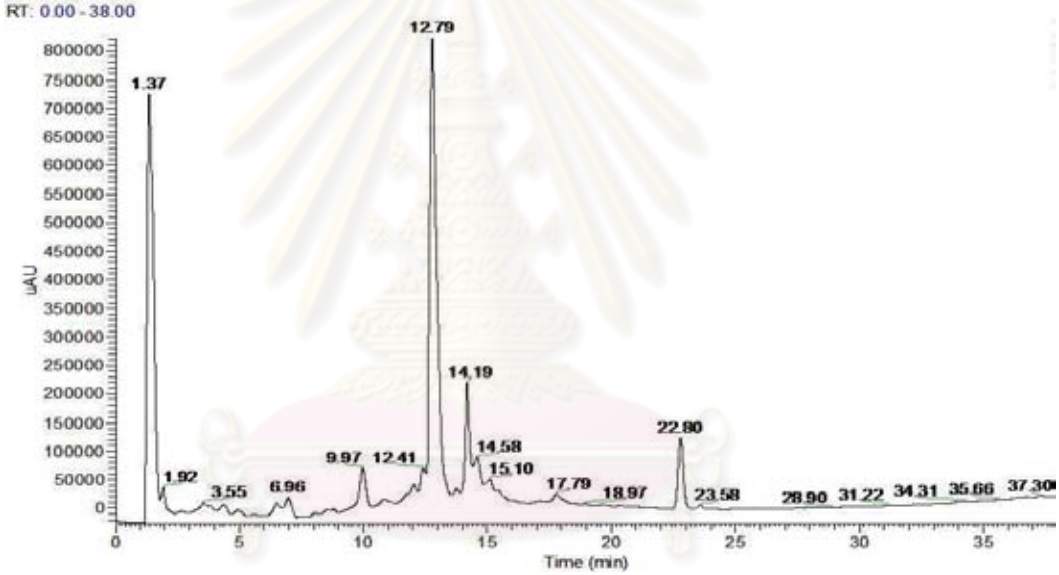
18. *Acacia catechu* branch



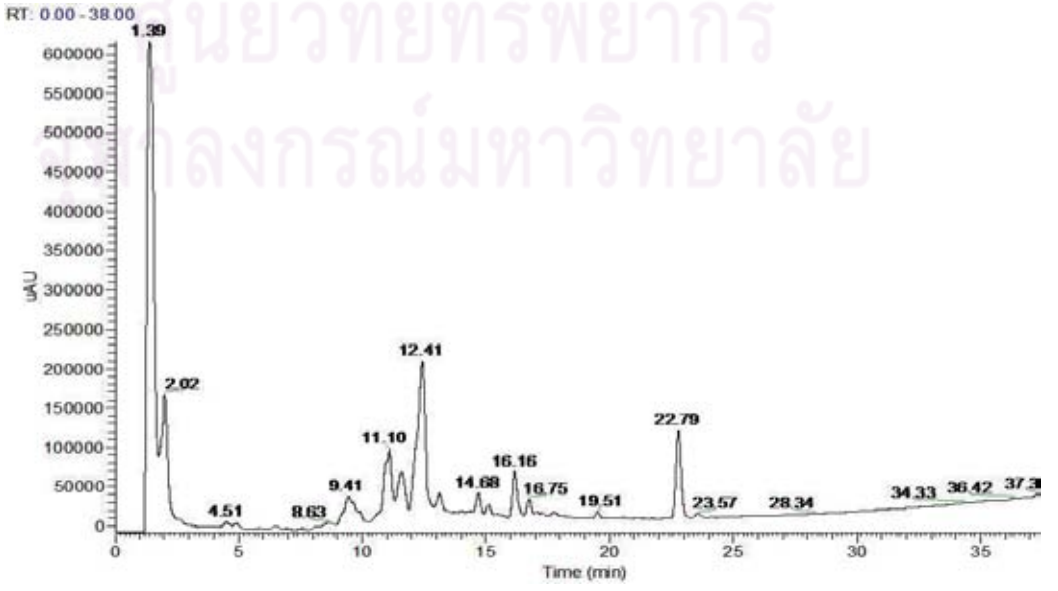
19. *Acacia catechu* leaves



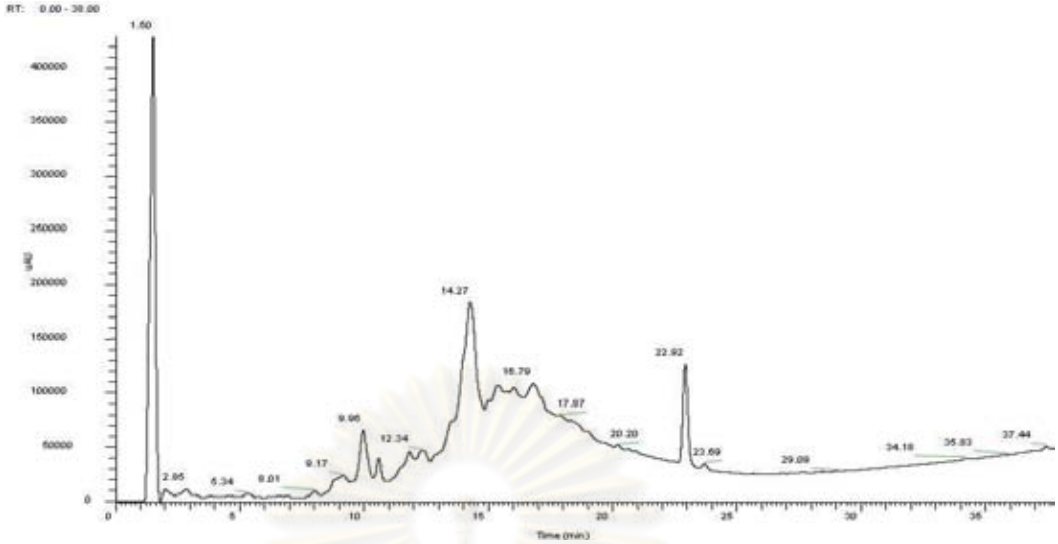
20. *Albizia lebbeckoides* leaves



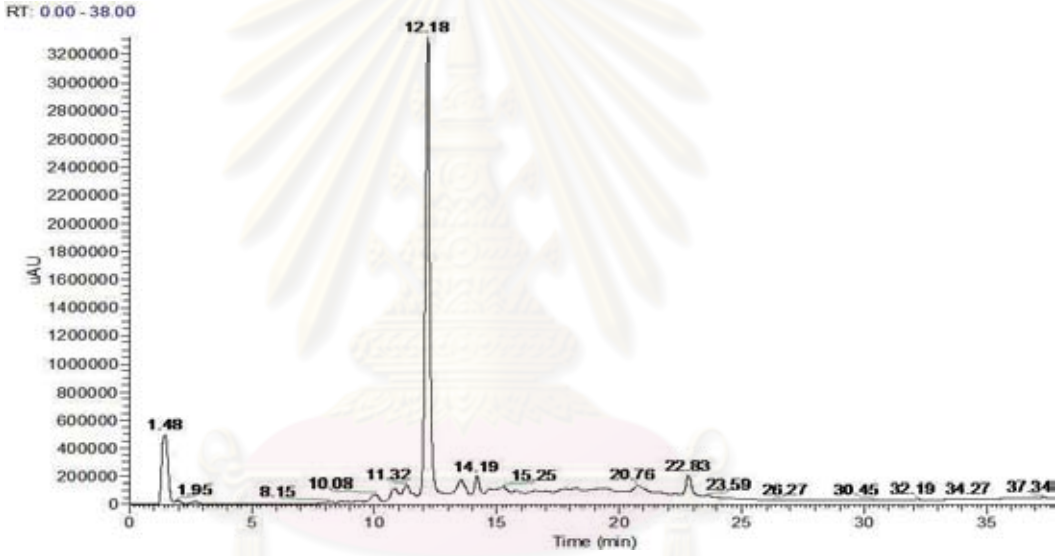
21. *Adenanthera pavonina* pericarp



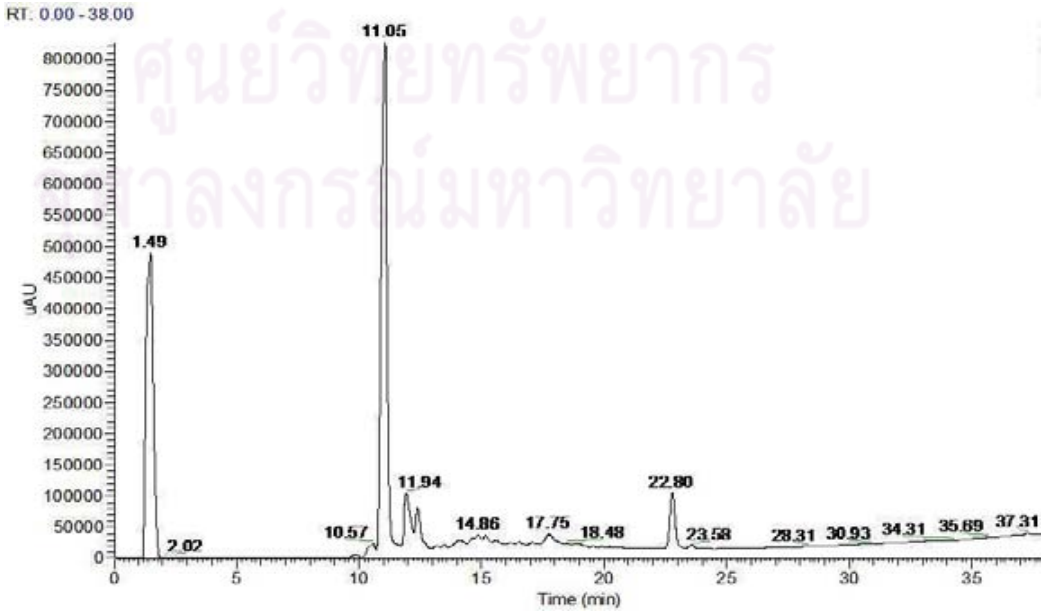
22. *Xylia xylocarpa* stem



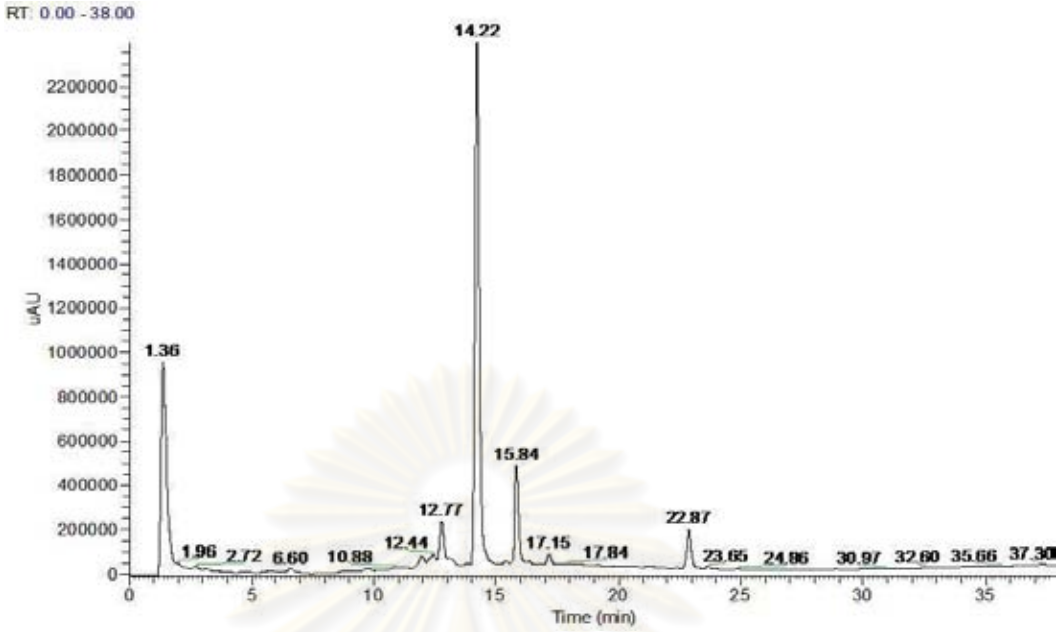
23. *Xylia xylocarpa* leaves



24. *Samanea saman* leaves



25. *Pithecellobium dulce* pericarp



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