Pharmacognostic specifications and quantitative analyses of (+)-catechin and (-)-epicatechin in Thai crude drugs under the name of Si-Siad

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A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Public Health Sciences
College of Public Health Sciences
Chulalongkorn University
Academic Year 2014

ข้อกำหนดทางเภสัชเวทและการวิเคราะห์หาปริมาณสาร (+)-แคทเทชินและสาร (-)-อีพิแคทเทชิน ในสมุนไพรไทยภายใต้ชื่อสีเสียด

นางสาวธิดารัตน์ ดวงยอด

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
สาขาวิชาวิทยาศาสตร์สาธารณสุข
วิทยาลัยวิทยาศาสตร์สาธารณสุข จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2557

Thesis Title	Pharmacognostic specifications and quantitative analyses of (+)-catechin and (-)-epicatechin in Thai crude drugs under the name of Si-Siad
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ธิดารัตน์ ดวงยอด : ข้อกำหนดทางเภสัชเวทและการวิเคราะห์หาปริมาณสาร (+)-แคทเทชินและสาร (-)-อีพิแคทเทชินในสมุนไพรไทยภายใต้ชื่อสีเสียด (Pharmacognostic specifications and quantitative analyses of (+)-catechin and (-)-epicatechin in Thai crude drugs under the name of Si-Siad) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. ชนิดา พลานุเวช, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. นิจศิริ เรืองรังษี, หน้า.

การศึกษาข้อกำหนดทางเภสัชเวท การต้านอนุมูลอิสระ ปริมาณฟีโนลิครวม ปริมาณฟีโนลิคที่ไม่ใช่แทนนิน และปริมาณแทนนินรวมของเครื่องยาสมุนไพรไทยภายใต้ชื่อสีเสียดจากหลากหลายแหล่งทั่วประเทศไทย รวมทั้งการหาปริมาณสาร (+)-แคทเทชินและสาร (-)-อีพิแคทเทชินด้วยเครื่องมือไฮเพอร์ฟอแมนซ์ลิควิดโครมาโตกราฟีซึ่งง่ายและน่าเชื่อถือ การประเมินลักษณะทางมหทรรศน์ของเครื่องยาสมุนไพรด้วยการวาดภาพลายเส้นแสดงลักษณะทั้งต้น การประเมินลักษณะทางจุลทรรศน์ของผงสีเสียดเปลือกพบว่าประกอบด้วยชิ้นส่วนของเส้นใย เรซิน แทนนิน เม็ดแป้ง ผลึกแคลเซียมออกซาเลท และชิ้นส่วนของพาเรนไคมา เอกลักษณ์ทางกายภาพเคมีของสีเสียดไทยและสีเสียดเทศแบ่งออกเป็น 2 กลุ่มตามปริมาณเถ้าที่พบ ตัวอย่างบางส่วนของสีเสียดไทย ทุกตัวอย่างของสีเสียดเทศและสีเสียดเปลือกมีฤทธิ์การต้านอนุมูลอิสระได้ดี อีกทั้งมีฟีโนลิครวมและฟีโนลิคที่ไม่ใช่แทนนินในปริมาณสูง จากผลการทดลองแสดงให้เห็นว่าฟีโนลิครวมในปริมาณสูงส่งผลให้ฤทธิ์การต้านอนุมูลอิสระสูงขึ้นด้วย นอกจากนี้ตัวอย่างของสีเสียดจากแหล่งที่ต่างกันจะมีความแตกต่างกันทั้งฤทธิ์การต้านอนุมูลอิสระและปริมาณฟีโนลิครวม ตัวอย่างบางส่วนของสีเสียดไทยประกอบด้วยสาร (+)-แคทเทชินและสาร (-)-อีพิแคทเทชินเป็นหลัก สาร (+)-แคทเทชินเป็นสารประกอบหลักของสีเสียดเทศ ในขณะที่สาร (-)-อีพิแคทเทชินเป็นสารประกอบหลักของสีเสียดเปลือก ค่าตัวแปรของการตรวจสอบความใช้ได้ของวิธีทดสอบประเมินโดยการใช้แนวทางของไอซีเอช (ICH guideline) จากการตรวจสอบความใช้ได้ของวิธีทดสอบพบว่าวิธีไฮเพอร์ฟอแมนซ์ลิควิดโครมาโตกราฟีที่ใช้มีความไวและมีความถูกต้องในการวิเคราะห์หาปริมาณสาร (+)-แคทเทชินและสาร (-)-อีพิแคทเทชินในสมุนไพรไทยภายใต้ชื่อสีเสียด ดังนั้นวิธีไฮเพอร์ฟอแมนซ์ลิควิดโครมาโตกราฟีนี้สามารถนำไปประยุกต์ใช้ในการหาปริมาณสาร (+)-แคทเทชินและสาร (-)-อีพิแคทเทชิน ในส่วนประกอบของพืชได้

สาขาวิชา วิทยาศาสตร์สาธารณสุข ลายมือชื่อนิสิต .....

ปีการศึกษา 2557 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

5479058353 : MAJOR PUBLIC HEALTH SCIENCES
KEYWORDS: ACACIA CATECHU / UNCARIA GAMBIR / PENTACE BURMANICA / (+)-CATECHIN / (-)-EPICATECHIN / ANTIOXIDANT ACTIVITY / TOTAL PHENOLIC / TOTAL TANNIN / HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

THIDARAT DUANGYOD: Pharmacognostic specifications and quantitative analyses of (+)-catechin and (-)-epicatechin in Thai crude drugs under the name of Si-Siad. ADVISOR: ASST. PROF. CHANIDA PALANUVEJ, Ph.D., CO-ADVISOR: ASSOC. PROF. NIJSIRI RUANGRUNGSI, Ph.D., pp.

Pharmacognostic specifications, antioxidant activities, total phenolic, non-tannin phenolic, and total tannin contents of Thai crude drugs under the name of Si-Siad from various sources throughout Thailand were investigated. A simple and reliable method to determine (+)-catechin and (-)-epicatechin contents was performed by high performance liquid chromatography (HPLC). Macroscopic evaluations of three plants were illustrated as whole plant drawing. Microscopic evaluation of *Pentace burmanica* Kurz stem bark powders showed fragment of fiber, resin masses, tannin masses, starch grain, calcium oxalate crystals, and fragment of parenchyma. The physico-chemical of *Acacia catechu* (Linn.f.) Wild. water extracts and *Uncaria gambir* (Hunter) Roxb. water extracts were divided into two classes of purity according to ash contents. Some of *Acacia catechu* water extracts and all of *Uncaria gambir* water extracts and *Pentace burmanica* stem bark extracts exhibited good antioxidant activities and showed high contents of total phenolics and non-tannin phenolics. The results demonstrated that greater amount of phenolic contents lead to more potent antioxidant effects. Moreover, the different sources of the Si-Siad samples showed the variation in both antioxidant activities and phenolic contents. Some of *Acacia catechu* water extract samples consisted of (+)-catechin and (-)-epicatechin as major compounds. (+)-Catechin was the main compound of *Uncaria gambir* water extracts; whereas (-)-epicatechin was the main compound of *Pentace burmanica* stem bark extracts. The validation parameters were investigated according to ICH guideline. The validation of the method revealed that the HPLC method showed good sensitivity and accuracy for (+)-catechin and (-)-epicatechin quantitations in Thai crude drugs under the name of Si-Siad. Hence, this HPLC method could be applied to determine (+)-catechin and (-)-epicatechin in plant materials.

Field of Study: Public Health Sciences
Academic Year: 2014

Student's Signature .....
Advisor's Signature .....
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ACKNOWLEDGEMENTS

The author wishes to express her heartily gratitude and appreciation to her thesis advisor, Assistant Professor Dr. Chanida Palnuvej, for her continuous guidance, suggestion, and support throughout the course of this study.

The author is sincerely grateful to her thesis co-advisor, Associate Professor Dr. Nijsiri Ruangrungsi, for his kindness and valuable suggestion to complete the study.

Gratitude is grateful to thesis committee members, Professor Dr. Surasak Taneepanichskul, Associate Professor Dr. Uthai Sotanaphun, Assistant Professor Dr. Naowarat Kanchanakhan, and Assistant Professor Dr. Kanchana Rungsrihirunrat for their important and constructive suggestion in finalizing this thesis.

The author is thankful to the Herbal Remedies and Alternative Medicine Task Force of STAR: Special Task Force for Activating Research under 100 Years Chulalongkorn University Fund.

Appreciation is also extended to College of Public Health Sciences, Chulalongkorn University and all the staff members for necessary assistance and instrumental support.

The author's final thank goes to her family, especially her parents for their love, understanding, encourage, and support her to concentrate on her study.

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LIST OF ABBREVIATIONS

°C	Degree Celsius
ABTS	2, 4, 6-azinobis (3-ethylbenzothiazoline-6 sulfonic acid)
ACN	Acetonitrile
BHT	Buthylated hydroxytoluene
$C_{15}H_{14}O_6$	(+)-Catechin, (-)-Epicatechin
C_{18}	A chain of 18 carbon atoms
CE	Catechin equivalent
cm	Centimetre
DPPH	2, 2-diphenyl-1 picryl hydrazyl
DW	Dry weight
EDTA	Ethylenediaminetetraacetic acid
Fe^{2+}	Iron (II)
Fe^{3+}	Iron (III)
$FeSO_4$	Iron (II) sulfate
FRAP	Ferric reducing antioxidant power
g	Gram
GC	Gas chromatography
H_2O_2	Hydrogen peroxide
HCl	Hydrochloric acid
HPLC	High performance liquid chromatography
IC_{50}	Half maximal inhibitory concentration
ICH	International Conference on Harmonization

kg	Kilogram
l	Litre
LOD	Limit of detection
LOQ	Limit of quantitation
m	Metre
mg	Milligram
min	Minute
ml	Millilitre
mM	Millimolar
nm	Nanometre
ODS	Octadecylsilane (C_{18})
OH	Hydroxyl group
ORAC	Oxygen radical absorbing capacity
pH	Potential of hydrogen ion
PTFE	Polytetrafluoroethylene
R	Correlation coefficient
RNS	Reactive nitrogen species
ROS	Reactive oxygen species
RSD	Relative standard deviation
SD	Standard deviation
TLC	Thin layer chromatography
TPTZ	2, 4, 6-triyridyl-S-triazine
TRAP	Total radical-trapping antioxidant parameter
UV	Ultraviolet

v/v	Volume in a volume
WHO	World Health Organization
µg	Microgram
µl	Microlitre

CHAPTER I
INTRODUCTION

Background and significance of the study

The use of plants and plant products to treat several diseases has been practice for a long time. Si-Siad, a well-known crude drug in Thailand, has been previously used as one of the most potent medicinal plant used for treatment of diarrhea and sore throat in Thai traditional medicine [1]. There are 3 kinds of crude drugs under Thai name of Si-Siad including Si-Siad-Thai, Si-Siad-Ted, and Si-Siad-Pleuak. The crude drugs of Si-Siad-Thai and Si-Siad-Ted are also known as catechu which is applicable to black catechu and pale catechu [2]. The water extract of *Acacia catechu* (Linn. f.) Willd. heartwood is known in commerce as black catechu or cutch. A similar extract, known in pharmacy as pale catechu and in general commerce as gambir, is produced from leaves and shoots of *Uncaria gambir* (Hunter) Roxb. Black catechu which is round masses has a blackish-brown color internally, with the black on the outside. The texture is uniform and shining. It is heavier than pale catechu. Pale catechu is generally in small cylinder or cube of pale reddish-brown color, light and friable. It is rough fracture and has a bitter taste with a degree of sweetness. Both are often much adulterated with sand and other impurities [1, 3]. In addition, Si-Siad-Pleuak refers to Burma mahogany (*Pentace burmanica* Kurz). *Pentace burmanica* stem bark is one of the medicinal plants that use to treat diarrhea but it is rarely used. Nowadays, older people in Laos and Northeast Thailand use this stem bark as an ingredient in chewing betel (nuts of *Areca catehu*) for the strengthening teeth. According to market survey, crude drug of *Pentace burmanica* stem bark could be adulterated with other plants.

Phenolic compounds are one type of natural products that widely found in plants. Plant polyphenols, which have the ability to precipitate protein, are called tannins. Nevertheless, not all phenolic compounds are tannins. Catechins are polyphenols antioxidant plant metabolite which extracted from *Acacia cactechu* and *Uncaria gambir*. Several recent studies are reported on the application of several analytical methods for quantitation and isolation of catechins which presented in *Acacia catechu* and *Uncaria gambir* [4-6]. (+)-Catechin and (-)-epicatechin are the most common optical isomers that found in nature. *Pentace burmanica* extract consists of total tannins about 9.93% [7]. However, there have been no reports about antioxidant activity and the contents of (+)-catechin and (-)-epicatechin in *Acacia catechu* water extract, *Uncaria gambir* water extract and *Pentace burmanica* stem bark. The screening of bioactive compounds from the herbal extract, the standardization and quality control of raw herbal materials are more important to new drug development [8]. Moreover, the standardization is essential measure for purity and authentication of herbal drugs. To control the quality of raw herbal material, establishment of standardization parameter is needed. Hence, this research is attempted to develop the specific standardization parameters of *Acacia catechu* water extract, *Uncaria gambir* water extract and *Pentace burmanica* stem bark in Thailand markets, to investigate the antioxidant activities, total phenolic and total tannin contents, as well as to determine the contents of (+)-catechin and (-)-epicatechin in these herbal medicines by high performance liquid chromatography (HPLC).

Objectives

1. To develop the pharmacognostic specification parameters of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica* stem bark.

2. To investigate the antioxidant activities, total phenolic, and total tannin contents of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica* stem bark.

3. To determine (+)-catechin and (-)-epicatechin contents of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica* stem bark using HPLC analysis.

Expected benefits

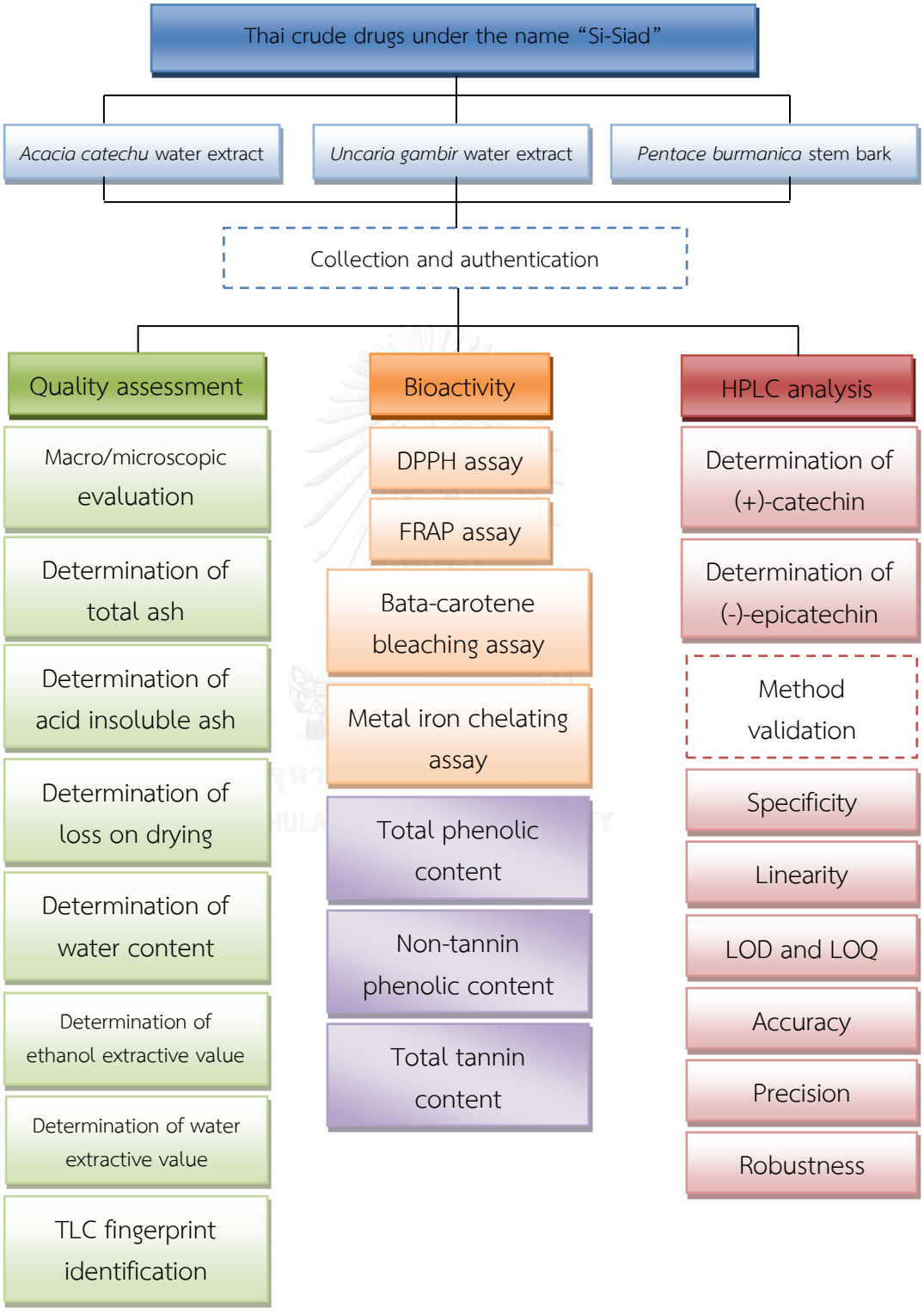
1. This research provides the pharmacognostic specification of Thai crude drugs under the name of Si-Siad which needed for drug standardization and drug quality improvement.

2. This research provides the antioxidant activities and chemical constituents of Thai crude drugs under the name of Si-Siad.

3. This research provides the methodology to determine the contents of (+)-catechin and (-)-epicatechin in plant material.

4. This research provides the quality specification with reference to bioactivities and active chemical contents of Thai crude drugs under the name of Si-Siad.

Conceptual Framework



CHAPTER II
LITERATURE REVIEW

Catechu

Catechu is considered as one of the most plant drugs used for anti-diarrhea and anti-ulcer. There are two varieties; black catechu and pale catechu. Although black catechu and pale catechu have the name catechu in common, they are from different plants. Black catechu is obtained from the tree *Acacia catechu* belongs to Fabaceae family while pale catechu obtained from the tree *Uncaria gambir* which is a member of Rubiaceae family [1, 9].

Acacia catechu (Linn. f.) Willd.

Botanical classification

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Rosidae

Order: Fabales

Family: Fabaceae

Genus: *Acacia*

Species: *catechu*

Botanical name: *Acacia catechu* (Linn. f.) Willd.

Synonyms

Acacia catechu (Linn. f.) Willd. var. *catechuoides* (Roxb.) Prain

Acacia catechuoides (Roxb.) Benth.

Acacia sundra (Roxb.) Bedd.

Acacia wallichiana DC.

Mimosa catechu Linn. f.

Mimosa catechuoides Roxb.

Vernacular names

Assamese: Kharira, Khara, Khayar

Bengali: Khera, Khayera

Burmese: Sha

English: Black catechu, Cutch tree

French: Acacie au cachou

Gujirati: Khair, Kathe, Kher

Hindi: Khair

Kannada: Kaggali, Kaggalinara, Kachinamara, Koggigida

Kashmiri: Kath

Malayalam: Karingali

Marathi: Khaira, Khair

Oriya: Khaira

Punjabi: Khair

Tamil: Karungali, Karungkali

Telugu: Chandra, Kaviri

Thai: Si-Siad, Si-Siad-Thai, Si-Siad-Nua

Urdu: Chanbe Kaath

Plant description

Acacia catechu is a moderate sized spiny deciduous tree which grows up to 15 m in height. Leaves are bipinnate and having 10-30 pairs of pinnae up to 5 cm long, each having many small, narrow, pale green hairy leaflets. There are two short recurved spines at the base of each leaf. Flowers are small, pale yellow or white in cylindrical axillary spikes, 10-12.5 cm long. Pods are flat and brown (Figure 1). Seeds are 3-10 per pod [1, 9-11].

Figure 1 *Acacia catechu*

Crude drug is a dried extract from the heartwood of *Acacia catechu* which also known as black catechu or cutch. The crude drug is prepared by boiling heartwood pieces in water for several hours until concentrated to syrup. After that it set to cool in leaf-lined wood trays. It is then cut into small size and dried slowly in the shade. Black catechu is blackish-brown, shining, and heavy (Figure 2). It is odorless but has a strong astringency [1, 10].

Figure 2 Black catechu

Macroscopic study

The color of *Acacia catechu* heartwood is light red and turning brownish red to near black with age. The heartwood is attached with whitish sapwood. It is very hard, moderately heavy and fairly straight-grained to slightly interlocked grained. It has characteristic odor and astringent taste [12].

Microscopic study

Transverse section of *Acacia catechu* heartwood is a ring porous wood. The wood shows vessels which are solitary or arranged in small radial groups and often blocked with tylosis impregnated with tannin. In longitudinal section, the wood exhibits numerous closely arranged minute bordered pits and slit like pores. The wood consists of fibers which are thin walled and usually arranged in tangentially running bands and often associated with metatrachieal parenchyma embedded with prismatic crystals of calcium oxalate. Medullary rays are biserriate to multiserrite. In transverse section, the medullary rays run parallel and straight to each other except where they bend when pass around the vessel. In tangential longitudinal section, mulyiserriate rays are seen as vertically running linear bands, narrow horizontally

running bands crossing the vessels or at the places from narrow or broad tangentially running bands giving false annual rings. Cells are loaded with the simple starch grains and often contain prismatic crystals of calcium oxalate.

Several histological characters including fibers with prism, fibers with scleroids, fragment of border pitted vessels, starch grains with xylem along with prism of calcium oxalate, yellowish dark brown matter, lignified fibers, and fibers passing through medullar rays were found in powders of *Acacia catechu* heartwood [12].

Pharmacognostic studies

Pharmacognostic specification of *Acacia catechu* heartwood was carried out in India. Various functional groups were presented in alcoholic extract of *Acacia catechu* heartwood powder including alkaloids, tannin, saponins, carbohydrate, starch, and protein. The physico-chemical study showed that loss on drying, total ash, acid insoluble ash, alcohol soluble extractive values, water soluble extractive values, and tannin content were of 7.15%, 2.80%, 0.04%, 15.78%, 12.00%, and 8.28% respectively [12].

Similar study was conducted to investigate the pharmacognostic evaluation of *Acacia catechu* heartwood. The biochemical analysis showed that the deposition of lipid and lignin was high in the vascular region compared to the deposition of starch and protein. The phytochemical screening showed the presence of catechins, tannins, flovones, and sugars. The physico-chemical investigation revealed that loss on drying, total ash, acid insoluble ash, water soluble ash, water soluble extractive value, alcohol extractive value, fiber content, total sugar, and reducing sugar were 9-

11%, 1.6-2%, 0.2-0.4%, 0.09-0.25%, 23-25%, 19-21%, 49-53%, 1.2-1.9%, and 0.7-1.5% respectively. Volatile oil content was not detected [13].

Chemical components

Acacia catechu wood contains 50% tannins, 20-35% catechutannic acid, 2-10% acacatechin, 13-33% catechin, 2.5% epicatechin, isorhamnetin, quercetin, 25-33% phlobatannins, 22-50% tannic acid, catechu-red, 20-35% gum [1, 5, 9].

Total phenolic content

The total phenolic content in water extract of *Acacia catechu* bark was found to be 67.40 ± 0.28 mg/ml gallic acid equivalent per 100 mg the extract [14].

Antimicrobial activities

Acacia catechu extract was found to be an effective antimicrobial agent. This extract was effective in both gram positive and gram negative bacteria as well as against some fungi.

Saini *et al*. conducted an antimicrobial study of *Acacia* species using disc diffusion method. The results demonstrated that methanolic extract of *Acacia catechu* bark exhibited activity against 3 bacterial strains including *Escherichia coli*, *Staphylococcus aureus,* and *Salmonella typhi* and 2 fungal strains (*Candida albicans* and *Aspergillus niger*) [15].

Similar study was conducted to investigate the antimicrobial property of the resin part (black catechu) of *Acacia catechu* using agar diffusion method. The resin was extracted with different polarity solvents (petroleum ether, chloroform, methanol, and

water). All extracts were effective against *Escherichia coli*, *Staphylococcus aureus, Pseudomonas aeruginosa,* and *Bacillus subtilis*. The water extracts showed good inhibition against these 4 strains of bacteria with the minimum inhibitory concentration (MIC) of 330, 40, 220, and 20 µg/ml for *E. coli*, *S. aureus*, *P. aeruginosa*, and *B. subtilis* respectively [16].

In Gulzar *et al*. study, antimicrobial activity of petroleum ether, ethanol, and ethanol: water (1:1) extracts of *Acacia catechu* leaves against gram positive bacteria, gram negative bacteria, and some pathogenic fungi was evaluated by disc diffusion method. All three extracts at different concentration showed the positive results against all bacterial strains tested and all fungal strains tested. The ethanolic extract was found to be potent antibacterial activity [17].

Lakshmi and Aravind also conducted a similar study on antibacterial activity of ethanolic bark extract of *Acacia catechu* against selected oral microbes by the macro broth dilution method. The results revealed that the ethanolic extract exhibited antibacterial activity against *Streptococcus mitis* with minimum bactericidal concentration (MBC) of 500 µg/ml whereas the MBC for *Streptococcus sanguis* and *Lactobacillus acidophilus* were found to be 1, 5, and 10 mg/ml respectively [18].

Antioxidant activities

Devi, V.G. *et al*. evaluated the qualitative and quantitative antioxidant capacity of *Acacia catechu* heartwood alcohol extract by Dot-blot assay and DPPH assay respectively. The results of Dot-blot assay showed that the extract was able to be antioxidant and the results of DPPH assay revealed that the extract gave very good

DPPH radical scavenging activity (IC_{50} = 61.72 μM) compared to ascorbic acid (IC_{50} = 66.12 μM) [13].

Alam *et al*. has conducted a similar study on the antioxidant properties of the water extract of *Acacia catechu* bark. The *in vitro* tests including DPPH assay, hydrogen peroxide assay, and reducing power assay were evaluated. For DPPH and hydrogen peroxide scavenging, IC_{50} values were found to be 177.53 and 455.20 μg/ml respectively. The *Acacia catechu* bark extract reduced the most of Fe^{3+} ions in reducing power investigation and the reducing power of the extract was increased with the quantity of the sample. In the *in vivo* test, the water extract of *Acacia catechu* bark at dose of 100 and 200 mg/kg btw showed significant increase in the level of superoxide dismutase, catalase, and glutathione-S-transferase whereas glutathione level was reduced. The levels of the toxicity marker enzyme were found to be in the normal range. The results indicated that the extract showed no toxicity [14].

The ethanolic extracts of *Acacia catechu* leaves and branches were good DPPH radical scavenger (IC_{50} = 2.73 and IC_{50} = 10.45 μg/ml) that were compared to quercetin (IC_{50} = 0.45 μg/ml) and buthylated hydroxytoluene (BHT) (IC_{50} = 3.47 μg/ml) respectively [19].

Similar study was carried out to investigate the antioxidant and iron chelating properties of 70% methanolic extract of *Acacia catechu* resin (black catechu). IC_{50} values for different scavenging activities including DPPH radical scavenging, superoxide anion scavenging, nitric oxide scavenging, peroxynitrite scavenging, singlet oxygen scavenging, hypochlorous acid scavenging, and hydrogen peroxide scavenging

were 17.80, 39.55, 55.31, 746.85, 57.50, 155.48 µg/ml, and 44.20 mg/ml respectively. The extract had the ability to chelate iron with an IC_{50} of 810.80 µg/ml and it was found to inhibit lipid peroxidation with an IC_{50} of 12.35 µg/ml. The results provided evidence that the 70% methanolic extract of *Acacia catechu* resin acts as an antioxidant and iron chelator [20].

***Uncaria gambir* (Hunter) Roxb.**

Botanical classification

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: Asteridae

Order: Rubiales

Family: Rubiaceae

Genus: *Uncaria*

Species: *gambir*

Botanical name: *Uncaria gambir* (W. Hunter) Roxb.

Synonyms

Nauclea gambir Hunter

Ourouparia gambir (Hunter) Baill.

Uncaria gambir var. *latifolia* S. Moore

Uruparia gambir (Hunter) Kuntze

Vernacular names

Chinese: Er Cha

English: Gambir, Gambier, Pale catechu, White cutch

Malay: Kachu, Kekait, Gambir

Thai: Si-Siad-Ted

Plant description

Uncaria gambir is a shrub with square stems and erect main stems which bear horizontal branches. It is single leaf, opposite, oval form, jagged edges, base rounded, tapered tip, 8-13 cm long, and 4-7 cm wide. Leaves are green in color (Figure 3). Flowers are in numerous clusters and appeared from among the leaves. Fruits are egg shape and orange in color. Seeds are very tiny [1].

Figure 3 *Uncaria gambir* leaves

The crude drug of pale catechu is prepared as a dried extract from the leaves and young branches of *Uncaria gambir.* The extract is prepared by boiling leaves and young branches in water, evaporating this decoction, cooling into flat cake in moulds then cut into small pieces [21]. Pale catechu is cube or cylindrical shape and

externally brown, internally ocherey-yellow (Figure 4). It is easily broken with a dull earthy fracture. The taste is bitter and astringent [1].

Figure 4 Pale catechu

Chemical components

Uncaria gambir contains chalcane-flavan dimers, gambiriin A1, A2, B1, and B2, catechin, epicatechin, dimeric proanthocyanidins, procyanidin B1, procyanidin B3, gambiriin C, gambirine, ganbirdine, and isogambirdine [22].

Phytochemical screening

The screening tests of methanolic extract of *Uncaria gambir* resin were positive for alkaloid, sterol, carbohydrate, phenolic compound, flavonoid, resin, protein, and amino acid [23].

Total phenolic content

The total phenolic content of methanolic extract of *Uncaria gambir* resin was investigated. The content was found to be 18.37 mg gallic acid equivalent/ g dry weigh of the extract [23].

Similarly, total phenolic contents of the ethanolic extracts of 4 kinds of *Uncaria gambir* (gambir cubadak, gambir udang, gambir riau mancik, and gambir riau gadang) obtained from West Sumatra, Indonesia ranged from 13.58 – 13.90 g/ 100 g of crude sample [24].

The total phenolic contents of ethyl acetate, methanol, and hot water extracts of *Uncaria gambir* resin were evaluated by Folin-Ciocalteu assay. The *Uncaria gambir* extracts showed different contents for each solvent. The total phenolic contents of ethyl acetate, methanol, and hot water extracts were found to be 113.43, 99.25, and 76.75 mg gallic acid equivalents /g respectively [25].

Antioxidant activities

The antioxidant activities and reducing power of methanolic extract of *Uncaria gambir* resin were evaluated. IC_{50} values of different scavenging activities including superoxide anion, hydroxyl radical, nitric oxide, and DPPH radical assays were found to be 35.95, 14.83, 34.20, 18.27, and 11.75 µg/ml. The extract was potent reducing power and the power of the extract was increased with the quantity of the sample [23].

The DPPH radical scavenging activity of the water extracts of *Uncaria gambir* obtained from West Sumatra, Indonesia ranged from 92-93.1% [24].

Kassim *et al*. conducted a study to investigate the antioxidant activity of *Uncaria gambir* extracts by DPPH scavenging assay. The study showed that the ethyl acetate extract of *Uncaria gambir* at the concentration of 50 µg/ml had highest radical scavenging activity which was 88.63% followed by the methanolic extract

(85.98%) at the same concentration. The hot water extract showed the minimum radical scavenging activity as 82.23% [25].

Similar study reported that the water extract of *Uncaria gambir* at concentration of 30 µg/ml showed 92% DPPH inhibition. IC_{50} of ethanolic extract, ethyl acetate extract and the residue from ethanolic extract of *Uncaria gambir* ranged between 13.8-16.2 µg/ml for DPPH inhibition, whereas that from water extract was 27.4 µg/ml. For catechin standard, IC_{50} was 15.9 µg/ml. The results suggested that the ethanol and ethyl acetate extracts had higher DPPH inhibitory activity than the water extract [26].

Alpha-glucosidase inhibitory activity

The alpha-glucosidase inhibitory activity of water extract, ethanolic extract, ethyl acetate extract and the residue from ethanolic extract of *Uncaria gambir* was carried out. Koji was used as a standard compound. IC_{50} for koji extract was 4.1 µg/ml while the IC_{50} for all extracts ranged from 15.2-49.5 µg/ml [26].

Cytotoxicity activity

Intestinal epithelial cell line no. 6 (IEC-6) was used for toxicity test of *Uncaria gambir* extract. The extracts at the concentration of 1-200 µg/ml showed no negative effects against IEC-6, as indicated by more than 93% cell viability. The results revealed that the *Uncaria gambir* extract was safe [24].

Anthelmintic activity

The anthelmintic potential of *Uncaria gambir* leaves and shoots extract was evaluated. The alcoholic extract and its ethyl acetate fraction at different

concentrations of 25, 50, 75, and 100 mg/ml were tested on *Pheretima posthuma* (Indian earthworm). Time of paralysis and time of death of the worms were investigated. For the alcoholic extract, time of paralysis and time of death were 6.01 and 10.20 min whereas the ethyl acetate showed time of paralysis and time of death as 3.30 and 6.16 min. Time of paralysis and time of death for the reference standard, albendazole were found to be 1.2 and 1.33 min respectively. The results revealed that the ethyl acetate fraction of alcoholic extract of leaves and shoots of *Uncaria gambir* exhibited potent anthelmintic activity against Indian earthworm compared to alcoholic extract [27].

Pharmacognostic studies of black catechu and pale catechu

In 1986, the quality of black catechu and pale catechu in Thailand was evaluated. Four samples of black catechu and 4 samples of pale catechu sampling from traditional drug stores and a local factory were analyzed. The results showed that only one sample of black catechu from a local factory in Lampang province met the standard requirements whereas all others were substandard [28].

In 2009, 21 samples of black catechu and pale catechu (11 of black catechu and 10 of pale catechu) were studied to investigate the quality of catechu in Thai market. The result revealed that only one black catechu from Lampang province was complied with the standard specification. The other samples were inferior. Total ash and acid insoluble ash were found to be high which indicated that the most of catechu in Thai traditional drug stores had the adulterant problem. Moreover, this study reported that black catechu was adulterated with clay and pale catechu was adulterated with quart [29].

Standardization parameters

In Thailand, there were no reports about the standard requirements for black catechu whereas the standard requirements for pale catechu were given in Thai Pharmacopoeia volume 1 (1993) [30]. However, the standard for black catechu was stated in Indian pharmacopoeia (1966) [31].

Standard for black catechu

Water insoluble residue value is not more than 25%. Alcohol insoluble residue is not more than 30%. Loss on drying value is not more than 12%. Ash value is not more than 6% [31].

Standard for pale catechu

Water insoluble residue value is not more than 33%. Ethanol insoluble residue is not more than 34%. Ethanol extractive value is not less than 70%. Loss on drying value is not more than 15%. Ash value is not more than 8% or 6%. Acid insoluble ash value is not more than 1.5% [30, 32].

Pentace burmanica Kurz

Botanical classification

Domain: Eukaryota

Kingdom: Plantae

Subkingdom: Tracheobionta

Superdivision: Spermatophyta

Division: Magnoliophyta

Class: Magnoliopsida

Subclass: -

Order: -

Family: Malvaceae

Genus: *Pentace*

Species: *burmanica*

Botanical name: *Pentace burmanica* Kurz

Vernacular names

English: Burma mahogany

Burmese: Takalis

Thai: Si-Siad-Pleuak

Plant description

Pentace burmanica is a 5 -15 m tall tree and steep extensive buttresses. Its grey outer bark is 2 cm thick, and the inner bark is reddish with a sticky red sap. Branches and young parts are reddish brown and hairy. The soft oval leaves measure 8 cm-15 cm x 4 cm-8 cm, with a white-green lower blade and jagged edges. The leaf stalk petiole and leaf nerves are hairy (Figure 5). Inflorescences consist of a 5-10 cm long cluster of white, hairy, bell-shaped flowers, each about 5 mm long. The fruit is a five-winged and green capsule, 4-5 cm long and 5-5.5 cm wide with a hairy seed, 1-1.5 cm long [33].

Figure 5 *Pentace burmanica*

Chemical component

Tannin content of *Pentace burmanica* bark extract was 9.93% [7].

Antimicrobial activity

The water extract and 50% ethanol extract of *Pentace burmanica* inhibited activity against 3 bacterial strains (*Escherichia coli*, *Staphylococcus aureus*, and *Streptococcus mutans*) [34].

Phenolic compounds

Phenolic compounds are the biggest group of phytochemicals and they are chemical compounds that have one or more hydroxyl groups (OH) attached directly to an aromatic ring [35, 36]. Hence, plant constituents that possesses a phenol group; an aromatic ring bearing hydroxyl groups are classified as phenolic compounds. Plants produce phenolic compounds as secondary metabolites to interact with the environment [37]. In addition, chemically, polyphenols is a collective term for several sub-groups of phenolic compounds [38].

Classification of phenolic compounds

Phenolics or polyphenols constitute one of the most numerous and widely distributed groups of natural products in the plant kingdom. Although polyphenolic compounds are chemically characterized as compounds with phenolic structure, this group of nature products is highly diverse and consists of several sub-groups of phenolic compounds [38]. Therefore, the phenolic compounds can be classified into groups based on the number of carbons in the molecules (Table 1) [36].

Table 1 Classification of phenolic compounds

Structure	Class
C_6	Simple phenolics
C_6-C_1	Phenolic acids and related compounds
C_6-C_2	Acetophenones and phenylacetic acids
C_6-C_3	Cinnamic acids, cinnamyl aldehydes, cinnamyl alcohols, coumarins, isocoumarins, and chromones
C_{15}	Chalcones, aurones, dihydrochalcones
C_{15}	Flavans, flavones, flavanones, flavanonols, anthocyanidins, anthocyanins
C_{30}	Biflavonyls
C_6-C_1-C_6, C_6-C_2-C_6	Benzophenones, xanthones, stilbenes
C_6, C_{10}, C_{14}	Quinones
C_{18}	Betacyanins
Lignans, neolignans	Dimer or oligomers
Lignan	Polymers
Tannins	Oligomers or polymers
Phlobaphenes	Polymers

Flavonoids

Flavonoids constitute one of the most important groups of phenolic compounds in plant. Flavonoids have the C_6-C_3-C_6 (C_{15}) general structural backbone in which the two C6 units (Ring A and Ring B) are phenolic nature (Figure 6). According to the hydroxylation pattern and variations in the chromane ring (Ring C), flavonoids can be further divided into different sub-groups such as flavan-3-ols, flavonols, flavones, flavanones, and anthocyanins [38].

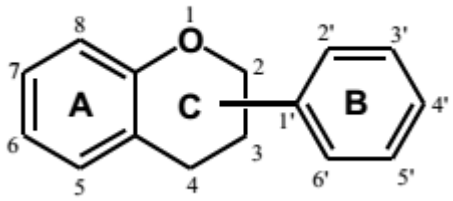


Figure 6 Flavonoid structure

Catechin ($C_{15}H_{14}O_6$)

Flavan-3-ols or flavanols are commonly known as catechins. Difference from most flavonoids, there is no double bond between C_2 and C_3 and no C_4 carbonyl in Ring C of flavanols. This structure and the hydroxylation at C_3 allow flavanols to have two chiral centers on the molecules (on C_2 and C_3), leading to four possible diastereoisomers. Catechin and epicatechin have the same molecular formula but have different spatial configurations of those atoms. Catechin is the isomer with *trans* configuration and epicatechin is the one with *cis* configuration. Each of two configurations has two stereoisomers including (+)-catechin, (-)-catechin, (+)-epicatechin, and (-)-epicatechin (Figure 7). (+)-Catechin and (-)-epicatechin are the most common optical isomers which found in nature. The pharmacological

properties of chiral substances like (+)-catechin and (-)-epicatechin and their enantiomers are different depend on the conformation of the molecules [38-40].

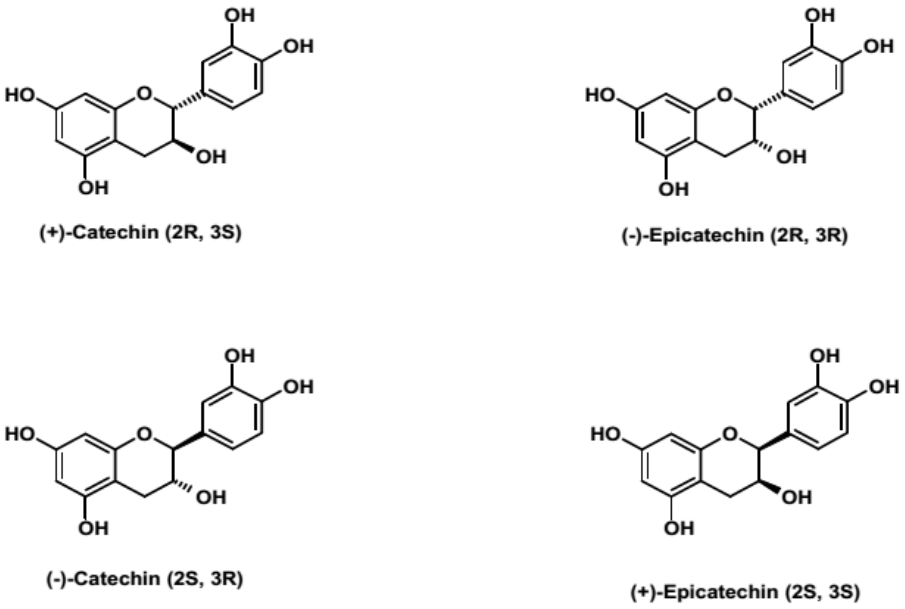


Figure 7 The structures of catechin and epicatechin

Standardization parameters [41]

Macroscopic examination

Macroscopic examination is the simplest and quickest method to determine the characteristics of medicinal plant materials or medicinal plant products by visual inspection. The macroscopic identity of herbal materials is based on shape, size, color, surface characteristics, texture, fracture characteristic, and appearance of the cut surface.

Microscopic examination

Microscopic examination is indispensable for the identification of broken or powdered crude drug. The powder of crude drug is examined for histological characters under microscope with 10X, 20X, and 40X objective lens magnifications and 10X eyepiece lens.

Determination of ashes

The ash remaining following ignition of herbal materials is determined by different methods including measurement of total ash and acid insoluble ash.

The total ash method is used to measure the total amount of materials remaining after ignition. It is mainly measured the presence of inorganic compounds in a crude drug, for example, silica, iron, titanium, phosphorus, alumina, calcium, magnesium, sulfur, sodium, potassium which found in oxide forms under combustion. Boiling of the ash in about 2N hydrochloric acid solution can solubilize most of them except silica [42].

Determination of water

An excess of water in herbal materials will encourage microbial growth, the presence of fungi or insects, and deterioration following hydrolysis. Limit of water content should therefore be set for every given herbal materials. This is important for materials that absorb moisture easily or deteriorate quickly in presence of water.

Toluene distillation method gives a direct measurement of water present in the material being examined.

The test for loss on drying determines both water and volatile matters.

Determination of extractable matters

This method determines the amount of active constituents extracted with solvents from a given amount of herbal material. It is employed for materials for which as yet suitable chemical or biological assay exists.

Thin-layer chromatography

Thin-layer chromatography is particularly valuable for the chemical fingerprint approach of plant materials. This technique is effective and easy to perform, and the equipment required is inexpensive. It is frequently used for evaluating herbal materials and their preparations.

Free radical

A free radical is a molecule or ion which has one or more unpaired (odd or single) electrons in its outer orbit, or valence shell [43, 44]. Free radicals are naturally produced during normal respiration and can be generated in response to exposure to toxic substances such as alcohol, UV rays, and ozone. Free radicals are highly reactive and can create a chain reaction that produces even more free radicals. This chain reaction occurs when a free radical reacts with a stable compound. If free radicals are produced in excess and not neutralized by the antioxidant system present in the human body, then the cellular damage can occur [44]. Free radicals, in form of reactive oxygen and nitrogen species (ROS and RNS), are an integral part of normal physiology. ROS has a greater impact on human both from within body and environment [45]. Oxygen is necessary for life-sustaining metabolic processes. These metabolic processes depend upon the chemical reactions of oxidation and reduction (a chemical reaction that adds electrons to the molecule) or the transfer of electrons [46]. The three major ROS that are of physiological significance are superoxide anion (O_2^-), hydroxyl radical ($^{\bullet}OH$), and hydrogen peroxide (H_2O_2) [45]. However, the effectiveness of an antioxidant depends on the free radical involved.

Antioxidants

Antioxidants are a group of compounds which have ability to prevent, delay, or remove oxidative damage to a target molecule [47]. Therefore, antioxidant activity is an important biological property of many phytochemicals that protects living organisms from oxidative damage thereby preventing several deleterious events and diseases in plants and animals [48]. There are many different methods to evaluate

the *in vitro* antioxidant activity of the medicinal plants which involve different mechanisms of antioxidation, based on chemically scavenging of ROS or RNS [49].

***In vitro* antioxidant activities**

2, 2-diphenyl-1 picryl hydrazyl (DPPH) assay

DPPH is a stable free radical with deep violet color. DPPH radical solution has the maximum absorbance at 517 nm. The assay directly investigates the ability of the extract or antioxidant to donate hydrogen and/or electrons to quench the DPPH radical leading to decolorization of DPPH (DPPH-H or reduced DPPH) to light yellow and the absorbance at 517 nm decreased [50]. However, DPPH can only be dissolved in organic solvent, this is an important limitation when interpreting the role of hydrophilic antioxidants [51].

2,2'-azinobis (3-ethylbenzothiazoline-6 sulfonic acid) (ABTS) assay

ABTS assay can be used to determine the antioxidant activity of the natural and other synthetic therapeutical compounds. The formation of $ABTS^{+}$ radical cation is induced by metmyoglobin and hydrogen peroxide. The antioxidant activity is determined by measuring the decolorization of the $ABTS^{+}$ (blue-green color) to represent the reduction of the radical cation. The absorbance at 734 nm of the reaction mixture of ABTS and an antioxidant is compared to that of the Trolox standard, and the results are expressed in term of Trolox equivalent antioxidant capacity (TEAC) [45, 52]. Nevertheless, the ABTS radical cation must be generated by enzymes or chemical reaction for 12 – 16 h before assay [51].

Ferric reducing antioxidant power (FRAP) assay

FRAP assay is an assay for reduction ability. Complex of ferric ion and 2, 4, 6-tripyridyl-S-triazine (TPTZ) are used as reagents in this assay. The FRAP mechanism is totally electron transfer. The assay relies upon the reduction from the ferric ion (Fe (III))-TPTZ complex to the ferrous ion (Fe (II))-TPTZ complex by antioxidants. (Fe (II))-TPTZ has an intensive blue color and can be monitored at 593 nm. Therefore, antioxidative activity of the antioxidant can be determined by the measurement of the absorption at 593 nm [53]. FRAP assay cannot detect species that act by radical quenching (H transfer), particularly SH group of antioxidants such as thiols and protein [54].

Reducing power assay

The reducing power assay is another form of reducing assay which used to investigate the ability of an antioxidant to donate an electron. This assay is based on the ability of the sample to reduce Fe^{3+} to Fe^{2+} [55]. The antioxidant samples which have reduction potential react with potassium ferricyanide (Fe^{3+}) to form potassium ferrocyanide (Fe^{2+}) which then reacts with ferric chloride to form ferric ferrous complex that has an absorption maximum at 700 nm [56, 57]. The solution is changed to various shades from green to blue, depending on the reducing power of the samples [55].

Metal ion chelating assay

The basic principle of this assay is based on the capacity to decolorize the iron - ferrozine complex [58]. Measurement of the color reduction determines the chelating agent activity to compete with the ferrozine for the ferrous ions. Ferrozine acts as a chelating agent and forms purple complex iron with Fe^{2+} giving maximum absorbance at 562 nm [59].

Beta-carotene bleaching assay

Beta-carotene bleaching assay measures the ability of an antioxidant to inhibit lipid peroxidation. In this assay, a model system made of beta-carotene and linoleic acid undergoes a rapid discoloration in the absence of an antioxidant. The free linoleic acid radical formed upon the abstraction of a hydrogen atom from one of its methylene groups attacked the beta-carotene molecules, which lost the double bonds and therefore, its characteristic orange color [59]. The reproducibility of the results highly depends on experiment variables. Moreover, the chemical and physical properties of emulsion components may affect the ultimate antioxidant activity [60].

Thiobarbituric acid reactive species (TBARS) assay

The TBARS assay is used to measure the anti-lipid peroxidation activity using egg yolk homogenates as lipid rich media. This assay concerns the spectrophotometric measurement of the pink pigment produced through reaction of thiobarbituric acid with malondialdehyde and other secondary lipid peroxidation products. The extent of lipid degradation is measured at 532 nm [61].

Oxygen radical absorbing capacity (ORAC) assay

The ORAC assay is another widely applied antioxidant assay. This assay is based on the ability of a sample to inhibit peroxyl radical by H atom transfer and thus reflects classical radical chain - breaking antioxidant activity. Briefly, the peroxyl radical reacts with a fluorescent probe to form a non-fluorescent product, which can be quantitated by time - dependent fluorescent intensity decreasing. B-phycoerythrin which is a protein isolated from *Porphyridium cruentum* is used as the fluorescent probe. The antioxidant activity is determined by a decreased rate in a loss of fluorescence. ORAC values are usually reported as Trolox equivalents. The ORAC is limited to measurement of hydrophilic chain breaking antioxidant activity against only peroxyl radicals. It ignores lipophilic antioxidants that are particularly important against lipid oxidation in all system as well as other radicals such as O_2^- and $^{\bullet}OH$ that are very relative physiologically. This assay requires fluorometer which may not be routinely available in analytical laboratories. In addition, temperature variation decreases reproducibility [54, 62].

Total radical-trapping antioxidant parameter (TRAP) assay

The TRAP assay monitors the ability of antioxidant compounds to interfere with the reaction between peroxyl radicals generated by 2,2'-azobis (2-amidinopropane) dihydrochloride (AAPH or ABAP) and a target probe. The reaction probes that commonly used in different variations of the method are oxygen uptake, fluorescent of R-phycoerythrin, or absorbance of ABTS. The basic reactions of the assay are similar to those of ORAC assay. The oxidation of the probe is followed optically or by fluorescence. The antioxidant activity has been determined as time to consume all

of the antioxidant, by extension of the lag time for appearance of oxidized probe when antioxidants are present, and by the percent reduction of a reaction. TRAP values are usually expressed as a lag time or reaction time of the sample compared to corresponding time for Trolox. The TRAP assay involves the initiation of lipid peroxidation by generating water-soluble peroxyl radicals and is sensitive to all known chain breaking antioxidants, but it is relatively complex and time consuming to perform, requiring a high degree of expertise and experience [62].

Superoxide anion scavenging activity assay

The superoxide anion scavenging activity assay is based on the inhibition of the production of nitroblue tetrazolium formazan by the sample. Superoxide anions are generated in a non-enzymatic phenazine methosulfate-nicotinamide adenine dinucleotide (PMS-NADH) system [63]. The superoxide anions are subsequently made to reduce nitroblue tetrazolium which yields a chromogenic product that is measured at 560 nm [64].

Hydrogen peroxide scavenging activity assay

The hydrogen peroxide scavenging activity assay is used to investigate the ability of the sample to scavenge hydrogen peroxide (H_2O_2) [65]. This is based on a decrease in absorbance of H_2O_2 at 230 nm upon oxidation of H_2O_2 [66].

Nitric oxide scavenging activity assay

The nitric oxide scavenging activity assay measures the ability of the sample to scavenge nitric oxide. The nitric oxide ($NO^{\bullet}$) is generated from sodium nitroprusside at physiological pH (7.2). Under the aerobic conditions, $NO^{\bullet}$ reacts with oxygen to produce stable products (nitrate and nitrite). The nitrate and nitrite react with sulphanilic acid and couple with naphthylethylenediamine (Griess reagent), producing pink color that can be measured at 546 nm [65, 67].

Total phenolic contents

There are several methods available for analyzing the total phenol contents in plant including precipitation with heavy metals, precipitation by the addition of organic compounds, oxidation under controlled conditions, and formation of colored products with various chemical elements. The most recently established procedure for analyzing total phenolic content is the Folin-Ciocalteu assay developed by Singleton and Rossi [68]. Folin-Ciocalteu assay is based on the oxidation of a phenolate ion from the sample and the reduction of the phosphotungstic-phosphomolybdic reagent, which also known as Folin-Ciocalteu reagent. The result of this reaction turns the chromophore moiety from a yellow complex to a blue phosphotungstic-phosphomolybdic complex. The reaction must take place under alkaline conditions in order to aid with the uptake to oxygen by the phenol, which occurs most efficiently near the pH 10 of the system to control through the addition of sodium cyanide, sodium hydroxide, or more commonly with sodium carbonate. The blue color is relative stable and has a broad light absorption peak that can be measured on a spectrophotometer. The concentration of the total phenolics is proportional to the intensity of the light absorption near 760 nm [69]. The

absorbance values of the samples are then compared to a standard phenolic compound such as gallic acid, tannic acid, catechin, and tyrosine. Therefore, the total phenolic contents for the sample are expressed in term of standard equivalent [70]. The Folin-Ciocalteu assay suffers from a number of interfering compounds particularly sugars, aromatic amines, sulfurdioxide, ascorbic acid, enediols, reductones, organic acid, and Fe (II). These compounds may react with Folin-Ciocalteu reagent to give elevated apparent phenolic contents [62].

Total tannins

Numerous methods have been devised to quantitatively or qualitatively determine the presence of tannins and related phenols in plant materials. The total tannins determination may be based on precipitation with protein, absorption of tannins on insoluble protein (hide powder or polyvinyl-pyrrolidone), precipitation with heavy metal salts, formation of colored products by oxidation of the tannins, and UV measurement [71].

High performance liquid chromatography (HPLC)

Chromatography is a separation technique that is mostly employed in chemical analysis. This technique separates compounds in a mixture due to the different time taken for each compound to travel through a stationary phase when carried through it by a mobile phase [72].

HPLC is a chromatographic technique which involves a solid or liquid stationary phase coated on solid supporter, normally placed inside a stainless steel column, and a liquid mobile phase. It is currently the most commonly used analytical technique for quantitative and qualitative analysis of chemical compounds in plant extracts. The separations of analytes or compounds are represented by the peak in the chromatogram. Moreover, the detection of analytes can be performed using a variety of instrument detectors such as UV photometric detector, photo diode array detector, fluorescence detector, differential refractive index detector, electrical conductivity detector, and mass spectroscopy detector. According to a recent survey, it is found that 85% of pharmaceutical analyzes use absorbance detectors such as UV photometric and photo diode array detectors. Therefore, HPLC analysis is applied to identify compounds based on retention time and absorbance spectrum of each chromatographic peak and to distinguish different plant extracts based on chromatogram fingerprints. The qualitative analysis data indicates which components are present in the sample. The quantitative analysis data provides the actual amounts of components in the sample. The goal of most HPLC analysis is the separation of one or more analytes from other components in the sample in order to obtain quantitative information for each analyte. The concentration of the analytes in the sample is obtained from the chromatographic peak area or peak

height. Peak areas or peak heights in the chromatogram are proportional to the concentration of the analytes and quantitation is done by using calibration curve of the standard compound [8, 72-74].

Reverse-phase HPLC (RP-HPLC) is the most common HPLC technique. A numerous of compounds can be separated by RP-HPLC. This type of HPLC is performed on a non-polar stationary phase with a polar mobile phase. Eighteen carbon atoms (C18) column is widely used as a stationary phase in RP-HPLC. The mobile is typically a mixture of organic solvent (acetonitrile, methanol, and isopropanol, etc.) and water. The mobile phase selection depends on the type of HPLC, the nature of the analyte, the choice of stationary phase, and the type of detector used for the analyte measurement [73].

However, the selection of equipment for analysis of chemical components in plant extracts depends on the purpose of the analysis and the properties of components in the extracts to be analyzed. Most phenolic compounds (i.e. flavonoids, anthroquinone, coumarins, and anthocyanins), alkaloids, amino acid, protein and other types of natural compounds can be qualitatively and quantitatively analyzed by HPLC. These compounds have strong UV absorption; therefore UV is a good detector for the analytes. Nevertheless, UV detector is not a good choice for saponins and other compounds that have no UV absorption due to the lack of conjugated double bond [8].

HPLC and gas chromatography (GC) are the most widely used analytical equipments for plant study because of their high resolving power and low limit of detection, good accuracy and reproducibility. GC is used only to separate

compounds that are naturally volatile or that can be converted to volatile derivatives. HPLC is particularly suitable for analysis of natural compounds including those with low volatility and poor thermal stability which GC is incapable of analyzing [74]. Consequently, the main advantages of HPLC are (1) it can be used for separation of any compounds that are soluble in liquid phase, (2) the retention time of compounds in HPLC depends on their interaction with both mobile phase and stationary phase; therefore retention of solutes can be varied by changing the solvent passing through it. One disadvantage of HPLC is that it is less resolution or produces broader peaks than GC [75-77].

Quantitative analysis of (+)-catechin and (-)-epicatechin

There are several reports on the application of HPLC for quantitation of catechins which were present in *Acacia catechu* and *Uncaria gambir*.

In 2006, LC/ESI-MS method under the selected ion monitoring (SIM) mode was developed to quantitate the predominant catechins ((+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, and (-)-epigallocatechin-3-*O*-gallate) in the *Acacia catechu*. HPLC separation was performed on a C18 column (5 μm, 4.6 x 250 mm). The mobile phase consisted of A (0.1% formic acid in water, v/v) and B (0.1% formic acid in ACN, v/v) in gradient. The flow rate was of 1.0 ml/min with the column temperature at 25 °C. *Acacia catechu* was found to be a rich source for catechins that accumulate in both leaves and heartwood. (+)-Catechin and (-)-epicatechin are the main secondary metabolites in *Acacia catechu* heartwood and catechu resin chunks with 2.46 % and 8.89 % of dry mass respectively. In contrast, the leaves consist of (+)-catechin, (-)-epicatechin, (-)-epicatechin-3-*O*-gallate, and (-)-epigallocatechin-3-*O*-gallate as major components [5].

In 2011, the aqueous extract of *Uncaria gambir* stems and leaves from west Sumatra, Indonesia were prepared with the traditional method to determine the catechin and epicatechin contents by HPLC. The analysis was used a Develosil ODS-HG-5 column (4.6 i.d. x 150 mm) and at a flow rate of 0.7 ml/min. The mobile phase was the mixture of ACN/water/acetic acid (10:88:2, v/v). The linear gradient was achieved within 30 min. The detection was set at 280 nm with a UV/Vis detector. The results demonstrated that high catechin contents were found in 4 kinds of gambir extract whereas epicatechin and caffeic acid were detected at very low concentrations. Catechin and epicatechin content of gambir extract ranged from 99.4-108.5 μg/ml and 0.49-0.80 μg/ml respectively [24].

Raw *Uncaria gambir* (pale catechu) was analyzed using HPLC to investigate the major chemical constituents. Moreover, 3 different solvent extracts (ethyl acetate, methanol, and hot water) of raw pale catechu were also analyzed to determine (+)-catechin content. The HPLC analyses were performed using Shimadzu AD-VP equipped with Chromolith SemiPrep RP-18 column (100-10 mm) at a flow rate of 0.5 ml/min. The detection was set at 280 nm using a UV detector. Two different mobile phases were solvent A, ACN/water (50:50, v/v) with 1% acetic acid and solvent B, ACN/water (5:95, v/v) with 1% acetic acid. Five standard compounds ((+)-catechin hydrate, (-)-epicatechin, (-)-epicatechin gallate, (-)-gallocatechin, and (-)-epigallocatechin gallate) were used in this study. The results from qualitative analysis showed that raw *Uncaria gambir* consisted of (+)-catechin as a major comnponent and (-)-gallocatechin and (-)-epigallocatechin gallate as minor components. For quantitative analysis, the ethyl acetate pale catechu extract gave the highest

catechin content (87.33 %) when compared to methanol (59.47 %) and hot water (55.26 %) extracts [25].

In 2012, epicatechin content in ethanolic bark extract of *Acacia catechu* using HPLC analysis was investigated. The estimation was performed using C18 column (Luna, 5µm, 4.9x 150mm) with an isocratic mode. The flow rate was of 1.0ml/min. The mixture of %5acetic acid, methanol with a ratio of 85:15 v/v was used as mobile phase. The detection was set at 280nm with UV/Vis detector. The results showed that epicatechin content present in *Acacia catechu* ethanolic bark extract was found to be %4.156 w/w [78].

Validation of analytical procedures

Method validation is a process used to confirm and demonstrate the performance characteristics of an analytical methodology. The purpose of method validation is to ensure that the methodology is accurate, specific, reproducible, and robust [79, 80].

According to International Conference on Harmonization (ICH) guideline for the validation of analytical procedures, the validation characteristics recommended are specificity, linearity, range, limit of detection (LOD), limit of quantitation (LOQ), accuracy, precision (repeatability and intermediate precision), and robustness [80].

Specificity

Specificity is the ability to determine impurities in the analyte. For chromatographic procedures, the representative chromatograms should be used to demonstrate specificity. Purity test is commonly used to ensure that all the analytical methods performed allow an accurate statement of the impurity content of an analyte. However, the procedures used to demonstrate specificity is depend on the analytical method.

Linearity

Linearity is the ability of analytical method within a given range to obtain test results which are directly proportional to the analyte concentration in the sample. According to ICH guideline, a minimum of 5 concentration levels is recommended for establishment of linearity. Acceptability of linearity data is estimated from examining the correlation coefficient (r^2) of the linear regression line for the response versus

concentration plot. The correlation coefficient of the linear regression line should be above 0.99.

Range

Range is the interval between the upper and lower concentration of analyte in the sample which have been demonstrated that the analytical method has a suitable level of accuracy, precision, and linearity. Moreover, the range is commonly expressed in the same unit as the test results obtained by the analytical method.

Limit of detection (LOD)

The limit of detection is the lowest concentration of an analytes in a sample which can be detected but not quantitated. There are several approaches for determining the LOD. Based on the visual evaluation, the LOD is estimated by the analysis of the sample with known concentration of the analyte and by determining the minimum level at which the analyte can be reliably detected. Based on signal-to-noise, it is expressed as a concentration at a specified ratio 3:1. In addition, the LOD is also calculated based on the standard deviation of the response ($\boldsymbol{\sigma}$) and the slope of the calibration curve (S) at levels approximating the LOD according to the formula: LOD = 3.3($\boldsymbol{\sigma}$)/S. The standard deviation of the response is determined based on the standard deviation (SD) of the blank, on the residual SD of a regression line, or the SD of y-intercepts of a regression lines.

Limit of quantitation (LOQ)

Limit of quantitation is the lowest concentration of an analyte in a sample which can be quantitatively determined with acceptable accuracy and precision. A

typical signal-to-noise ratio of 10:1 is used to determine LOQ. The calculation method is again based on the SD of the response (***σ***) and the slope of the calibration curve (*S*) according to the formula: LOD = 10(***σ***)/*S*. Again, the SD of the response is determined based on the standard deviation (SD) of the blank, on the residual SD of a regression line, or the SD of *y*-intercepts of a regression lines.

Accuracy

Accuracy is the closeness of the test results obtained by the analytical method to the true or accepted value. ICH guideline recommends that accuracy data is accessed using a minimum of 9 determinations over a minimum of 3 concentration levels covering the specified range for example 3 concentrations, 3 replicates each. Accuracy is reported as percent recovery by the assay of spiked sample with known amount of analyte. Briefly, spiked sample are prepared in triplicate at 3 levels over a range of target concentration. The analyte levels in the spiked sample are determined using the same quantitation procedure as used in the final analytical method.

Precision

Precision is the closeness of the degree of repeatability of an analytical method under normal operation. It is usually expressed as the percent relative standard deviation (RSD). The precision is considerate at 3 levels including repeatability, intermediate precision, and reproducibility.

Repeatability

Repeatability is the precision over the short time interval under the same conditions. It is also known as intra-assay precision. The repeatability is assessed from a minimum of 9 determinations covering the specified range (3 concentrations/ 3 replicates each) or from a minimum of 6 determinations at 100% of the test or target concentration.

Intermediate precision

Intermediate precision is the precision from within-laboratories variations due to random event such as different equipment, different analysts, and different days. Therefore, it is also termed as inter-assay precision.

Reproducibility

Reproducibility is the precision between laboratories. It is performed by testing homogeneous samples in multiple laboratories. However, the reproducibility is not recommended if the intermediate precision is performed.

Robustness

Robustness of an analytical method is a measure of its capacity to remain unaffected by small deliberated variations in method parameters. It is performed by varying method parameters such as pH buffer in mobile phase, different HPLC column, column temperature, flow rate etc. Robustness should be considerate early in the development of an analytical method.

CHAPTER III
MATERIALS AND METHODOLOGY

Chemicals and reagents

1. (-)-Epicatechin (CAS no. 490-46-0, purity ≥ 98 %) (Sigma-Aldrich, St. Louis, MO, USA)
2. (+)-Catechin (CAS no. 154-23-4, purity ≥ 99 %) (Sigma-Aldrich, St. Louis, MO, USA)
3. (+)-Catechin hydrate (CAS no. 225937-10-0, purity ≥ 98 %) (Sigma-Aldrich, St. Louis, MO, USA)
4. 2, 2-diphenyl-1-picylhydrazyl (DPPH) (Sigma-Aldrich, St. Louis, MO, USA)
5. Acetonitrile, HPLC grade (RCI Labscan, Thailand)
6. Beta-carotene (Fulka, USA)
7. Chloroform, HPLC grade (J.T. Baker Chemical, Phillipsburg, USA)
8. Ethyl acetate, A.R. grade (RCI Labscan, Thailand)
9. Ethylenediaminetetraacetic acid (EDTA) (Merk, Darmstadt, Germarny)
10. Ferrozine (Fulka, USA)
11. Folin-Ciocalteu reagent (Merk, Darmstadt, Germarny)
12. Formic acid (Fisher Scientific, Leicestershire, UK)
13. Hide powder (Sigma-Aldrich, St. Louis, MO, USA)
14. Hydrochloric acid 37%, A.R. grade (RCI Labscan, Thailand)
15. Iron (II) chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) (Sigma-Aldrich, St. Louis, MO, USA)
16. Iron (III) chloride ($FeCl_3 \cdot 6H_2O$) (Ajax Finechem, New Zealand)
17. Linoleic acid (Sigma-Aldrich, St. Louis, MO, USA)
18. Methanol, HPLC grade (RCI Labscan, Thailand)

19. Sodium acetate ($C_2H_3NaO_2$) (Sigma-Aldrich, St. Louis, MO, USA)
20. Sodium carbonate (Na_2CO_3) (Sigma-Aldrich, St. Louis, MO, USA)
21. Tween 20 (Merk, Darmstadt, Germarny)
22. Ultra-pure water (NW20VF, Heal Force, China)

Materials

1. 13 mm x 0.45 µm PTFE membrane syringe filters (ANPEL Scientific Instrument, China).
1. 46 mm x 0.45 µm nylon membrane filters (National Scientific, TN)
2. 96 wells plate (BRAND Plates®, Wertheim, Germany)
3. Disposable cuvettes 2.5 ml, visible range (Bibby Scientific, Staffordshire, UK)
4. Filter papers No. 4 (Whatman®, UK)
5. Filter papers No. 40 Ashless (Whatman®, UK)
6. Inersil ODS-3 column, 5 µm x 4.6 x 250 mm (GL Sciences, Tokyo, Japan)
7. Inertsil ODS-3 HPLC guard column, 5 µm x 4.0 x 10 mm (GL Sciences, Tokyo, Japan)
8. TLC silica gel 60 F_{254} (Merk, Darmstadt, Germarny)

Instrumentations

1. Chamber furnaces (Carbolite, Scientific Promotion, Bangkok, Thailand)
2. High performance liquid chromatograph (Shimadzu DGU-20A3, Shimadzu, Japan) equipped with photo diode array detector (Shimadzu SPD-M20A, Shimadzu, Japan)
3. Hot air oven (WTB binder, Scientific Promotion, Bangkok, Thailand)
4. Microplate reader (Biochrom Asys UVM 340, Bangkok, Thailand)
5. Ultra-pure water purification NW20VF (Heal Force, China)

6. Ultrasonic bath (Analytical Lab Science Co., LTD, Bangkok, Thailand)
7. UV-spectrophotometer (UV-1800 model, Shimadzu, Kyoto, Japan)

Sample collection

Twenty two samples of *Acacia catechu* water extract were purchased from 22 Thai traditional drug stores in 21 province located at four regions of Thailand as Bangkok Metropolis, Chachoengsao, Chanthaburi, Chiang Mai, Chiang Rai, Kamphaeng Phet, Kanchanaburi, Khon Kaen, Krabi, Lampang, Nakhon Pathom, Nakhon Phanom, Nakhon Si Thammarat, Phra Nakhon Si Ayutthaya, Ratchaburi, Rayong, Samut Sakhon, Samut Songkhram, Satun, Songkhla, and Tak.

Twenty samples of *Uncaria gambir* water extract were purchased from 20 Thai traditional drug stores in 18 province located at four regions of Thailand as Bangkok Metropolis, Chachoengsao, Chanthaburi, Chiang Mai, Kamphaeng Phet, Kanchanaburi, Khon Kaen, Krabi, Nakhon Pathom, Nakhon Phanom, Nakhon Si Thammarat, Phra Nakhon Si Ayutthaya, Ratchaburi, Rayong, Samut Sakhon, Samut Songkhram, Satun, and Songkhla.

Twelve samples of *Pentace burmanica* stem bark were collected from markets in 10 provinces as Buri Ram, Chaiyaphum, Chiang Rai, Nakhon Nayok, Nakhon Pathom, Phetchabun, Sa Kaeo, Si Sa Ket, Surin, and Ubon Ratchathani.

All sets of crude drugs were authenticated by Associate Professor Dr. Nijsiri Ruangrungsi. Voucher specimens were deposited at College of Public Health Sciences, Chulalongkorn University, Thailand.

Sample extraction

One milligram of *Acacia catechu* water extract or *Uncaria gambir* water extract was mixed with 1 ml of ultra-pure water. Then, the mixture was filtered and diluted to evaluate the antioxidant activities, total phenolic and total tannin contents at concentration of 100 µg/ml. The concentration of sample at 1 mg/ml was used for HPLC analysis.

Five grams of ground sample of *Pentace burmanica* stem bark was exhaustively extracted with 95% ethanol using a Soxhlet apparatus. The 95% ethanol extract was filtered through Whatman No. 4 and evaporated under vacuum. The extract yield was weighed, recorded and stored at -20 ℃ to avoid the possibility of degradation of active compound.

Standardization parameters

The standardization parameters were examined by standard methods of World Health Organization (WHO) [41].

Macroscopic examination

Each sample of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica* stem bark was identified by visual examination of the physical properties such as size, color, texture, and other visual inspection. Whole plants of *Acacia catechu*, *Uncaria gambir*, and *Pentace burmanica* were illustrated by hand drawing in proportional scale related to the real size.

Microscopic examination

The powder of *Pentace burmanica* stem bark is examined for histological characters under microscope with 10X, 20X, and 40X objective lens magnifications and 10X eyepiece lens. The powdered is mounted onto a slide in water to observation of tissue and cell structures. Pictures are taken with a digital camera and illustrated by hand drawing in proportional scale related to the original size.

Determination of loss on drying

The ground sample 3.0 g was accurately weighed in a pre-weighed small beaker and then dried at 105 °C to constant weight.

Determination of total ash

Three grams of the ground sample was placed in a pre-weighed crucible. The sample was spread in an even layer and ignited by gradually increasing the temperature to 500 °C and heated until white that ash was obtained. Then, the sample was cooled in a desiccator and weighed without delay.

Determination of acid insoluble ash

The crucible containing the total ash was added 25.0 ml of hydrochloric acid (70 g/l) then the mixture was boiled for 5 minutes. The insoluble matter was collected on an ashless filter-paper. The filter-paper containing the insoluble matter was transferred to the original crucible and ignited to constant weight. The residue was cooled in a desiccator for then weighed.

Determination of ethanol extractive value

The ground sample 5.0 g was macerated with 70 ml 95% ethanol in a closed conical flask in shaking bath for 6 hours and allowed to stand for 18 hours. The extract was filtered through Whatman No.4, washed the marc and then adjusted to 100 ml with 95% ethanol. Twenty milliliters of the filtrate was transferred to pre-weighed beaker and evaporated to dryness on a water bath. Then, the sample was dried at 105 ℃ for 6 hours, cooled in a desiccator and weighed.

Determination of water extractive value

The ground sample 5.0 g was macerated with 70 ml ultra-pure water in a closed conical flask in shaking bath for 6 hours and allowed to stand for 18 hours. The extract was filtered through Whatman No.4 washed the marc and adjusted to 100 ml with distilled water. The filtrate (20.0 ml) was transferred to pre-weighed beaker and evaporated to dryness on a water bath. Then, it was dried at 105 ℃ for 6 hours, cooled in a desiccator and weighed.

Determination of water content

Twenty five grams of the ground sample in 200 ml water-saturated toluene was subjected to an azeotropic distillation. As soon as the water was completely distilled, the inside of the condenser tube was rinsed with toluene and the distillation was continued for 1 more hours. The heat was then removed and receiving tube was allowed to cool to room temperature. The water and toluene layer were separated then the volume of water was read off.

The physico-chemical parameters including Loss on drying, total ash, acid insoluble ash, water content and extractive values were expressed as grand mean ± pooled SD [41].

Thin-layer chromatography identification

The water extract of *Acacia catechu* and *Uncaria gambir* were dissolved in methanol (5 mg/ml). Three milligrams of ethanolic extract of *Pentace burmanica* stem bark was dissolved in 1 ml methanol. Five microliters of each sample solution was applied on to a thin-layer plate coated with silica gel 60 F_{254}. The TLC plate was then placed in a chamber with chloroform, ethyl-acetate and formic acid (3: 6: 1, v/v/v) as mobile phase. After development, the plate was removed and allowed to dry at room temperature and examined under ultraviolet light with 254 nm and 365 nm. Then, the plate was sprayed with vanillin reagent and heated in an oven at 105 °C for 5 minutes.

Antioxidant activities

2, 2-diphenyl-1 picryl hydrazyl (DPPH) assay

Five hundred microliters of sample at concentration of 100 µg/ml in water was mixed with 500 µl of 120 µM DPPH solution in methanol. The incubation was performed in the dark at room temperature for 30 min. Absorbance at 517 nm was measured using a UV-spectrophotometer. A blank sample contained the same amount of distilled water and DPPH solution. (+)-Catechin hydrate was used as a positive control. Triplicate measurements were carried out. Percent scavenging activity was calculated from the following equation:

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

Ferric reducing antioxidant power (FRAP) assay

FRAP assay was performed in 96 well plates to assess antioxidant power. FRAP reagent was prepared according to the method of Benzie and Strain [81]. Briefly, the FRAP reagent was prepared by mixing 100 ml of 300 mM acetate buffer pH 3.6 with 10 ml of 10 mM 2, 4, 6-tris(2-pyridyl)-s-triazine (TPTZ) dissolved in 40 mM HCl and 10 ml of 20 mM $FeCl_3.6H_2O$ dissolved in ultra-pure water. Freshly prepared reagent was warmed at 37 °C before used. One hundred microliters of each sample (100 µg/ml) was mixed with 700 µl of the FRAP reagent for 30 min under the dark conditions. The absorbance was measured at 593 nm a microplate reader. Aqueous solutions of $FeSO_4$ in the range of 0.1-1.0 mM were used for calibration curve. The FRAP value of the sample was calculated using the following linear ($y = ax + b$) relationship from the calibration curve. Results were expressed in mM Fe (II)/mg of dry sample. In order to make comparison, (+)-catechin hydrate was also tested under the same conditions as standard antioxidant compounds. All samples were performed in triplicate.

Metal iron chelating assay

The chelating activity of the sample on Fe^{2+} was measured according to the method of Gupta *et al*. [82]. One hundred microliters of sample at the concentration of 100 µg/ml was incubated with 7.5 µl of 2 mM $FeCl_2$ for 5 min. The reaction was started by addition of 30 µl ferrozine (5 mM). After 10 min, the absorbance of ferrous iron-ferrozine complex at 562 nm was measured using a microplate reader. EDTA served as positive control. All determinations were performed in triplicate. The ability of the sample to chelate ferrous ion was calculated using the following equation:

$$\text{Chelating activity (\%)} = [(\text{Absorbance}_{\text{control}} - \text{Absorbance}_{\text{sample}}) / \text{Absorbance}_{\text{control}}] \times 100$$

Beta-carotene bleaching assay

Beta-carotene bleaching assay was performed in cuvette to investigate the lipid peroxidation activity. Briefly, 1 mg of beta-carotene, 40 mg of linoleic acid, and 400 mg of Tween 20 were mixed in 4 ml of chloroform. Then chloroform was removed at 40 ℃ under vacuum. The mixture was immediately diluted with 100 ml of ultra-pure water then the mixture was vigorous agitated for 5 min using ultrasonic bath to form an emulsion. Aliquots of the emulsion (1 ml) were transferred into different cuvettes which contained 250 µl of sample (100 µg/ml). The mixture was then gently mixed and placed in a water bath at 50 ℃ for 180 min. Absorbance of the sample was recorded at 0 min and 180 min at 470 nm using a UV-spectrophotometer. All determinations were performed in triplicate. (+)-Catechin hydrate was used as positive controls. The negative control was ultra-pure water. The degradation bleaching rates of beta-carotene was evaluated as the percent of antioxidant capacity using the following equation:

Antioxidant capacity (%) = $[1-(A_0-A_{180})/(C_0-C_{180})] \times 100$

A_0, A_{180} : absorbance at zero time and end time of incubation for test sample respectively

C_0, C_{180} : absorbance at zero time and end time of incubation for test control respectively

Total phenolic content

The total phenolic content of sample was determined using the Folin-Ciocalteu reagent. Eight hundreds microliters of sample extracts (100 µg/ml) and 200 µl of 15% Folin-Ciocalteu reagent were added in the test tube then adjusted the volume to 2.0 ml with ultra-pure water. The mixture was left for 5 min. After that 1.0 ml Na_2CO_3 (0.106 g/ml) is added. The incubation was performed in the dark at room temperature for 60 min. The absorbance was measured at 756 nm using a UV-spectrophotometer. The total phenolic content in all sample extracts were calculated from the following linear ($y = ax + b$) relationship from the calibration curve of (+)-catechin hydrate and the results were expressed as micrograms of catechin equivalents (CE) per 100 µg dry weights of crude drug (DW). The data were reported as a mean ± standard deviation for three replications.

Total tannin content and non-tannin phenolic content

The total tannin content was estimated by Folin-Ciocalteu assay. Briefly, 3.5 mg of hide powder was weighed, and then 500 ml of sample (100 µg/ml) was added in the test tube. The mixture was shaken for 60 min afterwards centrifuged for 10 min and finally the supernatant was collected. The supernatant has only simple phenolic compounds other than tannins. The tannins would have been precipitated along with the hide powder. The phenolic content of the supernatant was then measured following the same procedure describe above. The content of non-tannin phenols was expressed as micrograms of catechin equivalents (CE) per 100 µg dry weights of

crude drug. Total tannin content was determined by subtraction of non-tannin phenolic content from total phenolic content. All samples were performed in triplicate and the data are reported as a mean ± standard deviation.

(+)-Catechin and (-)-epicatechin analysis by HPLC

The determination of (+)-catechin and (-)-epicatechin contents were performed by HPLC analysis.

Chromatographic conditions

Shimadzu DGU-20A3 HPLC (Shimadzu, Japan) consisted of a binary solvent delivery system, an auto-sampler, a column temperature controller, and a photo diode array detector (Shimadzu SPD-M20A, Shimadzu, Japan). System control and data analysis were processed with Shimadzu LC Solution software. The chromatographic separation was accomplished with an Inersil ODS-3 column (5 µm x 4.6 x 250 mm) and an Inertsil ODS-3 HPLC guard column (5 µm x 4.0 x 10 mm) using water containing 0.1% formic acid (A) and acetonitrile containing 0.1% formic acid (B) as mobile phase at a flow rate of 1 ml/min. The isocratic program was set at 20% B for 15 min. The mobile phases were filtered through 0.45 µm nylon membrane filters and degassed using an ultrasonic bath before analysis. The column temperature was maintained at 40 °C and the injection volume was 1 µl. The wavelength was set at 280 nm.

Preparation of standard solution

The stock solution of (+)-catechin and (-)-epicatechin were prepared by dissolving 1 mg of each compound in 1 ml of methanol. The solution was filtered through a 0.45 µm PTFE membrane syringe filter.

Preparation of sample solution

One miligram of sample extract was dissolved in 1 ml of methanol and vortex for 1 min. Then the sample was filtered through a 0.45 µm PTFE membrane syringe filter before chromatographic analysis.

Method validation

The validation of analytical procedures: text and methodology Q2(R1) guideline which established by the ICH (International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use) was employed for validation of analytical method [80].

Specificity

The specificity was evaluated by peak purity test. Peak purity index of the analyte was processed with Shimadzu LC Solution software. It was determined by caomparing all the spectra within the chromatographic peak to the reference spectrum at the peak apex.

Linearity

Linearity was determined by the calibration curves that obtain from the HPLC analysis of (+)-catechin and (-)-epicatechin. The stock solutions of (+)-catechin and (-)-epicatechin were dissolved in methanol to give concentrations of 5, 10, 50, 100, and 200 µg/ml for evaluate the calibration curves. The calibration curves of these two compounds were fitted by linear regression. The regression equation was calculate in the form of $y = ax + b$, where y and x were peak area and concentration respectively.

Limit of detection (LOD) and limit of quantitation (LOQ)

LOD and LOQ were calculated based on the residual standard deviation (Residual standard deviation = square roots $\Sigma(y-y_{est})^2/(n-2)$) of a regression lines (σ) and the slope of the calibration curve (S) as follows:

$$LOD = 3.3(\sigma)/S$$

$$LOQ = 10(\sigma)/S$$

Accuracy

The accuracy of each sample was determined by recovery method. The sample was spiked with (+)-catechin (50, 100, and 150 µg/ml) and (-)-epicatechin (50, 100, and 150 µg/ml) then the recoveries were calculated by comparing the amount of those standards with the amount of original added.

Precision

The precision of each sample was evaluated at two levels including repeatability and intermediate precision. The relative standard deviation (RSD) of 9 determinations covering the specific range (3 concentrations and 3 replicates each) was evaluated and analyzed on one day and three consecutive days.

Robustness

(+)-Catechin and (-)-epicatechin were used to evaluate the robustness of the analytical method [83]. The robustness was determined for variations in flow rates (0.995 and 1.005 ml/min) and variations in column temperature (39 and 41 °C). The percentage of RSD was calculated to evaluate whether the flow rate and temperature variations alter the results of HPLC analysis.

CHAPTER IV
RESULTS

Acacia catechu water extract

Macroscopic examination

Black catechu (*Acacia catechu* water extract) was blackish-brown, shining, and heavy. The taste was bitter. Most of samples were round shape (3.5-5.0 cm) but the samples which purchased from Kanchanaburi and Chiang Rai were cylindrical shape (Figure 8, 9). The whole plant of *Acacia catechu* was illustrated in figure 10.

Figure 8 *Acacia catechu* water extracts (Round shape)

Figure 9 *Acacia catechu* water extracts (Cylindrical shape)

Figure 10 The whole plant of *Acacia catechu*

Physico-chemical evaluation

Loss on drying, total ash, acid insoluble ash, water content and extractive values parameters were performed to evaluate the pharmacognostic specifications of *Acacia catechu* water extract. The physico-chemical parameters of *Acacia catechu* water extract from 22 different sources throughout Thailand were demonstrated in table 2. Each sample was performed in triplicate.

Table 2 Physico-chemical parameters (% by weight) of *Acacia catechu* water extract

No. of sample	Loss on drying	Total ash content	Acid insoluble ash content	Ethanol extractive value	Water extractive value	Water content
1	2.95 ± 0.06	88.37 ± 0.48	28.08 ± 2.03	0.26 ± 0.08	3.17 ± 0.17	2.80 ± 0.40
2	3.99 ± 0.01	66.78 ± 0.04	58.86 ± 0.22	20.59 ± 0.34	28.17 ± 0.76	5.60 ± 3.18
3	10.27 ± 0.10	17.66 ± 0.44	8.76 ± 0.63	62.69 ± 2.39	75.07 ± 5.08	9.07 ± 0.23
4	2.49 ± 0.03	83.79 ± 0.20	75.95 ± 0.05	4.23 ± 0.30	10.22 ± 0.83	4.53 ± 2.44
5	10.38 ± 0.05	2.60 ± 0.01	0.54 ± 0.02	79.37 ± 4.14	68.44 ± 5.50	9.87 ± 1.22
6	16.01 ± 0.05	5.28 ± 0.11	2.88 ± 0.13	64.03 ± 2.28	88.39 ± 3.64	10.00 ± 0.69
7	2.94 ± 0.02	79.72 ± 0.06	71.81 ± 0.62	11.11 ± 0.64	18.17 ± 1.24	3.87 ± 1.01
8	8.39 ± 0.04	37.71 ± 0.16	29.59 ± 0.76	44.63 ± 0.62	52.31 ± 3.30	6.80 ± 0.69
9	2.00 ± 0.013	82.13 ± 0.09	74.06 ± 0.35	9.84 ± 0.29	15.51 ± 0.31	2.93 ± 0.46
10	1.69 ± 0.06	90.22 ± 0.11	82.43 ± 0.02	0.62 ± 0.05	4.88 ± 0.33	2.93 ± 0.23
11	17.56 ± 0.55	3.00 ± 0.12	0.37 ± 0.07	87.03 ± 7.69	68.98 ± 10.85	9.73 ± 1.85
12	2.83 ± 0.05	83.28 ± 0.33	75.18 ± 0.39	5.42 ± 0.35	12.16 ± 0.14	2.67 ± 0.61
13	1.88 ± 0.06	88.48 ± 0.53	23.73 ± 0.41	1.00 ± 0.07	6.19 ± 0.79	2.27 ± 0.46
14	1.45 ± 0.01	89.68 ± 0.14	81.21 ± 0.69	0.12 ± 0.03	6.47 ± 0.46	3.33 ± 1.15
15	2.69 ± 0.00	82.19 ± 0.36	74.49 ± 0.26	3.72 ± 0.55	11.79 ± 1.16	2.53 ± 0.61
16	3.23 ± 0.03	75.90 ± 0.16	68.04 ± 0.42	15.48 ± 0.63	19.62 ± 1.71	3.07 ± 0.23
17	1.75 ± 0.05	90.77 ± 0.26	83.18 ± 0.24	0.20 ± 0.09	2.83 ± 0.23	2.27 ± 1.01
18	1.93 ± 0.02	88.81 ± 0.24	80.64 ± 0.38	0.22 ± 0.06	5.55 ± 0.20	3.33 ± 0.61

Table 2 Physico-chemical parameters (% by weight) of *Acacia catechu* water extract (Continue)

No. of sample	Loss on drying	Total ash content	Acid insoluble ash content	Ethanol extractive value	Water extractive value	Water content
19	2.01 ± 0.04	81.49 ± 0.21	73.80 ± 0.06	10.09 ± 1.40	15.26 ± 1.38	2.80 ± 0.40
20	2.26 ± 0.02	90.86 ± 0.05	82.85 ± 0.06	0.03 ± 0.02	3.13 ± 0.29	2.93 ± 1.29
21	6.40 ± 0.80	55.82 ± 7.21	16.62 ± 1.07	62.87 ± 5.19	37.23 ± 4.55	3.73 ± 0.61
22	6.59 ± 0.02	29.67 ± 0.15	23.18 ± 0.17	6.68 ± 0.54	44.04 ± 5.04	9.07 ± 1.62

Thin-layer chromatography identification

TLC fingerprint of *Acacia catechu* water extract was shown in figure 11.

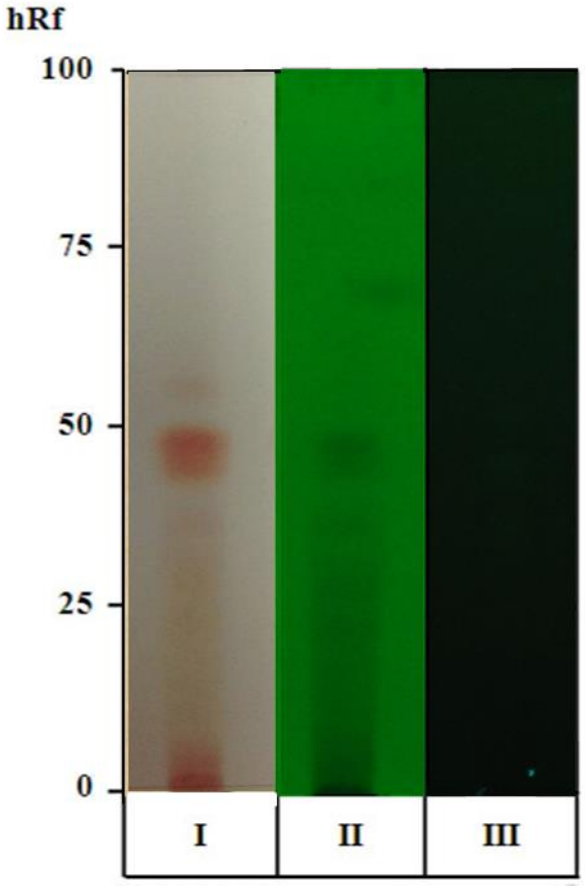


Figure 11 TLC fingerprint of *Acacia catechu* water extract

Solvent system Chloroform: ethyl acetate: formic acid (3: 6: 1)

Detection

I: Detection with vanillin reagent

II: Detection under UV light 254 nm

III: Detection under UV light 365 nm

Antioxidant activities

The different mechanisms of antioxidant activities including free radical scavenging of DPPH in the DPPH assay, the reduction of ferric ions in the FRAP assay, the chelation of ferrous ions in the metal ion chelating assay, and the peroxidation inhibition in the beta-carotene bleaching assay were evaluated. The percentage of free radical scavenging activity varied from 2.62 - 75.47 in *Acacia catechu* water extracts. FRAP values of *Acacia catechu* water extracts were of 0.0 - 0.57 mM $FeSO_4$/100 µg DW. The percentage of chelating activity of *Acacia catechu* water extracts were ranged between 1.97 - 16.12; while the chelating activity of EDTA standard was of 98.39%. The peroxidation inhibition of *Acacia catechu* water extracts were of 13.07 – 40.96%. The percentage of free radical scavenging activity, chelating activity and FRAP value of (+)-catechin hydrate were found to be 82.66 ± 0.24%, 2.59 ± 1.87% and 0.542 ± 0.003 mM $FeSO_4$/100 µg DW respectively. The peroxidation inhibition of catechin hydrate (100 µg/ml) was found to be 18.12 ± 3.62% (Table 3).

Total phenolic, non-tannin phenolic and total tannin contents of commercial *Acacia catechu* ranged from 0.0-68.77, 0.0-67.51, and 0.0-4.43 µg CE/100 µg DW respectively (Table 4).

The values of antioxidant activities, total phenolics, non-phenolics, and total tannins were found to be different among different sources of the samples. The results showed that the phenolic contents of *Accacia catechu* water extracts were correlated with the antioxidant power as shown in Figure 12.

Table 3 The antioxidant activities of *Acacia catechu* water extracts from 22 different sources throughout Thailand

No. of sample	DPPH Inhibition (%)	FRAP value*	Ferrous ion chelating activity (%)	Beta-carotene bleaching (%)
1	3.73 ± 0.96	0.007 ± 0.003	8.47 ± 1.08	14.64 ± 1.39
2	44.68 ± 1.12	0.112 ± 0.001	7.30 ± 5.18	20.28 ± 2.28
3	71.37 ± 1.77	0.432 ± 0.017	7.80 ± 2.46	28.53 ± 2.73
4	16.21 ± 1.01	0.031 ± 0.003	7.28 ± 4.49	23.48 ± 3.73
5	69.77 ± 0.78	0.566 ± 0.010	2.14 ± 1.26	30.18 ± 1.68
6	70.08 ± 2.48	0.500 ± 0.019	3.97 ± 2.35	31.65 ± 1.71
7	36.36 ± 0.42	0.081 ± 0.001	7.87 ± 2.68	26.80 ± 3.24
8	75.47 ± 0.64	0.305 ± 0.008	3.09 ± 5.01	29.84 ± 0.98
9	39.16 ± 0.82	0.055 ± 0.003	3.35 ± 1.75	27.04 ± 0.85
10	4.33 ± 0.51	nd	3.95 ± 3.92	26.62 ± 0.97
11	68.58 ± 4.33	0.543 ± 0.010	4.35 ± 0.66	38.14 ± 0.88
12	24.42 ± 0.43	0.050 ± 0.001	7.63 ± 1.54	30.16 ± 0.58
13	6.99 ± 0.45	0.013 ± 0.001	2.97 ± 1.80	26.88 ± 1.41
14	5.69 ± 1.78	nd	3.54 ± 0.38	26.24 ± 0.40
15	24.29 ± 0.83	0.044 ± 0.004	16.12 ± 4.01	13.07 ± 2.48
16	38.88 ± 0.92	nd	2.09 ± 0.65	34.36 ± 1.46
17	3.65 ± 0.37	0.085 ± 0.001	1.97 ± 0.50	32.79 ± 1.03
18	2.62 ± 0.29	nd	4.38 ± 0.58	32.34 ± 0.37

* mM $FeSO_4$/100 µg crude drug

Table 3 The antioxidant activities of *Acacia catechu* water extracts from 22 different sources throughout Thailand (Continue)

No. of sample	DPPH Inhibition (%)	FRAP value*	Ferrous ion chelating activity (%)	Beta-carotene bleaching (%)
19	29.62 ± 2.08	0.006 ± 0.005	6.37 ± 1.62	36.76 ± 0.50
20	10.56 ± 2.11	0.056 ± 0.003	10.77 ± 6.16	36.23 ± 3.48
21	24.29 ± 0.73	nd	7.25 ± 4.45	39.31 ± 1.73
22	68.92 ± 0.45	0.035 ± 0.002	9.49 ± 2.86	40.96 ± 1.84
(+)-Catechin hydrate	82.66 ± 0.24	0.542 ± 0.003	2.59 ± 1.87	18.12 ± 3.62
EDTA	-	-	98.39 ± 0.16	

* mM $FeSO_4$/100 µg crude drug

Table 4 The total phenolics, non-tannin phenolics and total tannins of *Acacia catechu* water extracts from 22 different sources throughout Thailand

No. of sample	Total phenolics*	Non-tannin phenolics*	Total tannins*
1	nd	nd	nd
2	5.16 ± 0.02	5.02 ± 0.04	0.14
3	59.77 ± 0.11	56.61 ± 0.20	3.16
4	nd	nd	nd
5	68.77 ± 0.03	67.51 ± 0.09	1.27
6	55.05 ± 0.14	50.62 ± 0.18	4.43
7	4.66 ± 0.04	4.21 ± 0.02	0.45
8	31.25 ± 0.20	29.56 ± 0.11	1.70
9	3.64 ± 0.05	3.50 ± 0.08	0.15
10	nd	nd	nd
11	60.23 ± 0.23	55.87 ± 0.16	4.36
12	3.20 ± 0.03	3.17 ± 0.04	0.03
13	nd	nd	nd
14	nd	nd	nd
15	nd	nd	nd
16	1.64 ± 0.03	1.67 ± 0.33	nd
17	nd	nd	nd
18	nd	nd	nd
19	0.91 ± 0.03	0.71 ± 0.03	0.19
20	nd	nd	nd

*μg CE/100 μg crude drug, nd: not detected

Table 4 The total phenolics, non-tannin phenolics and total tannins of *Acacia catechu* water extracts from 22 different sources throughout Thailand (Continue)

No. of sample	Total phenolics*	Non-tannin phenolics*	Total tannins*
21	0.73 ± 0.02	0.65 ± 0.03	0.08
22	21.71 ± 0.08	18.50 ± 0.13	3.21

*µg CE/100 µg crude drug, nd: not detected

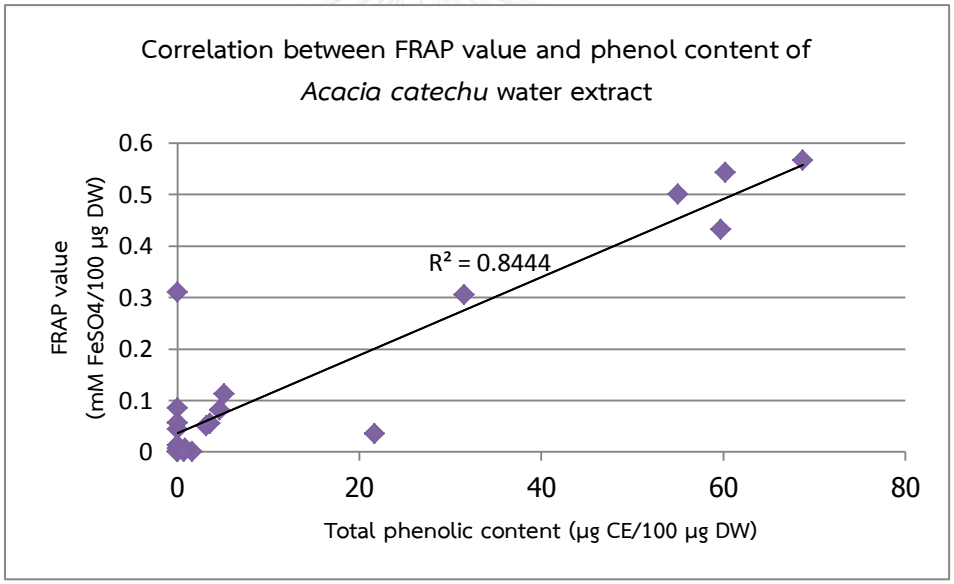


Figure 12 Correlation between FRAP value and phenolic content of *Acacia catechu* water extract

Quantitative analysis of (+)-catechin and (-)-epicatechin

Quantitative analysis of (+)-catechin and (-)-epicatechin in Thai crude drugs under the name of Si-Siat were performed by HPLC analysis. (+)-Catechin and (-)-epicatechin were identified by comparing the retention time and UV spectrum of each peak with those of standard compounds. The quantitation of catechins was evaluated by comparing the area under peak with the calibration curve.

The HPLC chromatograms of *Acacia catechu* water extract (Figure 13) showed both (+)-catechin and (-)-epicatechin peaks. Some *Acacia catechu* samples were found to be rich source for (+)-catechin and (-)-epicatechin. The concentrations of (+)-catechin and (-)-epicatechin in *Acacia catechu* water extract were range from 0 – 236.28 µg/mg and 0 – 160.12 µg/mg of crude drug respectively (Table 5).

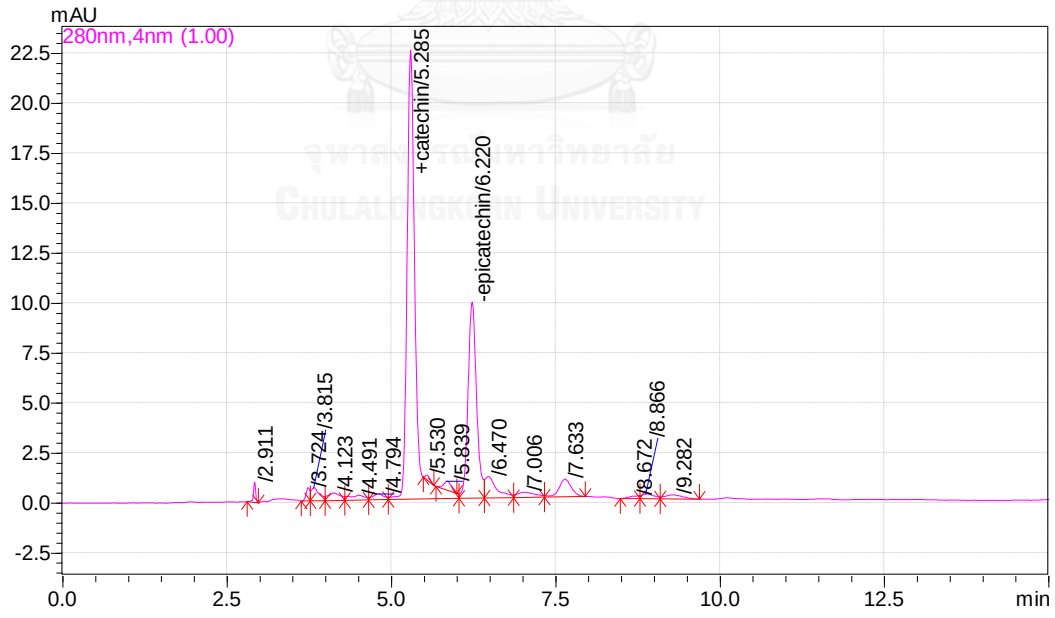


Figure 13 HPLC chromatograms of *Acacia catechu* water extract

Table 5 (+)-Catechin and (-)-epicatechin contents of *Acacia catechu* water extracts from 22 different sources throughout Thailand

No. of sample	(+)-Catechin*	(-)-Epicatechin*
1	< LOQ	nd
2	30.70 ± 0.40	< LOQ
3	132.13 ± 0.18	84.22 ± 0.66
4	< LOQ	< LOD
5	182.51 ± 1.67	152.51 ± 3.46
6	168.04 ± 0.55	116.07 ± 0.09
7	23.14 ± 0.37	< LOQ
8	24.45 ± 0.28	< LOQ
9	< LOQ	< LOQ
10	< LOD	nd
11	236.28 ± 1.06	160.12 ± 2.43
12	14.86 ± 0.09	< LOQ
13	< LOD	nd
14	< LOD	nd
15	< LOQ	< LOD
16	26.12 ± 0.21	< LOQ
17	< LOD	nd
18	nd	nd
19	17.08 ± 0.02	< LOQ
20	nd	nd

*μg/mg of crude drug, nd: not detected

Table 5 (+)-Catechin and (-)-epicatechin contents of *Acacia catechu* water extracts from 22 different sources throughout Thailand (Continue)

No. of sample	(+)-Catechin*	(-)-Epicatechin*
21	< LOQ	< LOD
22	< LOQ	nd

*μg/mg of crude drug, nd: not detected

***Uncaria gambir* water extract**

Macroscopic examination

Pale catechu (*Uncaria gambir* water extract) was small cylindrical in shape around 2.0-3.0 cm. The external was brown and internal was light brown or pale orange (Figure 14). It was easy to break and bitter taste. Figure 15 was illustrated the whole plant of *Uncaria gambir*.

Figure 14 *Uncaria gambir* water extract

Figure 15 The whole plant of *Uncaria gambir*

Physico-chemical evaluation

Loss on drying, total ash, acid insoluble ash, water content and extractive values parameters were performed to evaluate the pharmacognostic specifications of *Uncaria gambir* water extract. Table 6 illustrated the physico-chemical parameters of *Uncaria gambir* water extracts from 20 different sources throughout Thailand.

Table 6 Physico-chemical parameters (% by weight) of *Uncaria gambir* water extract

No. of sample	Loss on drying	Total ash content	Acid insoluble ash content	Ethanol extractive value	Water extractive value	Water content
1	11.68 ± 0.56	26.95 ± 2.35	10.04 ± 1.91	66.00 ± 2.71	41.03 ± 4.34	8.53 ± 1.22
2	11.61 ± 0.06	21.82± 0.54	14.67 ± 0.57	66.68 ± 4.61	45.13 ± 1.77	10.00 ± 1.74
3	9.93 ± 0.06	35.29 ± 0.40	29.89 ± 1.52	52.12 ± 2.57	42.00 ± 0.09	5.53 ± 1.67
4	10.49 ± 0.11	29.35 ± 1.08	21.36 ± 0.43	55.84 ± 3.23	64.63 ± 3.23	9.47 ± 1.62
5	10.41 ± 0.15	29.26 ± 0.79	22.74 ± 0.47	53.28 ± 12.08	50.52 ± 1.97	9.47 ± 0.61
6	13.11 ± 0.06	4.50 ± 0.17	1.20 ± 0.18	94.76 ± 6.49	45.30 ± 2.02	12.80 ± 1.60
7	9.33 ± 0.26	31.93 ± 0.10	24.66 ± 0.13	45.98 ± 8.41	45.12 ± 2.92	7.87 ± 0.83
8	11.71 ± 0.03	20.91 ± 0.10	13.95 ± 0.22	72.28 ± 7.92	47.94 ± 3.29	11.33 ± 1.22
9	14.06 ± 0.03	5.93 ± 0.23	2.44 ± 0.23	87.87 ± 11.02	38.27 ± 4.55	13.47 ± 1.15
10	12.13 ± 0.11	12.04 ± 0.78	6.48 ± 0.49	87.73 ± 4.98	46.15 ± 3.66	9.73 ± 1.29
11	8.98 ± 0.27	35.12 ± 1.45	19.21 ± 1.56	58.69 ± 9.35	34.2 ± 3.60	8.80 ± 0.80
12	8.50 ± 0.07	34.68 ± 058	27.45 ± 0.81	58.25 ± 4.00	40.46 ± 0.49	9.47 ± 1.51
13	12.80 ± 0.21	5.46 ± 0.30	1.41 ± 0.02	84.03 ± 9.87	42.93 ± 2.66	13.60 ± 0.80
14	13.09 ± 0.04	4.57 ± 0.06	1.22 ± 0.21	91.37 ± 5.03	49.44 ± 4.27	13.33 ± 1.01
15	8.15 ± 0.11	44.33 ± 0.25	39.82 ± 0.29	42.96 ± 6.38	43.90 ± 1.78	8.27 ± 2.60
16	13.18 ± 004	23.54 ± 056	17.19 ± 1.02	64.42 ± 1.84	50.74 ± 3.00	12.00 ± 1.06
17	12.62 ± 0.03	5.54 ± 0.06	1.78 ± 0.07	84.87 ± 7.54	47.02 ± 0.68	12.80 ± 0.40
18	8.54 ± 0.17	44.90 ± 0.34	38.87 ± 0.21	50.68 ± 2.44	42.76 ± 6.21	8.40 ± 1.06
19	11.27 ± 0.08	22.96 ± 0.84	5.89 ± 0.25	71.13 ± 3.13	40.58 ± 1.05	9.87 ± 1.67
20	10.23 ± 0.14	34.20 ± 0.31	26.78 ± 0.20	41.59 ± 3.11	40.02 ± 0.18	8.53 ± 0.83

Thin-layer chromatography identification

TLC fingerprint of *Uncaria gambir* water extract was shown in figure 16.

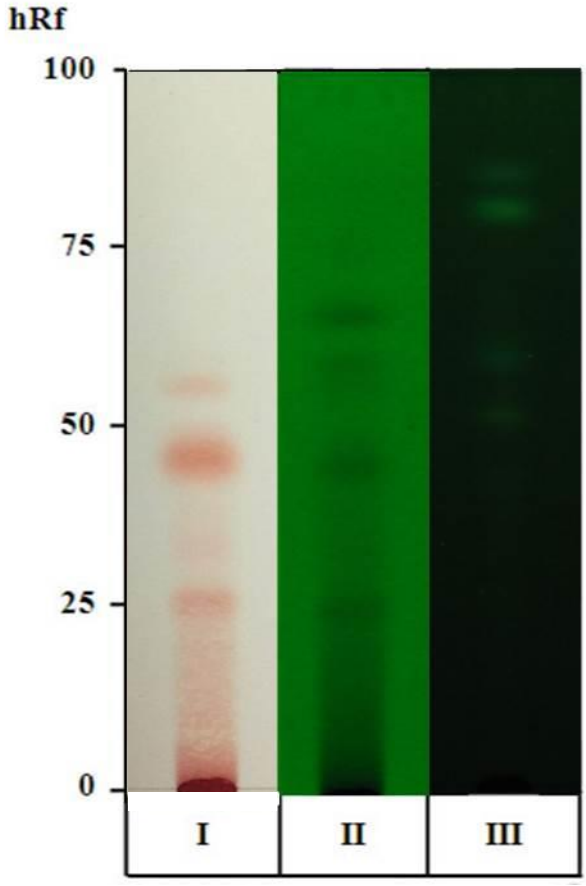


Figure 16 TLC fingerprint of *Uncaria gambir* water extract

Solvent system Chloroform: ethyl acetate: formic acid (3: 6: 1)

Detection

I: Detection with vanillin reagent

II: Detection under UV light 254 nm

III: Detection under UV light 365 nm

Antioxidant activities

Free radical scavenging of DPPH in the DPPH assay, the reduction of ferric ions in the FRAP assay, the chelation of ferrous ions in the metal ion chelating activity, and the peroxidation inhibition in the beta-carotene bleaching assay were evaluated. The antioxidant activities of *Uncaria gambir* water extracts from 20 different sources throughout Thailand were depicted in table 7. The percent of free radical scavenging activity in *Uncaria gambir* water extracts varied from 74.18 - 77.06. FRAP values of *Uncaria gambir* water extracts were of 0.17 - 0.39 mM $FeSO_4$/100 µg DW. The chelating activity (%) of *Uncaria gambir* water extracts were ranged between 0.65 - 7.59 and the percent chelating activity of EDTA standard was of 98.39. The peroxidation inhibition of *Uncaria gambir* water extracts were of 14.80% - 49.19%. (+)-Catechin hydrate showed the percentage of free radical scavenging activity, chelating activity, peroxidation inhibition and FRAP value of were found to be 82.66 ± 0.24%, 2.59 ± 1.87%, 18.12 ± 3.62% and 0.542 ± 0.003 mM $FeSO_4$/100 µg DW.

Total phenolic, non-tannin phenolic, and total tannin contents of *Uncaria gambir* water extract ranged from 19.55-58.64, 18.21-58.18, and 0.09-1.54 µg CE/100 µg DW respectively (Table 8).

Table 7 The antioxidant activities of *Uncaria gambir* water extracts from 20 different sources throughout Thailand

No. of sample	DPPH Inhibition (%)	FRAP value*	Ferrous ion chelating activity (%)	Beta-carotene bleaching (%)
1	75.48 ± 1.23	0.228 ± 0.004	3.85 ± 3.49	14.80 ± 4.01
2	75.02 ± 1.32	0.256 ± 0.002	2.83 ± 2.48	19.47 ± 4.19
3	76.09 ± 0.17	0.185 ± 0.011	7.59 ± 4.12	24.94 ± 2.73
4	76.64 ± 0.60	0.219 ± 0.006	2.19 ± 2.45	22.21 ± 0.87
5	76.05 ± 0.24	0.246 ± 0.008	1.72 ± 1.65	25.06 ± 0.84
6	75.18 ± 1.02	0.350 ± 0.006	1.28 ± 2.35	30.59 ± 1.69
7	76.62 ± 0.39	0.214 ± 0.004	2.33 ± 1.46	27.91 ± 1.57
8	74.18 ± 0.67	0.250 ± 0.010	3.01 ± 0.43	30.96 ± 0.17
9	77.06 ± 0.37	0.389 ± 0.003	2.11 ± 1.12	34.07 ± 1.52
10	74.71 ± 0.54	0.319 ± 0.002	2.02 ± 1.19	34.91 ± 3.20
11	75.54 ± 0.95	0.235 ± 0.003	4.06 ± 1.79	32.60 ± 2.78
12	72.25 ± 0.48	0.169 ± 0.004	1.20 ± 2.35	32.57 ± 2.81
13	75.56 ± 0.45	0.362 ± 0.001	5.16 ± 3.10	18.29 ± 1.83
14	75.58 ± 0.31	0.297 ± 0.004	3.53 ± 1.67	38.88 ± 1.50
15	76.07 ± 0.39	0.232 ± 0.002	3.48 ± 1.37	42.94 ± 1.26
16	75.04 ± 1.81	0.212 ± 0.001	3.96 ± 2.29	39.95 ± 6.60
17	74.53 ± 1.01	0.340 ± 0.003	3.74 ± 4.66	41.50 ± 4.76
18	75.75 ± 1.17	0.224 ± 0.003	1.46 ± 0.35	42.80 ± 6.17

* mM $FeSO_4$/100 µg crude drug

Table 7 The antioxidant activities of *Uncaric gambir* water extracts from 20 different sources throughout Thailand (Continue)

No. of sample	DPPH Inhibition (%)	FRAP value*	Ferrous ion chelating activity (%)	Beta-carotene bleaching (%)
19	74.31 ± 1.14	0.269 ± 0.009	0.65 ± 0.73	42.57 ± 6.75
20	74.30 ± 1.88	0.226 ± 0.006	nd	49.19 ± 1.77
(+)-Catechin hydrate	82.66 ± 0.24	0.542 ± 0.003	2.59 ± 1.87	18.12 ± 3.62
EDTA	-	-	98.39 ± 0.16	

* mM $FeSO_4$/100 µg crude drug

Table 8 The total phenolics, non-tannin phenolics and total tannins of *Uncaria gambir* water extracts from 20 different sources throughout Thailand

No. of sample	Total phenolics*	Non-tannin phenolics*	Total tannins*
1	19.55 ± 0.03	18.21 ± 0.08	1.33
2	29.60 ± 0.05	28.09 ± 0.78	1.51
3	51.00± 0.14	49.60 ± 0.44	1.35
4	29.58 ± 0.14	28.72 ± 0.39	0.86
5	47.55 ± 0.14	46.02 ± 0.21	1.54
6	53.73± 0.20	52.68 ± 0.18	1.05
7	24.81 ± 0.10	23.59 ± 0.17	1.23
8	27.93± 0.18	27.09 ± 0.05	0.84
9	32.23± 0.11	31.77 ± 0.12	0.47
10	42.33 ± 0.11	41.06 ± 0.20	1.27
11	45.94± 0.28	44.76 ± 0.20	1.18
12	21.81 ± 0.11	21.17 ± 0.30	0.64
13	36.95± 0.09	36.05 ± 0.20	0.09
14	41.03 ± 0.18	39.82 ± 0.38	1.21
15	28.28± 0.05	27.47 ± 0.33	0.81
16	29.85± 0.19	28.90 ± 0.23	1.00
17	58.64 ± 0.03	58.18 ± 0.19	0.46
18	32.817± 0.40	31.56 ± 0.34	1.25
19	35.40± 0.12	34.07 ± 0.40	1.33
20	35.95 ± 0.22	34.94 ± 0.27	1.01

*μg CE/100 μg crude drug, nd: not detected

Quantitative analysis of (+)-catechin and (-)-epicatechin

The HPLC chromatograms *Uncaria gambir* showed high peak of (+)-catechin and small peak of (-)-epicatechin (Figure 17). Both (+)-catechin and (-)-epicatechin peaks were shown in 5 samples of *Uncaria gambir* but the others samples were shown only (+)-catechin peak in the chromatogram. High (+)-catechin contents (183.90 – 633.78 µg/mg of crude drug) were found in all *Uncaria gambir* samples whereas (-)-epicatechin were detected at very low concentrations (0 – 9.30 µg/mg of crude drug). The contents of (+)-catechin and (-)-epicatechin in *Uncaria gambir* water extracts from 20 different sources throughout Thailand were illustrated in table 9.

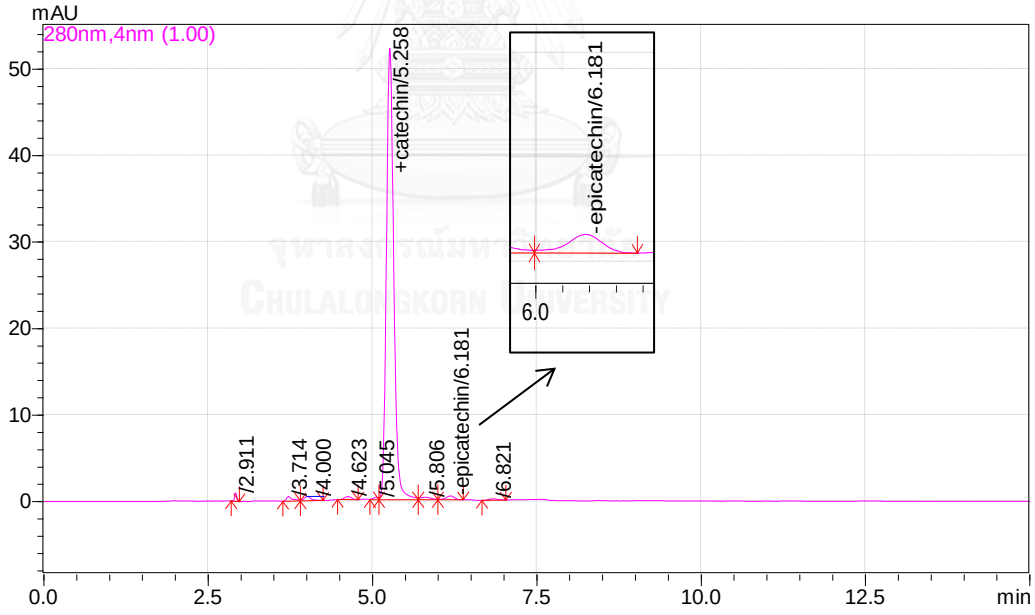


Figure 17 HPLC chromatograms of *Uncaria gambir* water extract

Table 9 (+)-Catechin and (-)-epicatechin contents of *Uncaria gambir* water extracts from 20 different sources throughout Thailand

No. of sample	(+)-Catechin*	(-)-Epicatechin*
1	283.33 ± 0.42	nd
2	312.02 ± 1.56	nd
3	248.66 ± 3.40	nd
4	328.88 ± 1.69	nd
5	220.77 ± 1.38	nd
6	450.30 ± 0.65	< LOQ
7	238.87 ± 1.14	nd
8	356.65 ± 9.11	nd
9	477.44 ± 0.43	< LOQ
10	409.59 ± 7.23	nd
11	277.88 ± 2.56	nd
12	183.90 ± 0.26	nd
13	633.78 ± 5.26	< LOQ
14	444.93 ± 1.69	< LOQ
15	218.61 ± 0.49	nd
16	205.08 ± 1.94	nd
17	387.87 ± 2.70	< LOQ
18	278.43 ± 1.44	nd
19	261.51 ± 0.98	nd
20	242.09 ± 2.10	nd

*μg/mg of crude drug, nd: not detected

Pentace burmanica

Macroscopic examination

The figure 18 showed reddish brown to brown color in dried stem bark of *Pentace burmanica*. The whole plant of *Pentace burmanica* was illustrated in figure 19.

Figure 18 *Pentace burmanica* stem bark

Figure 19 The whole plant of *Pentace burmanica*

Microscopic examination

The anatomical investigations of dried *Pentace burmanica* stem bark were demonstrated in the figure 20 and 21.

The histological investigation of *Pentace burmanica* stem bark powdered was illustrated in figure 22. Several histological characters including fragment of fibers, resin masses, tannin masses, starch grain, calcium oxalate, and fragment of parenchyma were found in powders of *Pentace burmanica* stem bark.

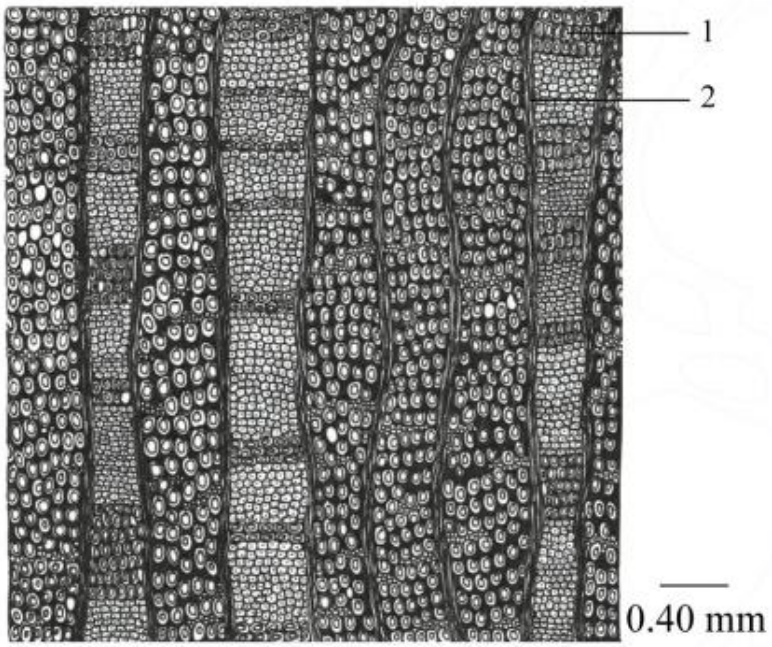


Figure 20 Transverse section of *Pentace burmanica* stem bark
1. Bordered pits 2. Pitted fiber

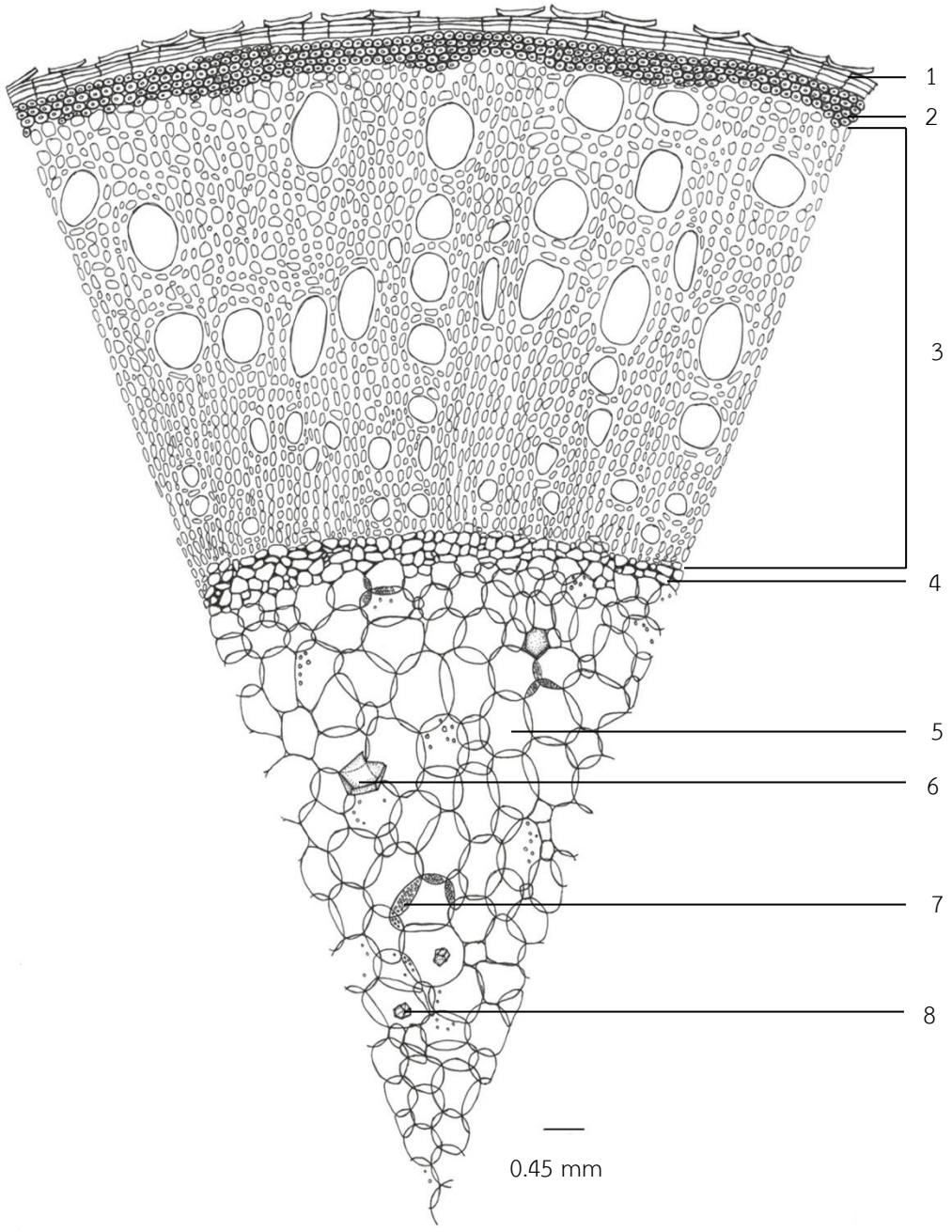


Figure 21 Transverse section of *Pentace burmanica* stem

1. Periderm 2. Cortical fiber 3. Cortex 4. Vascular cambium 5. Parenchyma 6. Resin mass 7. Parenchyma containing starch grain 8. Prism crystal of calcium oxalate

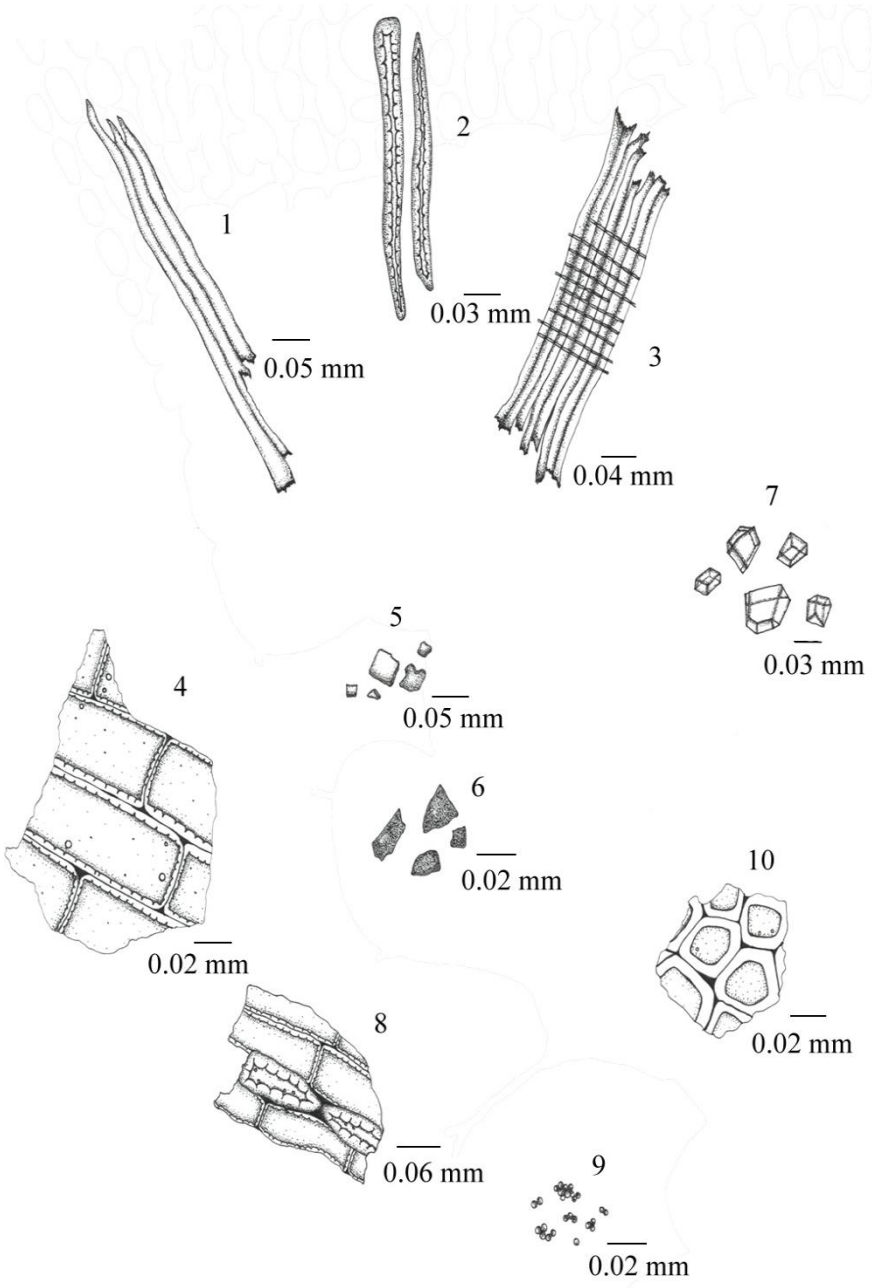


Figure 22 Powdered *Pentace burmanica* stem bark

1. Fragment of fiber 2. Sclerenchyma scleried 3. Fragment of xylem ray in radial longitudinal view 4. Parenchyma in longitudinal view 5. Resin masses 6. Tannin masses 7. Prism crystal of calcium oxalate 8. Parenchyma with sclerieds in longitudinal view 9. Starch grain 10. Cork in surface view

Physico-chemical evaluation

Physico-chemical parameters including loss on drying, total ash, acid insoluble ash, water content and extractive values parameters were performed to evaluate the pharmacognostic specifications of *Pentace burmanica* stem bark. The physic-chemical parameters of *Pentace burmanica* stem bark from 12 different sources throughout Thailand were shown in Table 10. The total ash, acid insoluble ash, loss on drying, water content, ethanol and water soluble extractive values were found to be 3.88, 0.54, 9.10, 10.54, 23.72 and 20.65 % of dry weight respectively.

Table 10 Physico-chemical parameters (% by weight) of *Pentace burmanica* stem bark

Parameters	**Mean ± SD***	**Range****
Total ash content	3.88 ± 0.07	3.66 – 4.09
Acid insoluble ash content	0.54 ± 0.02	0.47 – 0.61
Loss on drying content	9.10 ± 0.13	8.72 – 9.47
Water content	10.51 ± 1.34	6.49 – 14.54
Ethanol extractive value	23.72 ± 2.84	15.19 – 32.25
Water extractive value	20.65 ± 2.69	12.58 – 28.71

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, Samples were from 12 different sources throughout Thailand. Each sample was performed in triplicate.

Thin-layer chromatography identification

TLC fingerprint of *Pentace burmanic* stem bark was shown in figure 23.

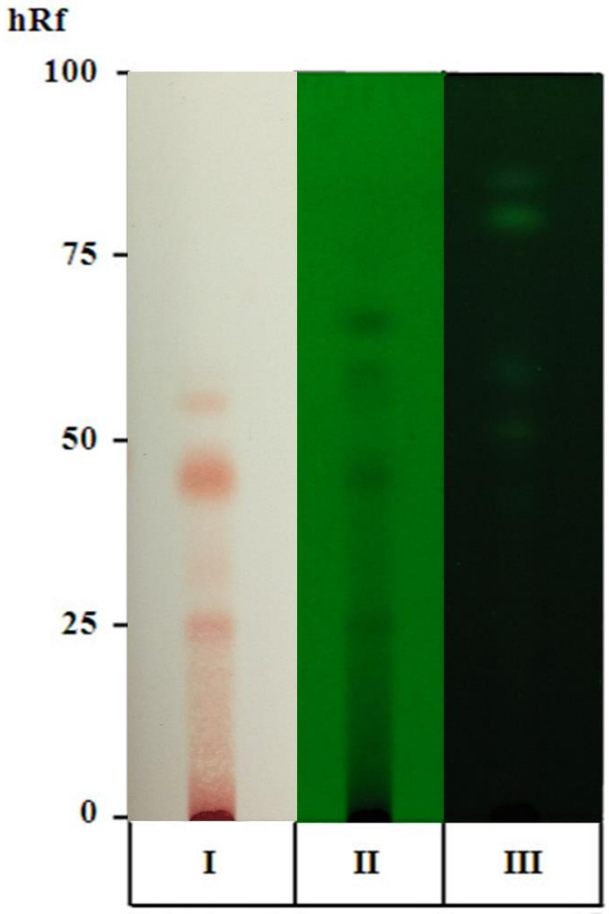


Figure 23 TLC fingerprint of *Pentace burmanica* stem bark

Solvent system Chloroform: ethyl acetate: formic acid (3: 6: 1)

Detection

I: Detection with vanillin reagent

II: Detection under UV light 254 nm

III: Detection under UV light 365 nm

Antioxidant activities

The antioxidant activities including free radical scavenging of DPPH in the DPPH assay, the reduction of ferric ions in the FRAP assay, the chelation of ferrous ions in the metal ion chelating activity, and the peroxidation inhibition in the beta-carotene bleaching assay were evaluated.

The ethanolic extract of *Pentace burmanica* stem bark at the concentration of 100 µg/ml showed free radical scavenging activity of 71.56% – 80.26% in DPPH assay. FRAP values were ranged between 0.09 – 0.29 mM $FeSO_4$/100 µg crude extract. The percentages of chelating activity of *Pentace burmanica* stem bark ethanolic extracts were of 4.24 – 12.14. The chelating activity of EDTA standard was of 98.39%. The ethanolic extract of *Pentace burmanica* stem bark (100 µg/ml) showed the peroxidation inhibition ranged between 22.76% - 41.06%. For (+)-catechin hydrate, the percentage of free radical scavenging, chelating activity, peroxidation inhibition, and FRAP value were found to be 82.66 ± 0.24%, 2.59 ± 1.87%, 18.12 ± 3.62%, and 0.542 ± 0.003 mM $FeSO_4$/100 µg DW respectively (Table 11).

Table 12 revealed that the ethanolic extract of *Pentace burmanica* stem bark contained phenolic, non-tannin phenolic and tannin contents with the range between 35.85 – 51.56, 14.08 – 40.66, and 10.90 – 21.77 µg CE/100 µg crude extract respectively. Moreover, the extract yields of *Pentace burmanica* stem bark from 12 different sources throughout Thailand were detailed in table 12.

Table 11 The antioxidant activities of *Pentace burmanica* extracts from 12 different sources throughout Thailand

No. of sample	DPPH Inhibition (%)	FRAP value*	Ferrous ion chelating activity (%)	Beta-carotene bleaching (%)
1	78.63 ± 1.64	0.18 ± 0.01	19.64 ± 4.88	22.76 ± 12.07
2	79.76 ± 0.50	0.16 ± 0.02	13.94 ± 1.53	32.59 ± 5.63
3	77.49 ± 1.24	0.14 ± 0.03	15.50 ± 1.00	33.27 ± 9.29
4	80.01 ± 1.63	0.18 ± 0.03	13.71 ± 1.63	31.66 ± 4.78
5	71.56 ± 1.04	0.10 ± 0.03	12.10 ± 2.42	41.06 ± 7.61
6	80.26 ± 0.48	0.22 ± 0.05	15.40 ± 0.95	28.11 ± 5.80
7	79.45 ± 1.33	0.19 ± 0.01	13.19 ± 5.59	32.28 ± 7.36
8	80.08 ± 0.22	0.19 ± 0.01	17.61 ± 5.55	39.09 ± 9.59
9	78.88 ± 0.58	0.29 ± 0.03	13.14 ± 2.12	32.13 ± 4.40
10	79.38 ± 0.82	0.09 ± 0.02	11.86 ± 1.17	32.96 ± 2.69
11	76.48 ± 1.61	0.21 ± 0.05	14.80 ± 2.95	27.40 ± 8.88
12	79.45 ± 1.84	0.21 ± 0.04	13.06 ± 4.65	34.45 ± 12.24
(+)-Catechin hydrate	82.66 ± 0.24	0.542 ± 0.003	2.59 ± 1.87	18.12 ± 3.62
EDTA	-	-	98.39 ± 0.16	

* mM $FeSO_4$/100 µg crude extract

Table 12 The total phenolics, non-tannin phenolics, total tannins, and the extract yields of *Pentace burmanica* from 12 different sources throughout Thailand

No. of sample	Total phenolics*	Non-tannin phenolics*	Total tannins*	Extract yield (% w/w)
1	42.20 ± 0.12	28.55 ± 0.61	13.65	33.65
2	45.58 ± 0.47	27.20 ± 0.21	18.38	42.36
3	39.80 ± 0.22	18.56 ± 0.20	21.24	18.15
4	46.66 ± 0.17	29.32 ± 0.37	17.34	32.49
5	35.85 ± 0.16	14.08 ± 0.08	21.77	22.13
6	46.85 ± 0.10	32.31 ± 0.53	14.54	34.90
7	47.16 ± 0.09	32.81 ± 0.07	14.35	34.26
8	44.84 ± 0.18	28.41 ± 1.09	16.43	32.54
9	45.41 ± 0.07	31.89 ± 0.25	13.51	37.80
10	51.56 ± 0.18	40.66 ± 0.92	10.90	35.89
11	50.38 ± 0.46	35.59 ± 0.02	14.80	33.37
12	47.67 ± 0.34	29.97 ± 0.06	17.70	34.90

* µg CE/100 µg crude extract, nd: not detected

Quantitative analysis of (+)-catechin and (-)-epicatechin

HPLC chromatogram of *Pentace burmanica* stem bark extract showed several chemical compounds containing in the extract (Figure 24). Both (+)-catechin and (-)-epicatechin peaks were found in the chromatogram. (+)-Catechinin was detected in *Pentace burmanica*, but it cannot be determined quantitatively due to low concentration (< LOQ); whereas (-)-epicatechin was found to be 59.74 ± 1.69 µg/mg of crude extract. The maximum content of (-)-epicatechin was 91.55 µg/mg of crude extract; while the minimum was 10.66 µg/mg of crude extract (Table 13).

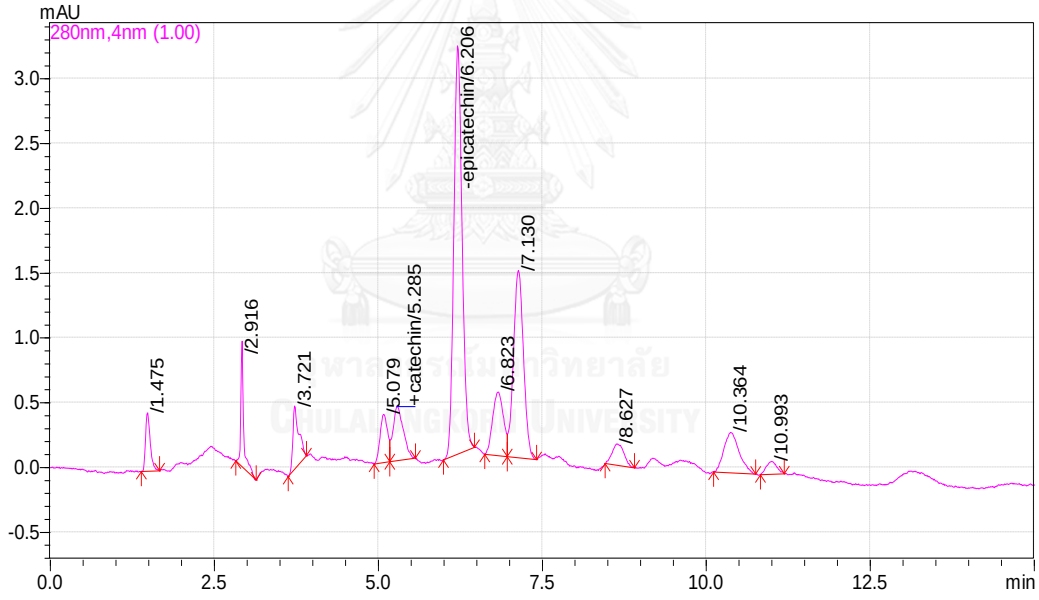


Figure 24 HPLC chromatograms of *Pentace burmanica* stem bark extract

Table 13 (+)-Catechin and (-)-epicatechin contents of *Pentace burmanica* extracts from 12 different sources throughout Thailand

No. of sample	(+)-Catechin*	(-)-Epicatechin*
1	< LOQ	43.60 ± 1.27
2	< LOQ	66.89 ± 2.24
3	< LOQ	21.37 ± 0.04
4	< LOQ	57.87 ± 1.86
5	< LOQ	10.65 ± 0.10
6	17.50 ± 0.31	89.84 ± 0.84
7	< LOQ	62.35 ± 2.81
8	18.09 ± 0.16	71.17 ± 2.07
9	< LOQ	83.23 ± 1.73
10	16.35 ± 0.13	91.55 ± 1.99
11	< LOQ	51.70 ± 2.02
12	15.88 ± 0.77	66.65 ± 0.57

*μg/mg of crude extract, nd: not detected

The antioxidant activities of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica* were demonstrated in table 14.

Table 14 The antioxidant activities of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica*

Sample	DPPH Inhibition (%)	FRAP value*	Ferrous ion chelating activity (%)	Beta-carotene bleaching (%)
Acacia catechu water extract	2.62 – 75.47	0.0 – 0.57	1.97 – 16.12	13.07 – 40.96
Uncaria gambir water extract	74.18 – 77.06	0.17 – 0.39	0.65 – 7.59	14.80 – 49.19
Pentace burmanica	71.56 – 80.26	0.09 – 0.29	4.24 – 12.14	22.76 – 41.06

* mM $FeSO_4$/100 µg crude extract

Method validation

The tests of linearity, LOD, LOQ, precision, accuracy, specificity, and robustness were investigated for the validation of an analytical method followed by ICH guideline [80].

(+)-Catechin and (-)-epicatechin at 5 concentration levels were investigated for linearity of the HPLC method. The calibration curves of both standard compounds were linear in the range of 5-200 µg/ml. The regression equation of (+)-catechin and (-)-epicatechin were $y = 746.29x - 2203.3$ and $y = 517.61x - 652.07$ respectively (Figure 25, 26). Correlation values of two standard compounds were above 0.99. The LOD values, taken as the lowest concentration of analyte in a sample which can be detected were found to be 4.80 µg/ml for (+)-catechin and 5.14 µg/ml for (-)-epicatechin. The LOQ values, taken as the lowest concentration of analyte in a sample which can be quantitively determined were 14.54 µg/ml for (+)-catechin and 15.57 µg/ml for (-)-epicatechin.

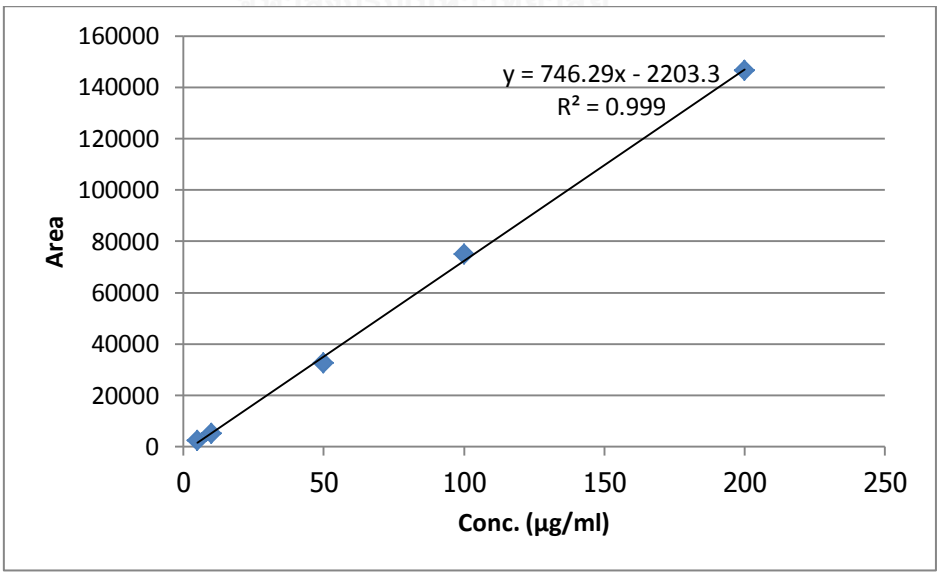


Figure 25 The calibration curve of (+)-catechin

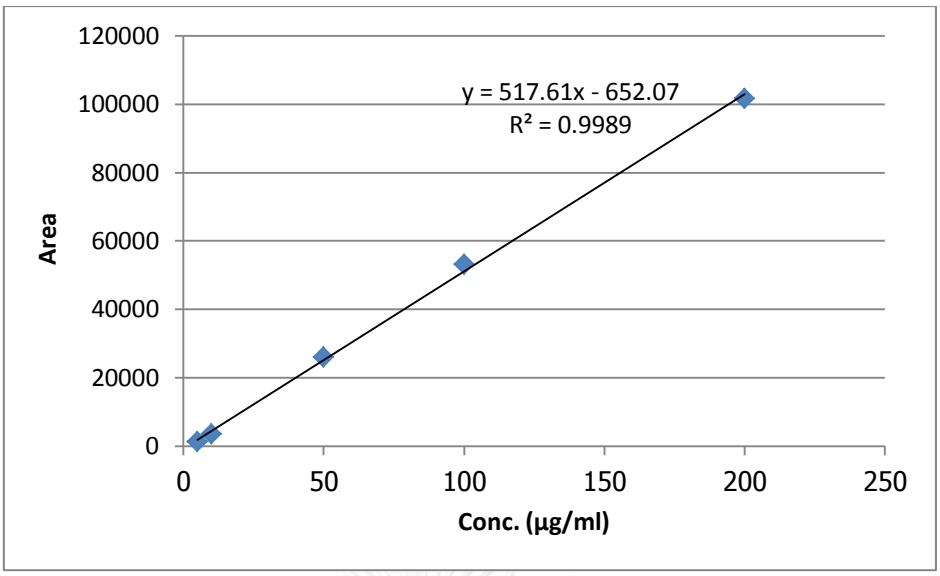


Figure 26 The calibration curve of (-)-epicatechin

The precision of all Si-Siad samples were conducted as % RSD of 9 determinations covering the specific range. The accuracy was determined by recovery test. The results of precision and accuracy of (+)-catechin and (-)-epicatechin of 3 Si-Siad samples were illustrated in Table 15-17. The percent RSD of repeatability and intermediate precision were found to be less than 3. The recoveries of (+)-catechin and (-)-epicatechin in *Acacia catechu* extract were ranged from 82.0 - 98.2% and 96.0 - 110.0% respectively. For *Uncaria gambir*, the recoveries of (+)-catechin were 80.0 – 111.8% and the recoveries of (-)-epicatechin were 91.3 – 114.3%. The recoveries of both (+)-catechin and (-)-epicatechin of *Pentace burmanica* extract were of 91.1 - 97.0% and 88.5 - 93. 8% respectively.

Table 15 Precision and accuracy of (+)-catechin and (-)-epicatechin in *Acacia catechu* water extract

Compound	Spike concentration (µg/ml)	%RSD		% recovery (n = 3)
		Repeatability precision (n = 9)	Intermediate precision (n = 3)	
(+)-Catechin	50	0.20	1.11	98.2
	100	0.36	1.10	97.6
	150	0.26	0.68	82.0
(-)-Epicatechin	50	0.26	0.67	96.0
	100	0.43	1.58	102.6
	150	0.14	0.91	110.0

Table 16 Precision and accuracy of (+)-catechin and (-)-epicatechin in *Uncaria gambir* water extract

Compound	Spike concentration (µg/ml)	%RSD		% recovery (n = 3)
		Repeatability precision (n = 9)	Intermediate precision (n = 3)	
(+)-Catechin	50	0.16	1.86	111.8
	100	0.68	1.44	96.9
	150	0.27	1.73	80.0
(-)-Epicatechin	50	0.79	2.46	114.3
	100	0.26	1.23	91.3
	150	0.29	2.71	102.4

Table 17 Precision and accuracy of (+)-catechin and (-)-epicatechin in *Pentace burmanica* stem bark water extract

Compound	Spike concentration (µg/ml)	%RSD		% recovery (n = 3)
		Repeatability precision (n = 9)	Intermediate precision (n = 3)	
(+)-Catechin	50	0.42	1.66	93.1
	100	0.37	2.07	97.0
	150	0.27	2.93	91.1
(-)-Epicatechin	50	0.33	0.97	87.1
	100	0.31	0.76	93.8
	150	0.62	1.13	88.5

The specificity was performed by peak purity checking. The peak purity test is useful to show that the analyte chromatographic peak is not attributable to more than one component. The results showed that peak purity index of both catechins were more than 0.99**.** The peak purity of (+)-catechin and (-)-epicatechin peaks of *Acacia catechu* water extract, *Uncaria gambir* water extract, and *Pentace burmanica* stem bark extract were illustrated in figure 27-32 respectively.

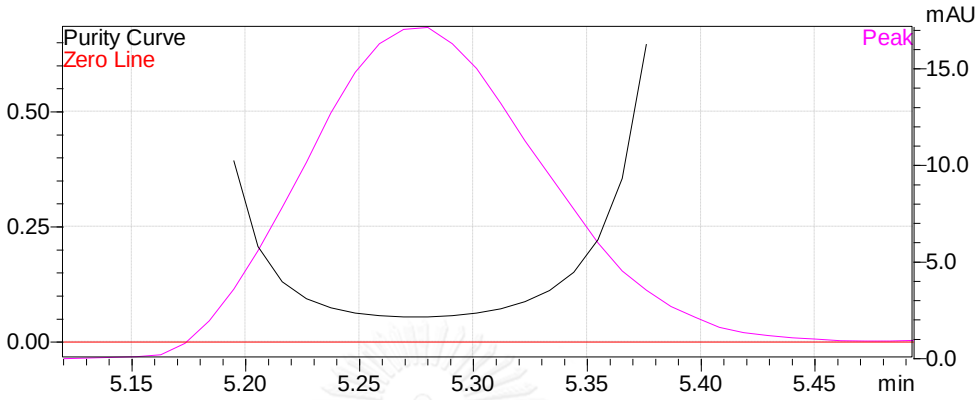


Figure 27 Peak purity of (+)-catechin in *Acacia catechu* water extract

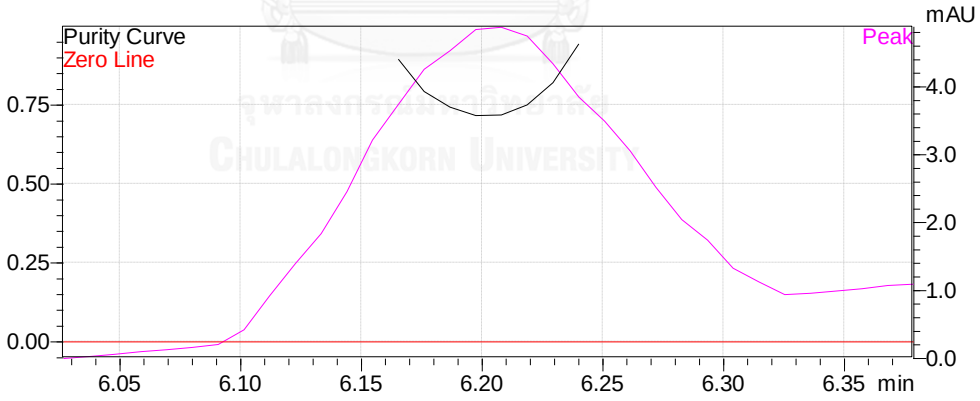


Figure 28 Peak purity of (-)-epicatechin in *Acacia catechu* water extract

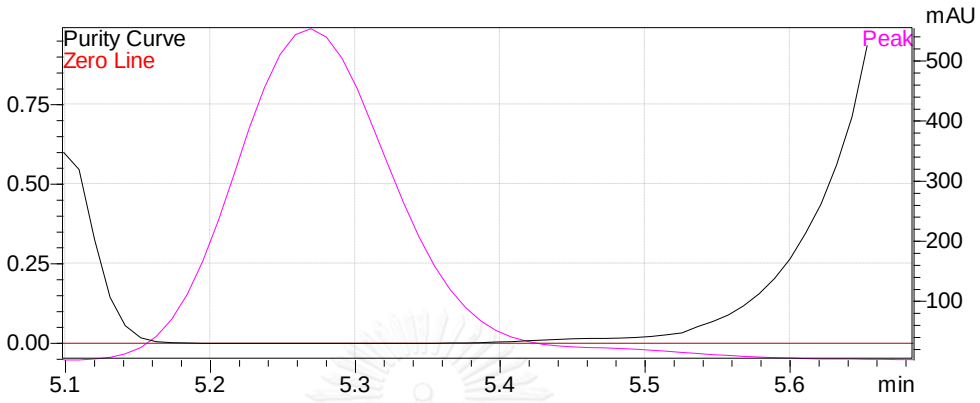


Figure 29 Peak purity of (+)-catechin in *Uncaria gambir* water extract

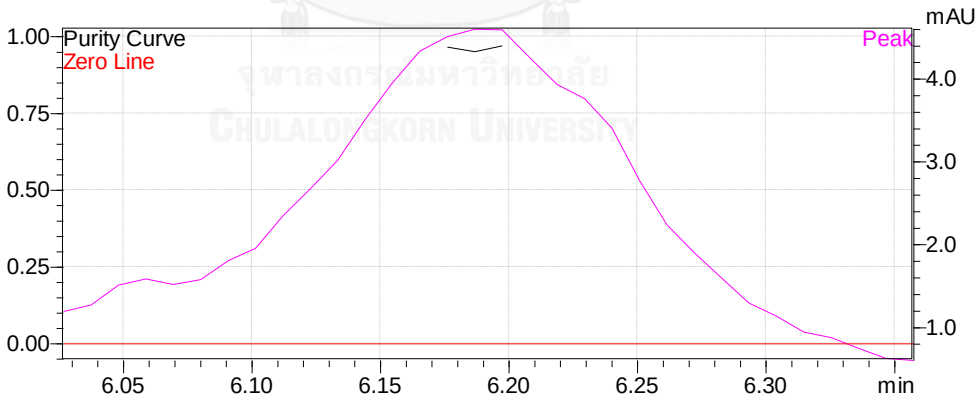


Figure 30 Peak purity of (-)-epicatechin in *Uncaria gambir* water extract

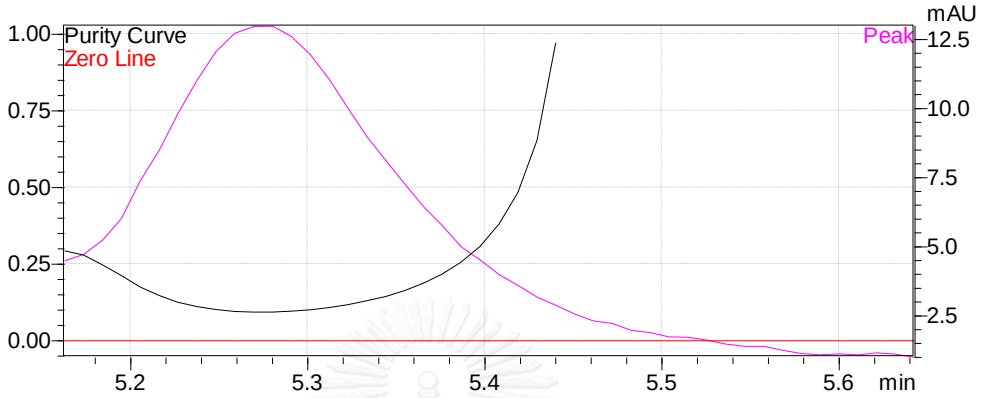


Figure 31 Peak purity of (+)-catechin in *Pentace burmanica* stem bark extract

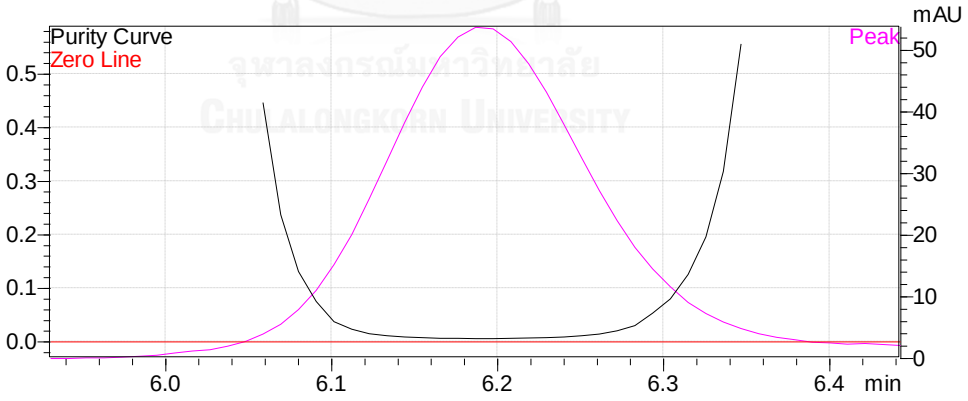


Figure 32 Peak purity of (-)-epicatechin in *Pentace burmanica* stem bark extract

The robustness was investigated during the analysis of HPLC method. The results revealed that there were no differences (%RSD < 5) in the area of the curve and retention time of (+)-catechin and (-)-epicatechin when the flow rate of mobile phase was varied from 0.995 – 1.005 ml/min and the column temperature was varied from 39 – 41 ˚C (Table 18, 19).

Table 18 Robustness investigation of (+)-catechin

		Retention time (min)			Area (unit)		
Flow rate (ml/min)	**0.995**	5.305	5.300	5.302	39183	36583	36393
	1.000	5.273	5.276	5.255	36971	40824	36562
	1.005	5.228	5.241	5.230	39008	36950	36403
% RSD			**0.58**			**4.27**	
Temp. (˚C)	**39**	5.318	5.332	5.316	36300	37075	36529
	40	5.273	5.276	5.225	36971	40824	36562
	41	5.213	5.203	5.219	36181	36705	37147
% RSD			**0.96**			**4.58**	

Table 19 Robustness investigation of (-)-epicatechin

		Retention time (min)			Area (unit)		
Flow rate (ml/min)	**0.995**	6.235	6.251	6.246	85087	84424	84800
	1.000	6.205	6.21	6.216	83409	81814	84668
	1.005	6.171	6.153	6.163	84336	83261	83242
% RSD			**0.58**			**1.24**	
Temp. (˚C)	**39**	6.285	6.263	6.279	84417	84907	85327
	40	6.205	6.21	6.216	83409	81814	84668
	41	6.115	6.118	6.13	85321	84095	85569
% RSD			**1.09**			**1.65**	

CHAPTER V
DISCUSSION

Si-Siad-Thai (black catechu) and Si-Siad-Ted (pale catechu) were generally water extracted from *Acacia catechu* and *Uncaria gambir* and common used in traditional medicine for treatment of diarrhea. They are components of Ya-Leong-Pid-Smut, Thai traditional medicine from the list of Herbal Medicine Product A.D. 2006. Si-Siad-Pleuak or *Pentace burmanica* stem bark was also used for anti-diarrhea in traditional Thai medicine. The majority of the information on the identity and quality of herbal medicine can be obtained from its macroscopy, microscopy, physico-chemical parameters, and TLC fingerprint [84]. The most common authentication methods of plant material are macroscopic and microscopic examination and TLC fingerprint identification [85]. In addition, TLC is widely used for a first screening. Macroscopic and microscopic methods are the simplest and cheapest method to establish the correct identification of plant materials [41]. It is useful for plant identification. In this study, only sample of *Pentece burmanica* stem bark was evaluated for microscopic evaluation. Although, this evaluation is used for powders or plant materials, it is not applicable to the extracts of plants. *Accacia catechu* and *Uncaria gambir* that used in this study were based on the extract of plant materials. Therefore, the water extracts of *Accacia catechu* and *Uncaria gambir* are not necessary for the microscopic evaluation. The physico-chemical evaluation of plant drugs is an important for detecting adulteration and quality of the drug [86]. The ash investigation is helpful to determine the quality and purity of powdered crude drug. A larger total value indicates that the crude drug contains more of inorganic compounds such as calcium oxalate crystals. If the crude drug contains a large

number of calcium oxalate crystals, the amount of substance remaining after acid treatment will be quite less [87]. In addition, a high ash value is indicative of contamination, substitution, adulteration, and carelessness in preparing the crude drug for marketing purpose [88, 89]. The physico-chemical parameters of *Acacia catechu* water extract from 22 different sources throughout Thailand were divided into 2 classes of purity according to the ash contents. The physico-chemical parameters of *Acacia catechu* water extracts (Class I) were illustrated in table 20. The total ash, acid insoluble ash, loss on drying, water content, ethanol and water soluble extractive values were found to be 3.62, 1.26, 14.65, and 9.87, 78.25 and 73.19 % of dry weight respectively. Table 21 showed the physico-chemical parameters of *Acacia catechu* water extracts (Class II). The total ash, acid insoluble ash, loss on drying, and water content, ethanol and water soluble extractive values were found to be 73.86, 58.55, 3.57, 4.03, 13.67 and 19.57 % of dry weight respectively.

Table 20 Physico-chemical parameters (% by weight) of *Acacia catechu* water extract (Class I)

Parameters	**Mean ± SD***	**Range****
Total ash content	3.62 ± 0.09	3.34 – 3.90
Acid insoluble ash content	1.26 ± 0.08	1.01 – 1.52
Loss on drying content	14.65 ± 0.32	13.70 – 15.60
Water content	9.87 ± 1.34	5.85 – 13.89
Ethanol extractive value	78.25 ± 3.09	68.97 – 87.53
Water extractive value	73.19 ± 3.85	61.64 – 84.73

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, Samples were from 3 different sources throughout Thailand. Each sample was performed in triplicate.

Table 21 Physico-chemical parameters (% by weight) of *Acacia catechu* water extract (Class II)

Parameters	Mean ± SD*	Range**
Total ash content	73.86 ± 1.67	68.84 – 78.88
Acid insoluble ash content	58.55 ± 0.65	56.60 – 60.50
Loss on drying content	3.57 ± 0.19	3.00 – 4.13
Water content	4.03 ± 1.18	0.48 – 7.58
Ethanol extractive value	13.67 ± 1.39	9.50 – 17.85
Water extractive value	19.57 ± 2.21	12.93 – 26.20

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, Samples were from 19 different sources throughout Thailand. Each sample was performed in triplicate.

Due to the ash contents, the physico-chemical parameters of *Uncaria gambir* water extracts from 20 different sources throughout Thailand were divided into 2 classes. For class I of *Uncaria gambir* water extracts, the total ash, acid insoluble ash, loss on drying, water content, ethanol and water soluble extractive values were found to be 5.20, 1.61, 13.14, 13.20, 91.66 and 44.59 % of dry weight respectively (Table 22). The physico-chemical parameters of class II *Uncaria gambir* water extracts were illustrated in table 23. The total ash, acid insoluble ash, loss on drying, and water content, ethanol and water soluble extractive values were found to be 73.86, 58.55, 3.57, 4.03, 13.67, and 19.57 % of dry weight respectively.

Table 22 Physico-chemical parameters (% by weight) of *Uncaria gambir* water extract (Class I)

Parameters	Mean ± SD*	Range**
Total ash content	5.20 ± 0.19	4.63 – 5.77
Acid insoluble ash content	1.61 ± 0.17	1.11 – 2.11
Loss on drying content	13.14 ± 0.10	12.84 – 13.44
Water content	13.20 ± 1.07	10.00 – 16.40
Ethanol extractive value	91.66 ± 5.16	76.18 – 107.14
Water extractive value	44.59 ± 3.18	35.05 – 54.13

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, Samples were from 5 different sources throughout Thailand. Each sample was performed in triplicate.

Table 23 Physico-chemical parameters (% by weight) of *Uncaria gambir* water extract (Class II)

Parameters	Mean ± SD*	Range**
Total ash content	29.80 ± 0.90	27.12 – 32.52
Acid insoluble ash content	21.27 ± 0.87	18.66 – 23.88
Loss on drying content	10.41± 0.20	9.82 – 11.00
Water content	9.35 ± 1.40	5.14 – 13.56
Ethanol extractive value	60.20 ± 5.25	44.44 – 75.95
Water extractive value	44.43 ± 2.99	35.46 – 53.40

*The parameters were shown as grand mean ± pooled SD. **mean ± 3SD, Samples were from 15 different sources throughout Thailand. Each sample was performed in triplicate.

From the results of pharmacognostic parameters, total ash and acid insoluble ash values of almost *Acacia catechu* and *Uncaria gambir* water extract samples were found to be high. It was suggested that the samples had adulterant problem. It might adulterate with sand and other impurities. The results were related with the previous studies in 1986 and 2009 which demonstrated that most of *Accacia catechu* water extracts and *Uncaria gambir* water extracts in Thailand were substandard [28, 29]. Moreover, this previous study reported that *Acacia catechu* water extract was adulterated with clay and *Uncaria gambir* water extract was adulterated with quartz [29]. Low extractive values were found in the samples with high ash values. The results indicated that the presence of specific component or group of specific components in the crude drugs were also low. Loss on drying value is used to determine the content of both volatile matters and water content in the crude drug [41]. Water content plays an important role in the stability of plant products. The water content should be minimized in order to prevent chemical degradation as well as microbial contamination [88]. The water content of *Pentace burmanica* stem bark (10.51%) was higher than loss on drying value (9.10%). This plant did not contain volatile substances; therefore, it might be due to the water within plant cells [90]. For *Acacia catechu* and *Uncaria gambir* water extracts, the loss on drying and water content value were quite high because they were based on the water extracts from the plants. Nevertheless, the physico-chemical parameters of 3 samples of *Acacia catechu* and 5 samples of *Uncaria gambir* were in accordant with standards [30-32]. This study proposed the first reports of pharmacognostic specification of *Pentace burmanica* stem bark in Thailand. The quantitative determination of some pharmacognostic parameters is useful for setting standards of crude drugs [86].

Consequently, the pharmacognostic investigations in this study can be used to set the standard parameters of Thai crude drugs under the name of Si-Siad in Thailand which be useful for authentication and quality control of these crude drugs. Quality control and standardization of herbal medicine are important for the production of high quality herbal product. Lack of the control may lead to health problems in the consumers [91].

The antioxidant activity and total phenolic contents were reversal related to the total ash values. The results revealed that greater amount of phenolic contents lead to more potent radical scavenging effect. The extracts of some *Acacia catechu*, all *Uncaria gambir*, and *Pentace burmanica* showed high antioxidant activities, total phenolic, and non-phenolic contents whereas total tannin contents were quite low. These results were related with previous reports [7, 13, 14, 19, 20, 23-26]. The values of antioxidant activities, total phenolics, non-tannin phenolics, and total tannins were found to be different for different sources of the catechus. These might be due to the impurity of both commercial *Acacia catechu* and *Uncaria gambir* [29]. Nevertheless, their phenolic contents were correlated with the antioxidant power. The extracts of some *Acacia catechu*, *Uncaria gambir*, and *Pentace burmanica* stem bark reduced the most of Fe^{3+} ions in the ferric reducing power investigation and the reducing power (FRAP values) of the extracts increased with the quantity of phenolics in the samples. The results were consistent with the finding of various researches that showed positive correlations between total phenolic content and antioxidant activity [82, 92-94]. The extracts at the concentration of 100 µg/ml had the ability to chelate iron but the percent chelating activities were quite low when compared to EDTA. The results might indicate that catechins or phenolic compounds presenting in

3 Si-Siad extracts might not be the main chelators of ferrous ions. Hider *et.al* stated that a sample containing high polyphenols might not chelate metal if the polyphenols present did not have suitable groups that could chelate the cations [95]. Catechin is one of the flavonoid compounds. The antioxidant activity of flavonoids is considered to be exerted by a combination of the reaction with free radicals and the metal ion chelating through phenolic hydroxyl groups in the flavonoid nucleus, which consists of A, B and C-rings [96]. Radical scavenging ability of flavonoid resides in the availability of free hydroxyls on the B-ring for hydrogen atom-donation. On the other hand, metal chelating ability is derived from three structural arrangements: 1) the 3′,4′-o-dihydroxycatechol on the B-ring; 2) the 3-hydroxyl in conjugation with a 4-oxo function on the C-ring; and 3) the 5-hydroxyl on the A-ring in conjugation with a 4-oxo function on the C-ring [97, 98]. Catechins are devoid of a C2-C3 double bond and a C4 carbonyl group on the C-ring (Figure 33). Hence, catechins lack the structural advantage of flavonols in regard to metal chelating ability [96].

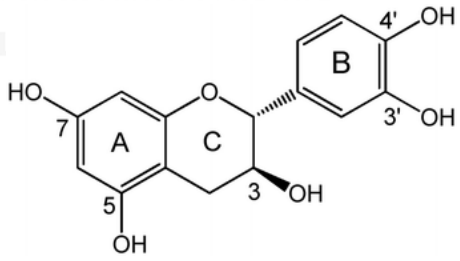


Figure 33 Catechin structure

In this study, almost of Si-Siad samples, which consisted of catechin as main compounds, had ability to inhibit lipid peroxidation in beta-carotene bleaching assay. The results were in accordant with previous study that measured the antioxidant activities of catechin mixture compound [99]. However, the previous studies reported

that non-polar antioxidants exhibited stronger antioxidative properties in emulsions because they were concentrated at the lipid: air surface, thus ensuring high protection of emulsion itself. On the other hand, polar antioxidants remaining in the aqueous phase are more diluted and are thus effective in protection the lipid [60, 100]. As previous reported the antioxidant activity depends on the chosen method, on the concentration and on the nature and physicochemical properties of studied antioxidants. The antioxidant capacities are influenced by many factors which cannot be fully described by a single method. It is necessary to perform more than one type of antioxidant activity measurement to take into account the various mechanisms of antioxidant action [101]. Therefore, four *in vitro* methods based on the different mechanisms of antioxidant activities including free radical scavenging of DPPH in the DPPH assay, the reduction of ferric ions in the FRAP assay, the chelation of ferrous ions in the metal ion chelating activity, and the peroxidation inhibition of linoleic acid in the beta-carotene bleaching assay were investigated in this present study.

HPLC is the primary analytical tool for quantifying chemical compounds in plant materials [85]. Photo diode array detector (PDA) and reverse phase (C_{18}) column were applied in this study. (+)-Catechin and (-)-epicatechin which are phenolic compounds have strong UV absorption; therefore PDA is a good detector for analyze. The PDA generates a large amount of spectral information without compromising sensitivity or wavelength resolution. This detector collects data with a maximum wavelength bandwidth of 190-800 nm [102]. The reverse phase HPLC is the most widespread form of chromatography because of its versatility. The C_{18} (ODS, Octadecylsilane) column is suitable for polar compounds analysis. Moreover, it has proven to be extremely versatile and reproducible [103]. Two solvent were used as

mobile phase in this study including water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid. The acidic mobile phase (pH 2.5) was chosen as it typically yield better peak shapes. Low concentrations of acid in mobile phase tend to markedly improve separation of phenolic compounds [8, 104]. A mobile phase at acidic pH of 2.5-3.0 is a good starting point for most pharmaceutical applications [74]. In addition, the acid is applied as a modifier solvent for mobile phase to maintain the free state of the isolated compounds in reversed phase [8]. Common acids used for mobile phase preparations are phosphoric acid, formic acid, and acetic acid [74, 104]. The mobile phase was filtered before analysis to help prevent particles from entering the chromatography system. The column temperature was maintained at 40 °C during analysis to improve the retention time precision. External standard method was used for quantifying (+)-catechin and (-)-epicatechin in Thai crude drugs under the name of Si-Siad because the external procedure was simple and extraneous peaks were in concern [105]. Additionally, the external standard method is the most general method for determination of the concentration of an analyte in an unknown sample [106]. The quantitation in this study is based on a comparison of peak area of standard or reference compound to the test analyte. The peak response used for quantitation can be either peak height or peak area. Peak height is usually used when incomplete resolution of the analyte peak in encountered, because the peak height measurement is subject to less interference from the adjacent overlapping peaks. On the other hand, peak area is less influenced by changes in instrumental or chromatographic parameters [106]. The results of HPLC analysis demonstrated that some *Acacia catechu* water extracts were found to be rich source for (+)-catechin and (-)-epicatechin. High (+)-catechin

contents were found in all *Uncaria gambir* water extracts whereas (-)-epicatechin were detected at very low concentrations. However, these findings were in accordant with the recent studies [5, 24, 25, 78]. (+)-Catechin and (-)-epicatechin contents of some *Acacia catechu* and *Uncaria gambir* samples cannot be determined quantitatively due to low concentration (< LOQ). Varied concentration of both (+)-catechin and (-)-epicatechin in different sources of the sample might be due to the impurity of both commercial *Acacia catechu* and *Uncaria gambir* water extracts as reported in previous study [29]. (+)-Catechinin was detected in *Pentace burmanica* stem bark extracts but it cannot be determined quantitatively due to low concentration (< LOQ); whereas (-)-epicatechin was found to be high concentration. Nonetheless, the contents of (-)-epicatechin were varied among samples. The varied concentration of (-)-epicatechin might be due to the different of geographical areas and the age of *Pentace burmanica*. Previous study reported that the age and height of *Pentace burmanica* were related with a quantity of tannin extract [34]. According to ICH guideline, the tests of specificity, linearity, LOD, LOQ, accuracy, precision, and robustness should be performed for the validation of an analytical method [80]. The specificity was conducted by peak purity test. The peak purity test is useful to show that the analyte chromatographic peak is not attributable to more than one component. It is based on spectra recorded by photo diode array detector. If all the individual spectra recorded during elution of a peak are identical, the peak is considered pure [107]. A homogeneous peak will produce a peak purity index of 100% (Peak purity index of 1.00), suggesting that all spectra are similar [108]. The results showed peak purity index of both catechins were more than 0.99 which can be suggested that no impurity detected in those peaks. Five concentration levels of

(+)-catechin and (-)-epicatechin were performed to evaluated the calibration curves. The calibration curves of both standard compounds were linear in the range of 5-200 μg/ml. Good correlation was obtained ($r^2 \geq 0.99$) in this study. An analytical method is acceptable, if the r^2 value obtained is 0.99 or better. The best result is obtained when the concentration of the sample is within the concentration range evaluated [109]. The percent RSD of repeatability and intermediate precision were found to be less than 3 which revealed that the HPLC method was precise [110]. Good agreement of recovery was ranged from 80 - 120% with the requirement for complex matrices [80]. Hence, the results indicated that this HPLC method was accurate for (+)-catechin and (-)-epicatechin quantification in Si-Siad crude drugs. The robustness should be investigated during the analysis of HPLC method, and it should demonstrate the reliability of analysis with the respect to deliberate variation in the parameters of the method [80]. The robustness was assessed by analysis of the results obtain after deliberate variation of the flow rate of mobile phase and the column temperature. This present study revealed that there were no differences (%RSD < 5) in the area of the curve and retention time of (+)-catechin and (-)-epicatechin. These results suggested that the HPLC method proved to be robust for catechins analyzed, under the condition evaluated. Moreover, the results were indicative of the reliability of the method. It is important to point that this present study represents the first report on the (+)-catechin and (-)-epicatechin quantitation in Thai crude drugs under the name of Si-Siad in Thailand by HPLC method.

CONCLUSION AND RECOMMENDATION

The pharmacognostic investigations can be used to set the standard parameters of Thai crude drugs under the name of Si-Siad which be useful for authentication and quality control of these crude drugs. The present study proposed the first reports of antioxidant activities as well as the contents of (+)-catechin and (-)-epicatechin from commercial *Acacia catechu*, *Uncaria gambir*, and *Pentace burmanica* in Thailand. The findings demonstrated high antioxidant activities related to non-tannin phenolic content in all *Uncaria gambir* and *Pentace burmanica* samples, but a few *Acacia catechu* samples. It revealed the inferiority of *Acacia catechu* crude drugs in Thai markets leading to insufficient phenolic components and inefficient antioxidant potential. In addition, this HPLC method showed good sensitivity and accuracy for (+)-catechin and (-)-epicatechin quantification in Thai crude drugs under the name of Si-Siad. Photo diode array detector was enabled evaluation of catechins peak purity. Hence, the HPLC method can be applied to determine (+)-catechin and (-)-epicatechin content in plant materials. Further researches are needed to establish the *in vivo* antioxidant activities and the toxicity of the 3 Thai Si-Siad crude drugs.

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APPENDICES

APPENDIX A

Data of antioxidant activities

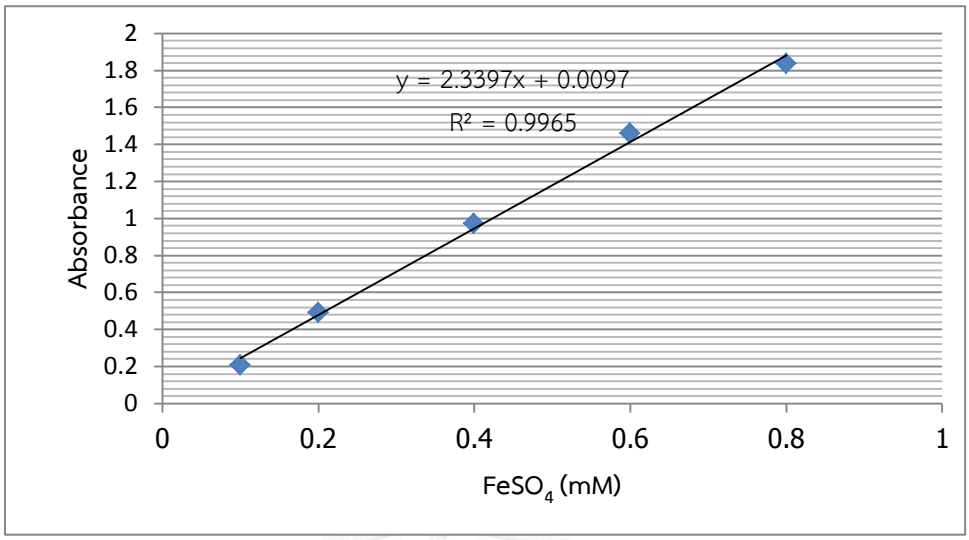

Figure 34 Calibration curve of $FeSO_4$ that used for calculate the FRAP value of *Acacia catechu* and *Uncaria gambir* stem bark extracts

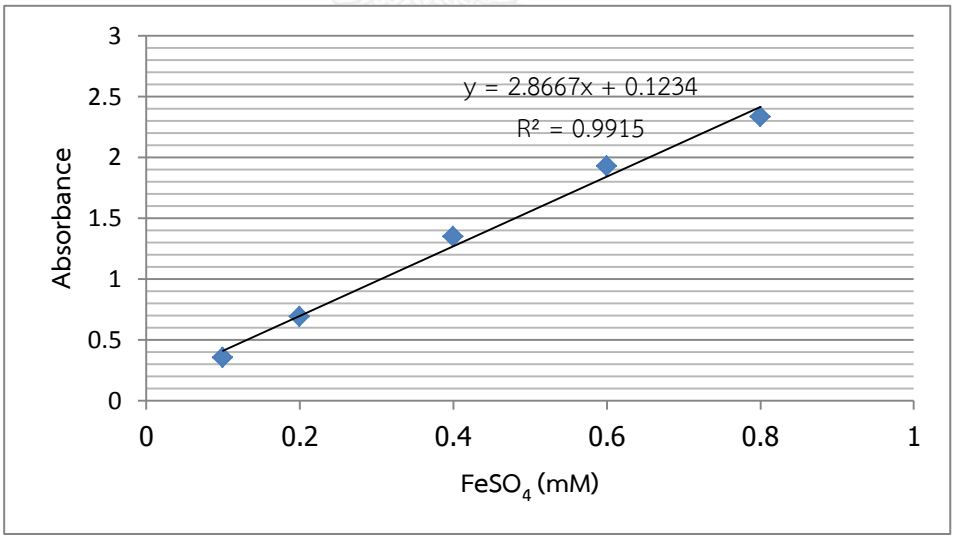

Figure 35 Calibration curve of $FeSO_4$ that used for calculate the FRAP value of *Pentace burmanica* stem bark extract

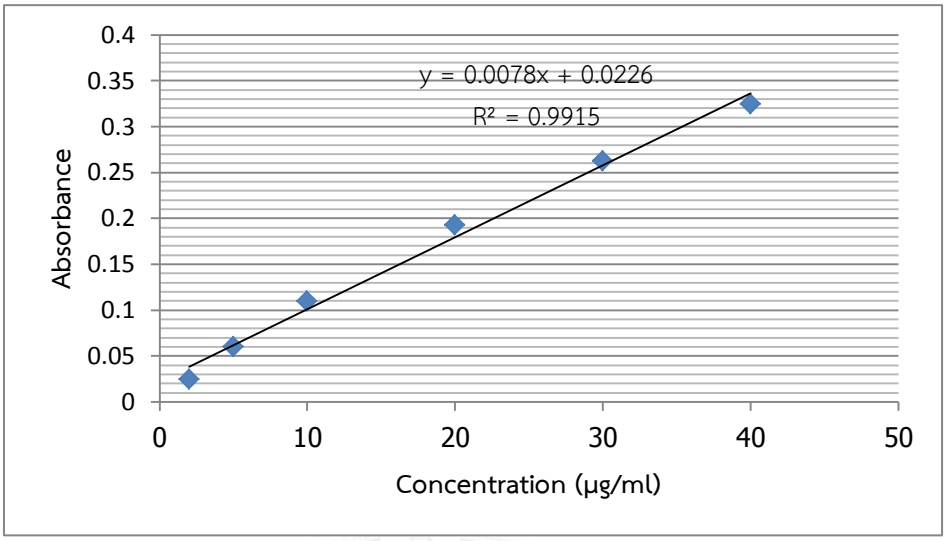

Figure 36 Calibration curve of (+)-catechin hydrate that used for calculate total phenolic and total tannin contents of *Acacia catechu* water extract

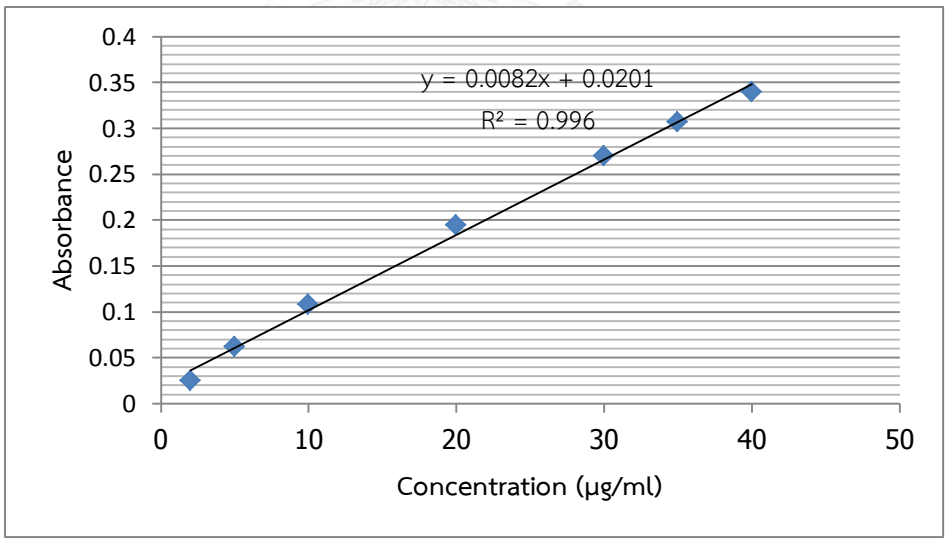

Figure 37 Calibration curve of (+)-catechin hydrate that used for calculate total phenolic and total tannin contents of *Uncaria gambir* water extract

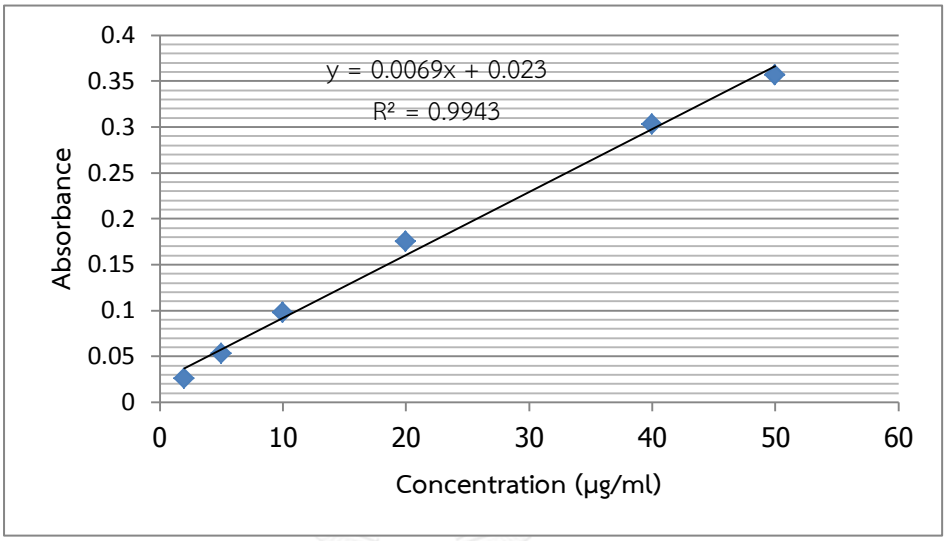

Figure 38 Calibration curve of (+)-catechin hydrate that used for calculate total phenolic and total tannin contents of *Pentace burmanica* stem bark extract

APPENDIX B

HPLC chromatogram of (+)-catechin and (-)-epicatechin

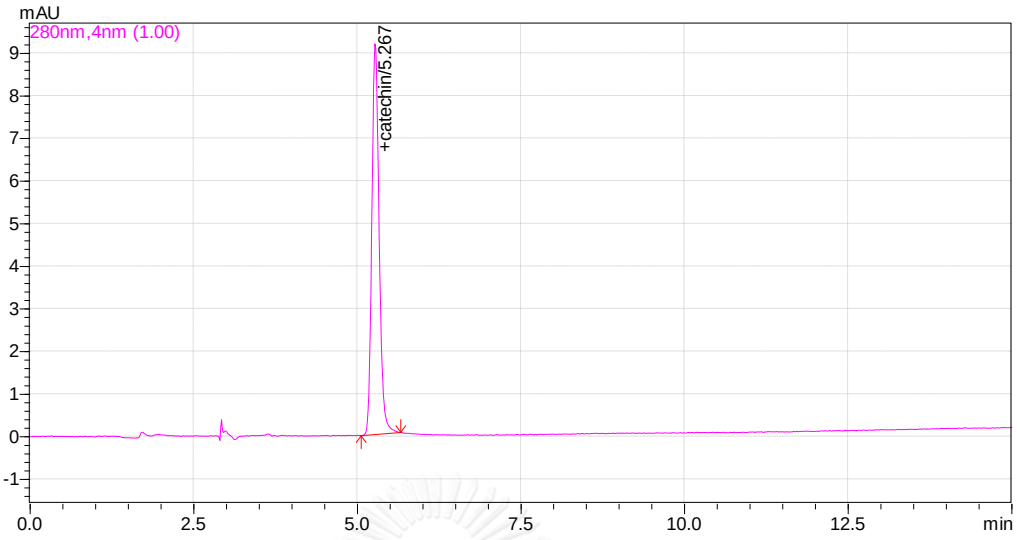

Figure 39 HPLC chromatogram of (+)-catechin

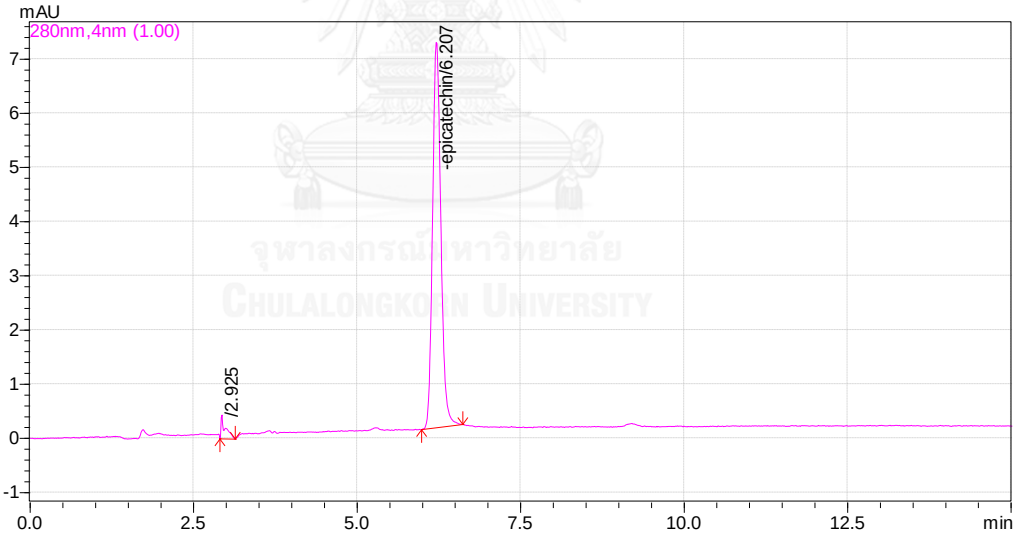

Figure 40 HPLC chromatogram of (-)-epicatechin

APPENDIX C

HPLC chromatogram of *Acacia catechu* water extract

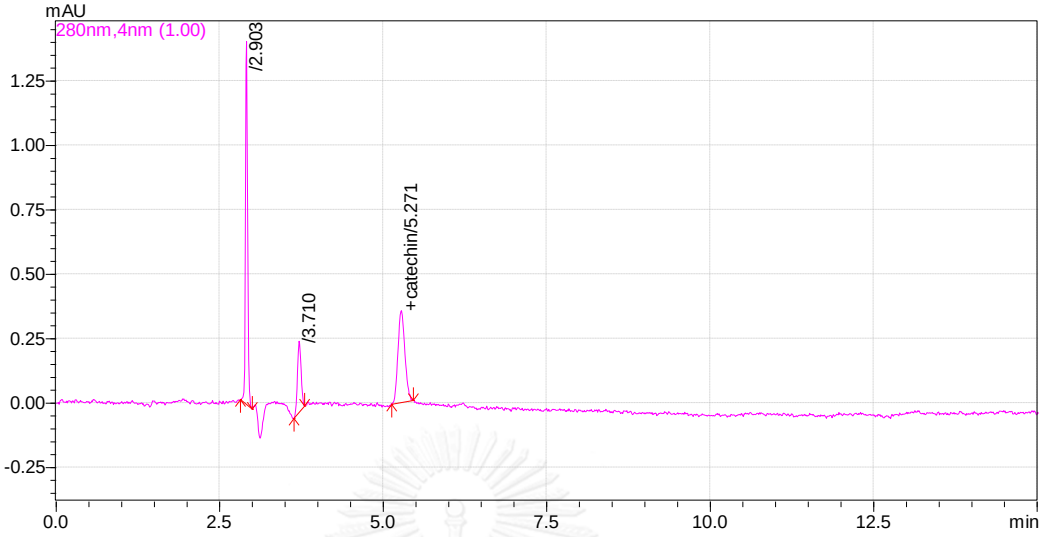

Figure 41 HPLC chromatogram of *Acacia catechu* water extract (Bangkok Metropolis)

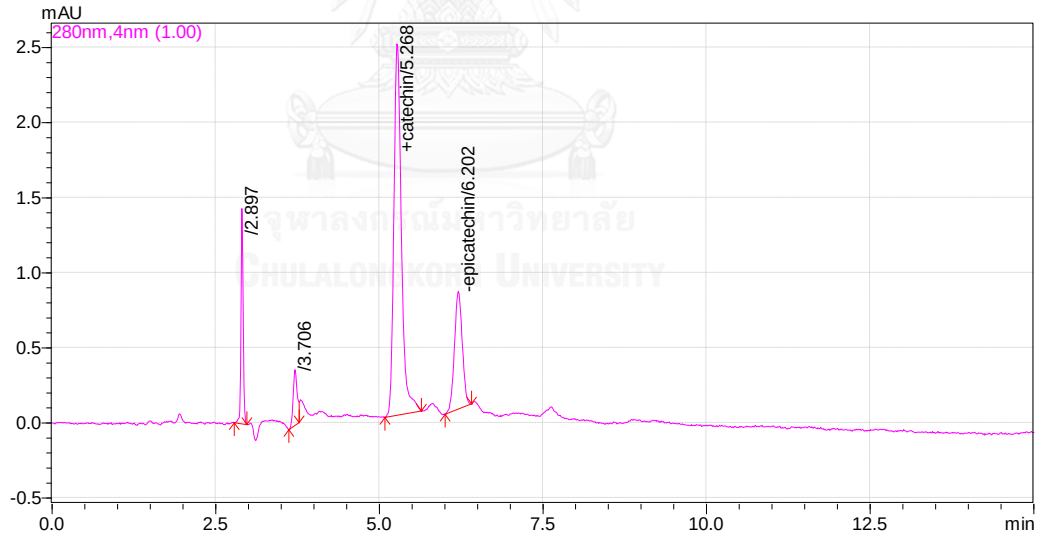

Figure 42 HPLC chromatogram of *Acacia catechu* water extract (Bangkok Metropolis 2)

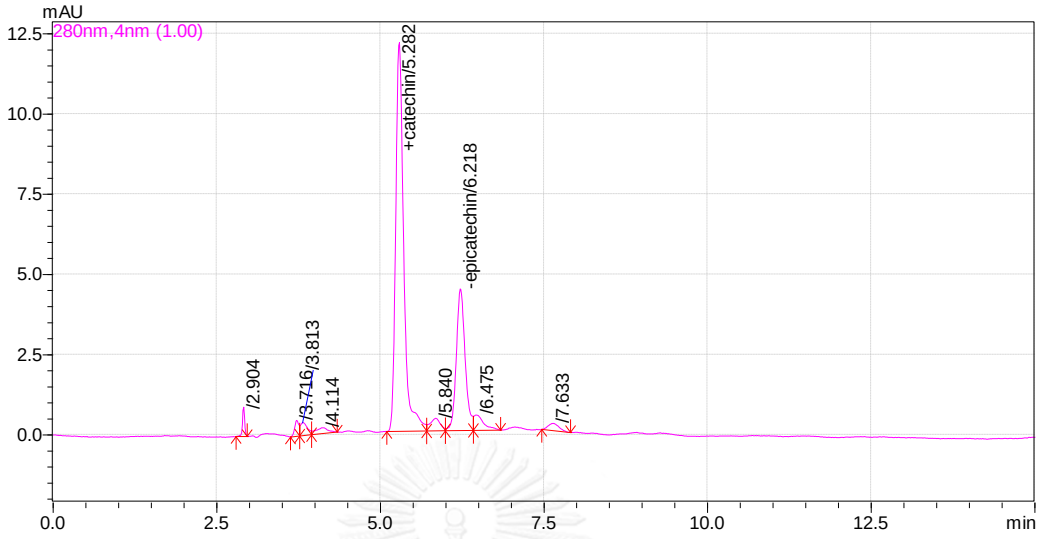

Figure 43 HPLC chromatogram of *Acacia catechu* water extract (Chachoengsao)

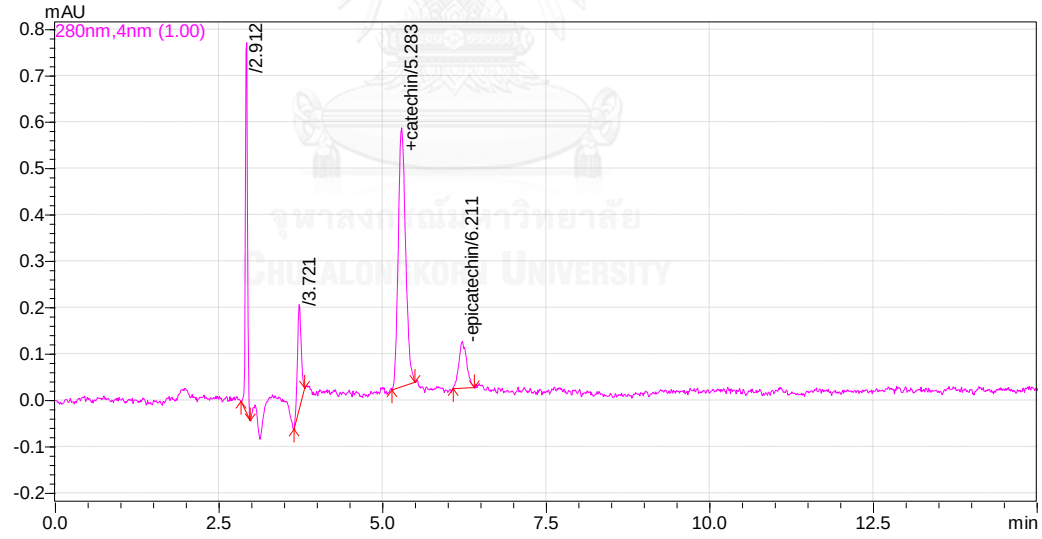

Figure 44 HPLC chromatogram of *Acacia catechu* water extract (Chanthaburi)

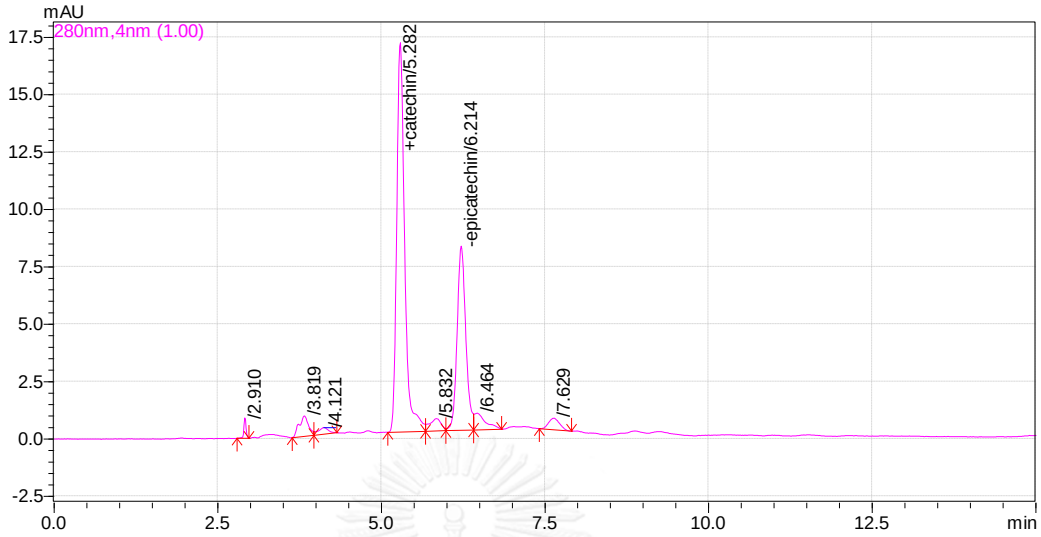

Figure 45 HPLC chromatogram of *Acacia catechu* water extract (Chiang Mai)

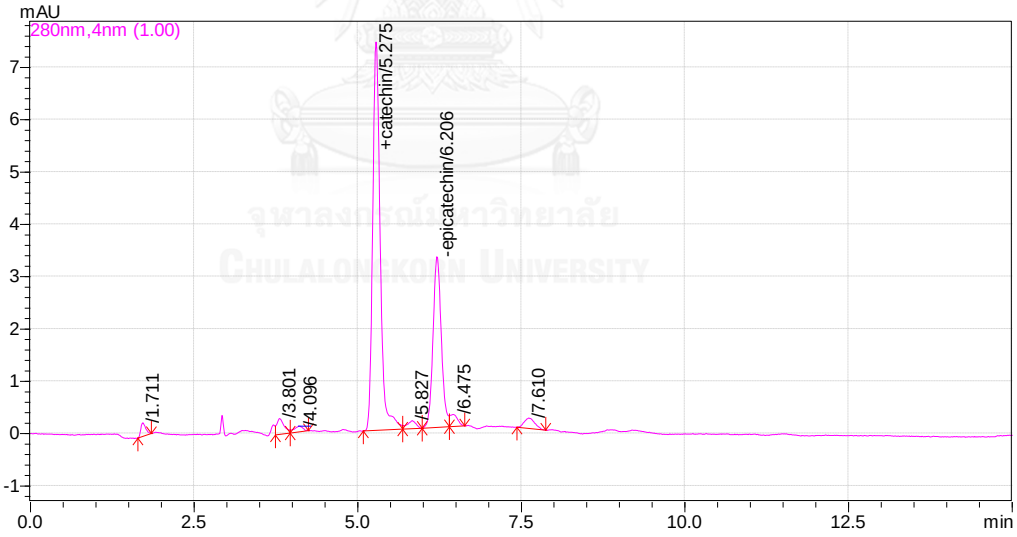

Figure 46 HPLC chromatogram of *Acacia catechu* water extract (Chiang Rai)

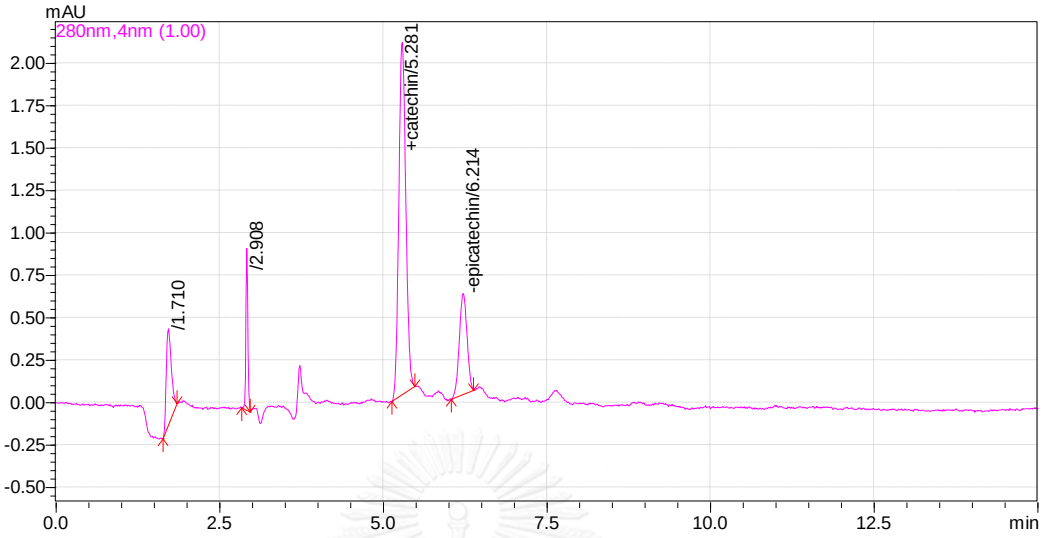

Figure 47 HPLC chromatogram of *Acacia catechu* water extract (Kamphaeng Phet)

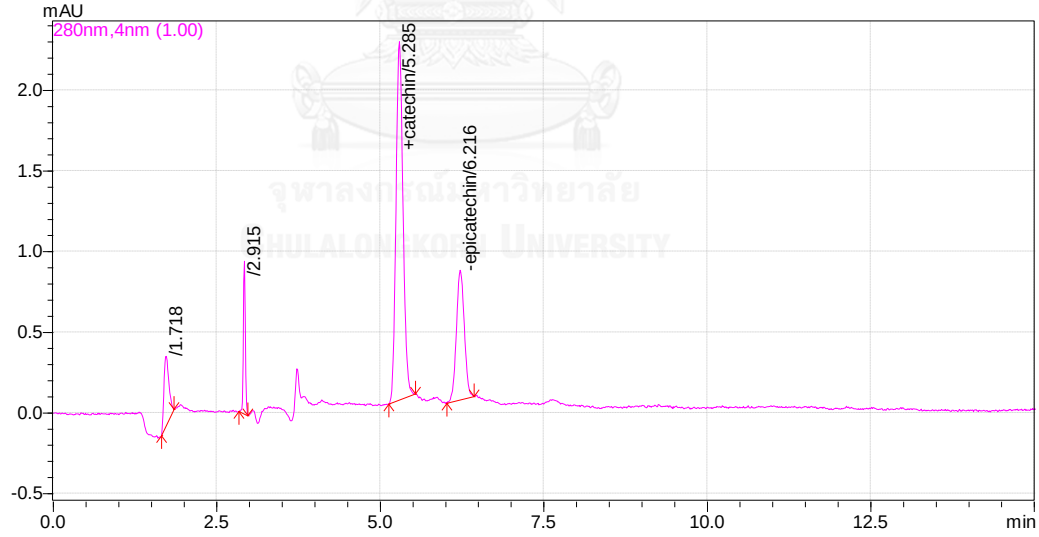

Figure 48 HPLC chromatogram of *Acacia catechu* water extract (Kanchanaburi)

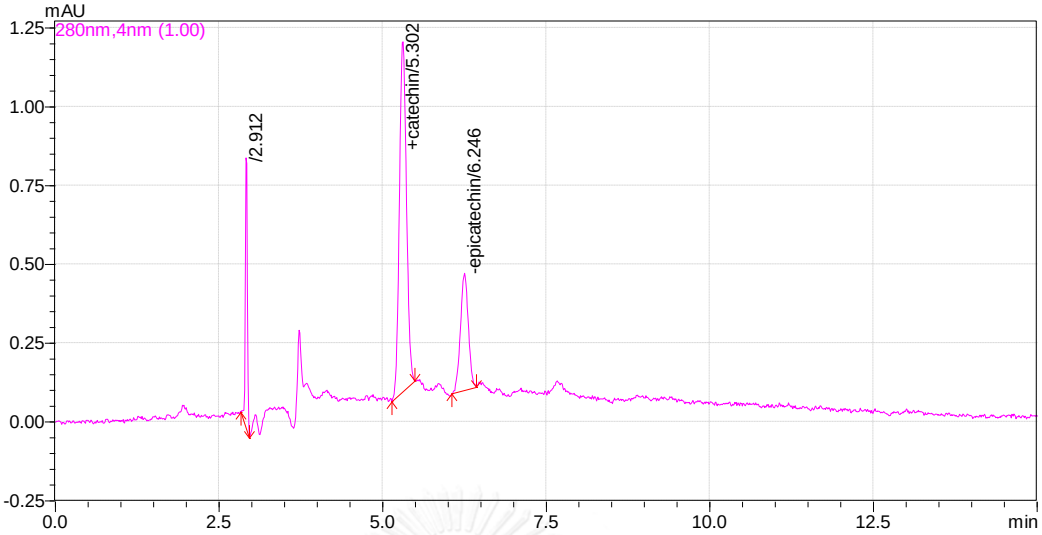

Figure 49 HPLC chromatogram of *Acacia catechu* water extract (Khon Kaen)

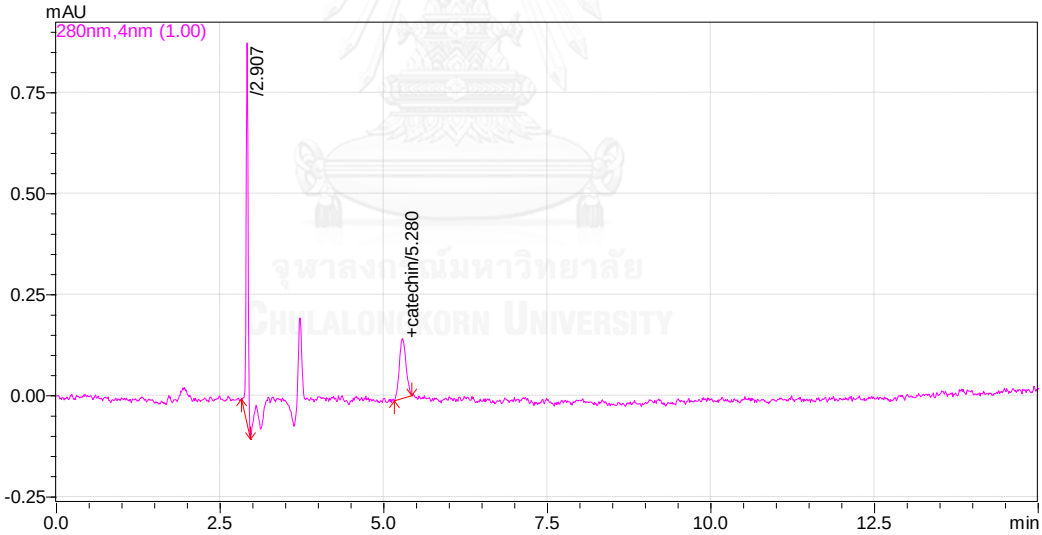

Figure 50 HPLC chromatogram of *Acacia catechu* water extract (Krabi)

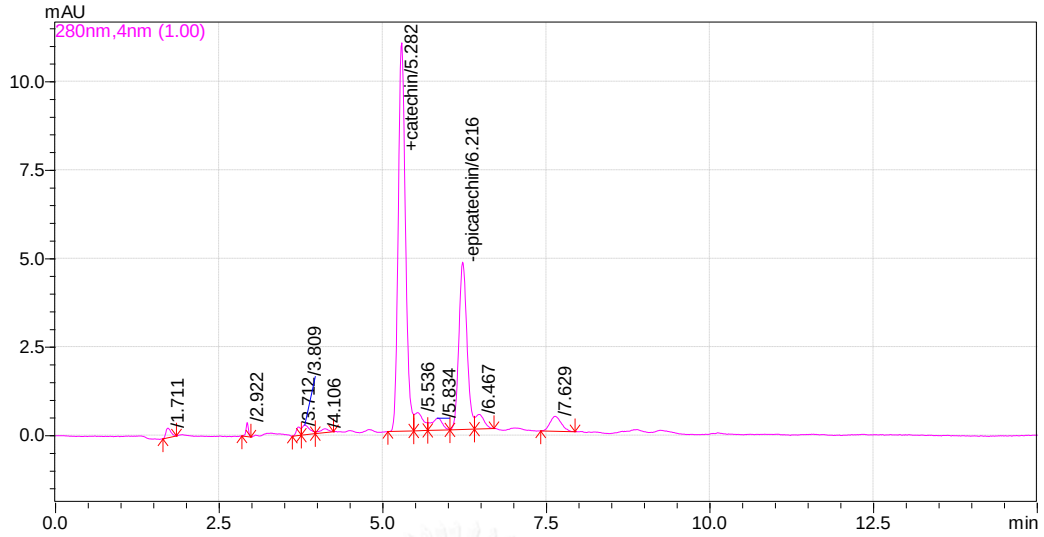

Figure 51 HPLC chromatogram of *Acacia catechu* water extract (Lampang)

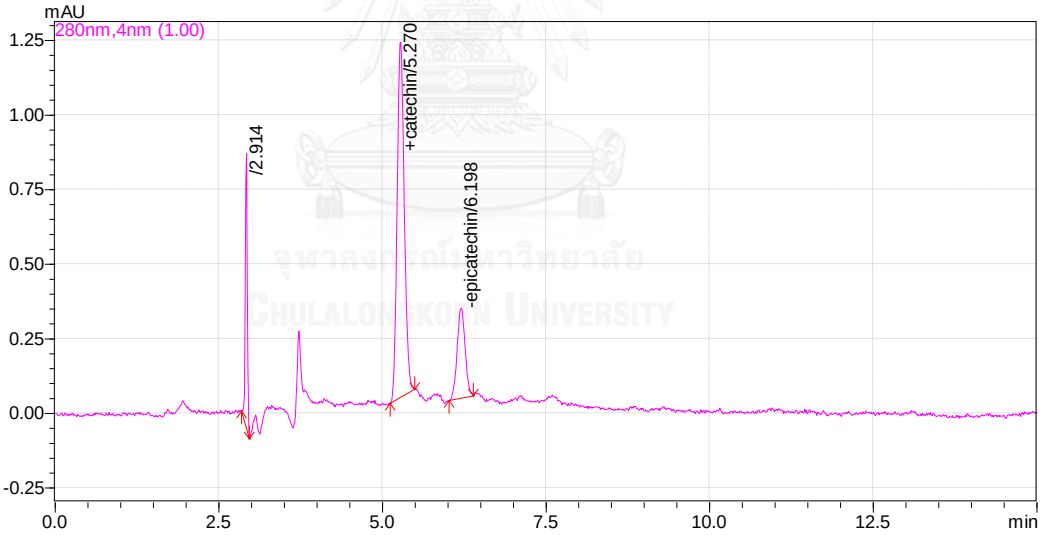

Figure 52 HPLC chromatogram of *Acacia catechu* water extract (Nakhon Pathom)

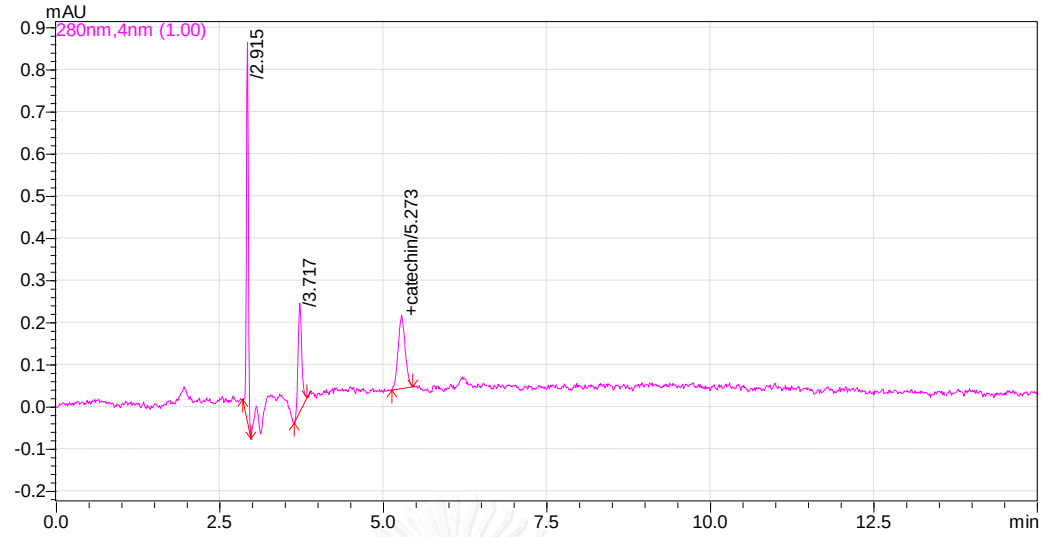

Figure 53 HPLC chromatogram of *Acacia catechu* water extract (Nakhon Phanom)

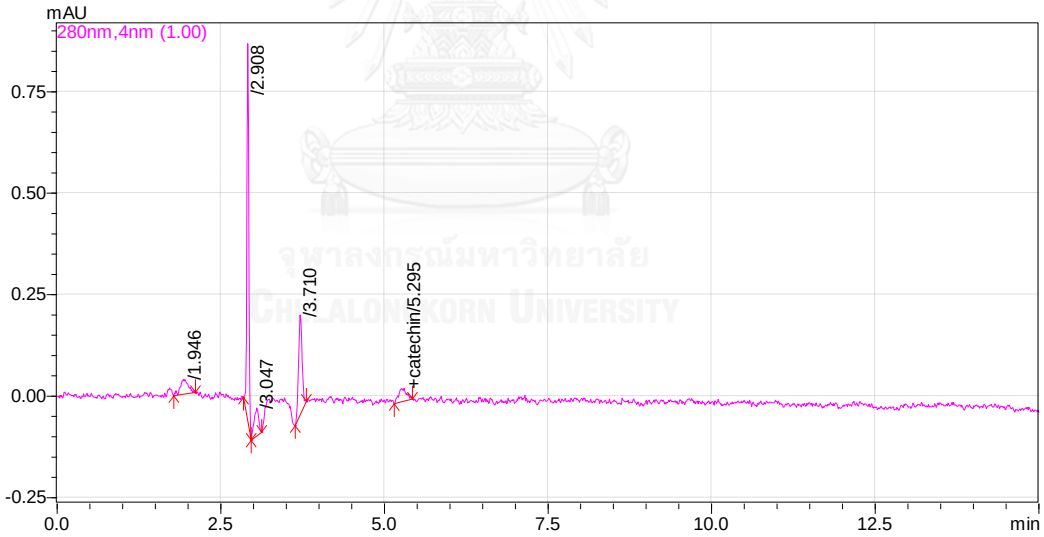

Figure 54 HPLC chromatogram of *Acacia catechu* water extract (Nakhon Si Thammarat)

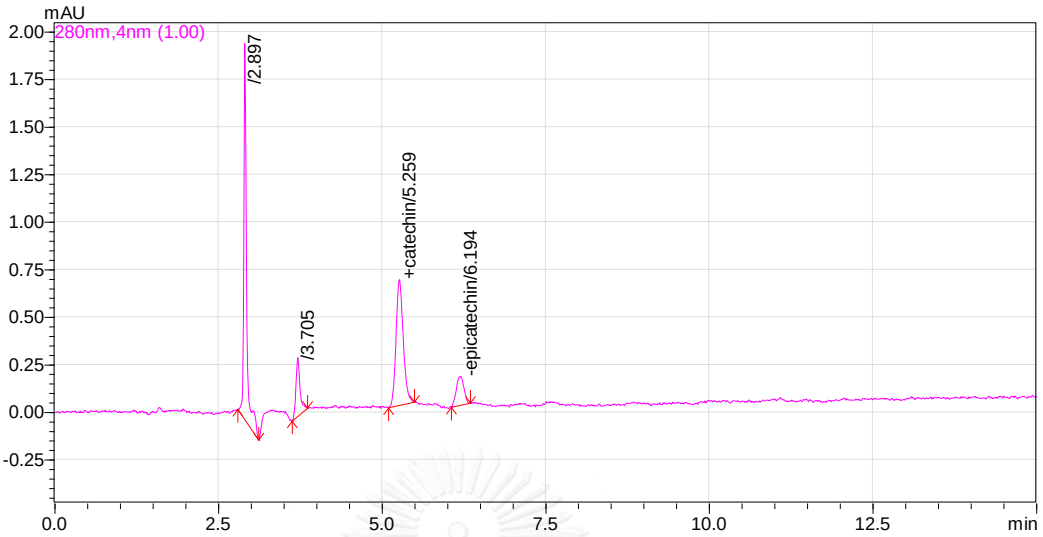

Figure 55 HPLC chromatogram of *Acacia catechu* water extract (Phra Nakhon Si Ayutthaya)

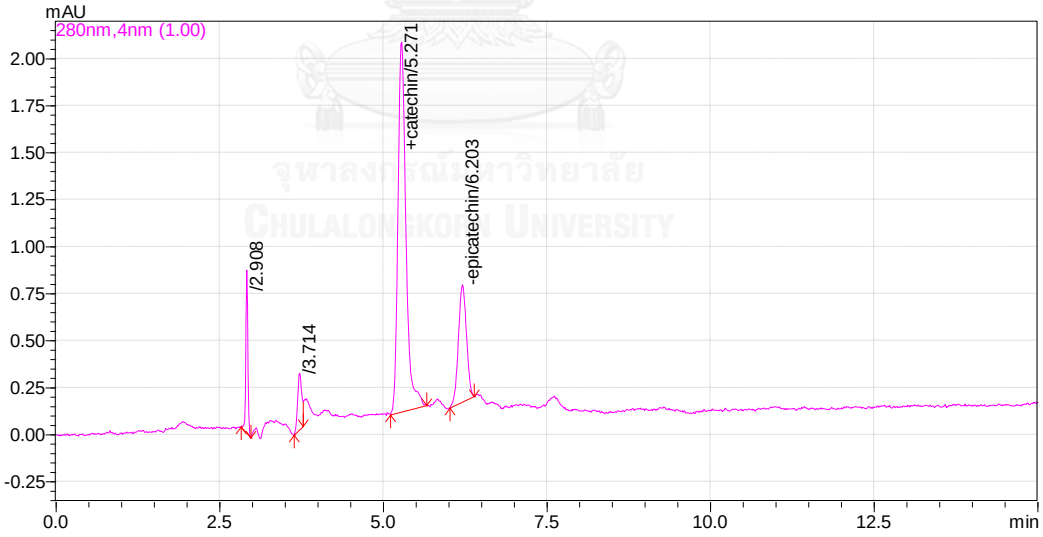

Figure 56 HPLC chromatogram of *Acacia catechu* water extract (Ratchaburi)

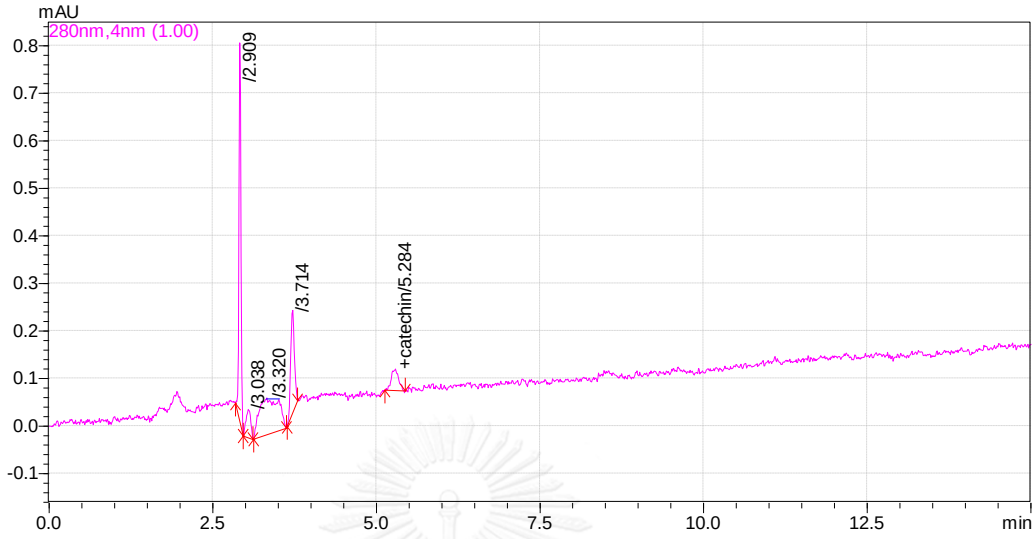

Figure 57 HPLC chromatogram of *Acacia catechu* water extract (Rayong)

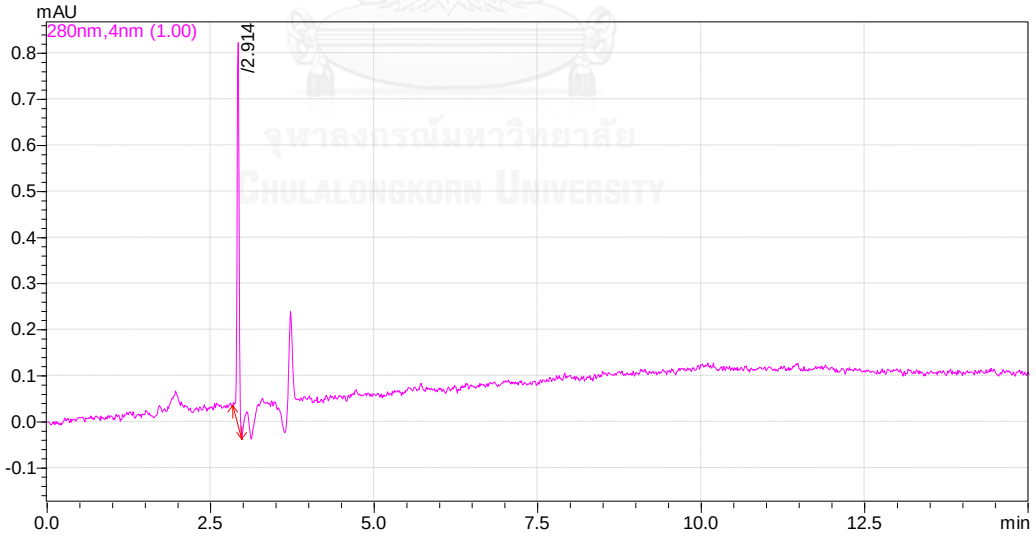

Figure 58 HPLC chromatogram of *Acacia catechu* water extract (Samut Sakhon)

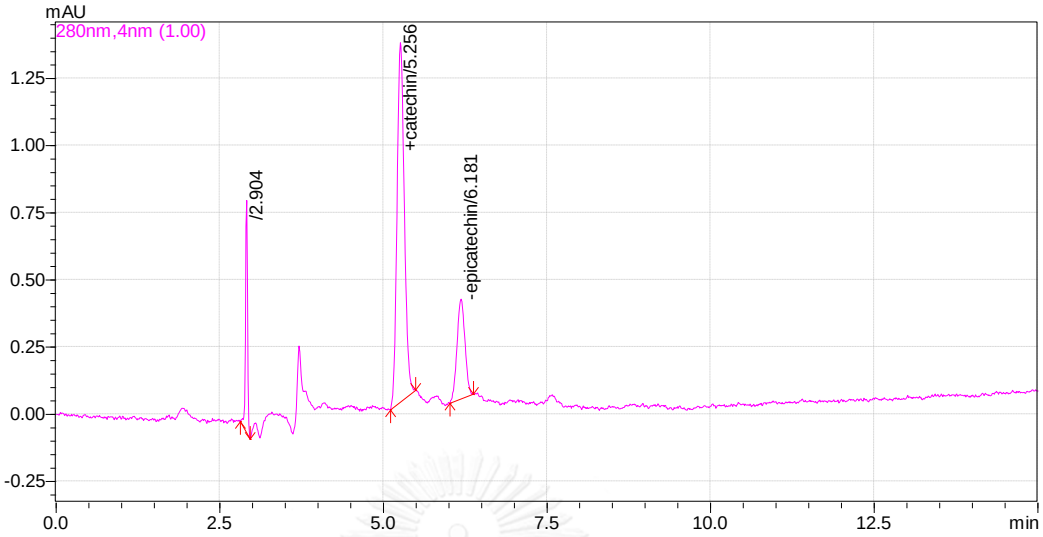

Figure 59 HPLC chromatogram of *Acacia catechu* water extract (Samut Songkhram)

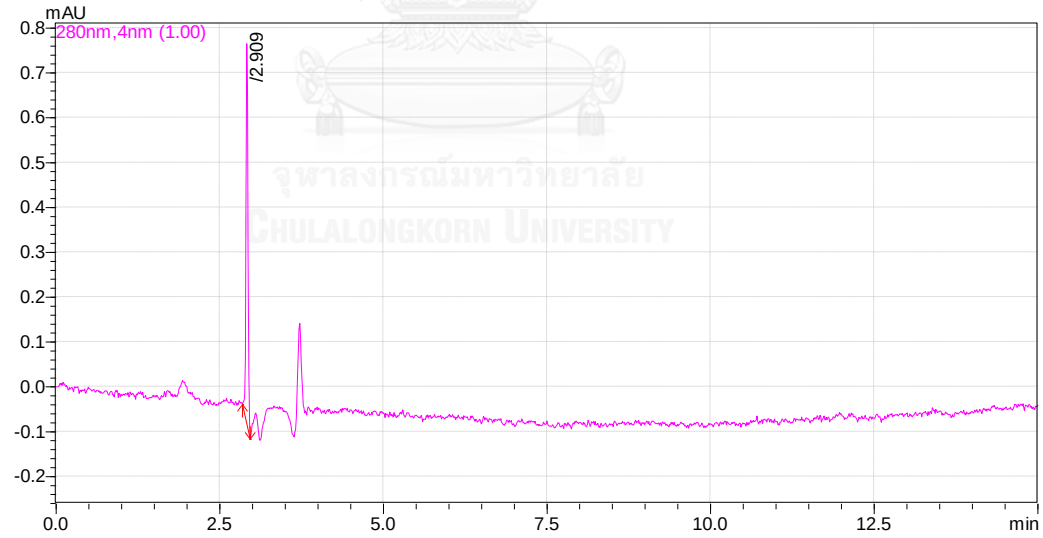

Figure 60 HPLC chromatogram of *Acacia catechu* water extract (Satun)

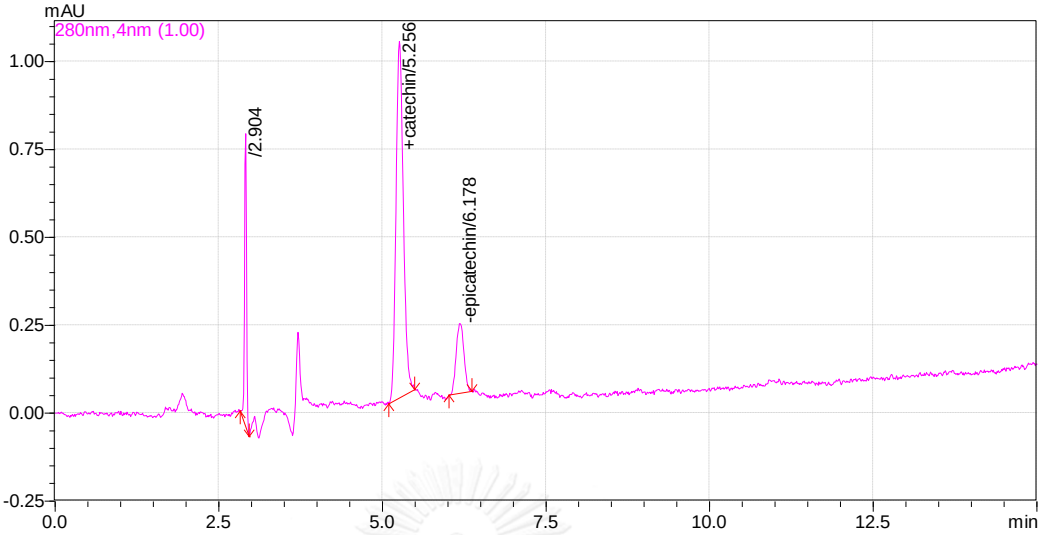

Figure 61 HPLC chromatogram of *Acacia catechu* water extract (Songkhla)

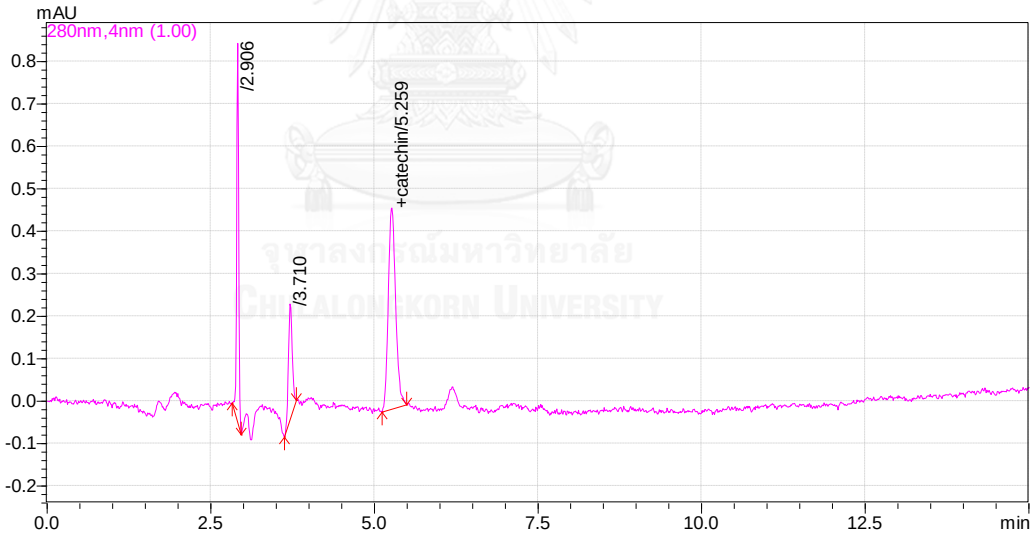

Figure 62 HPLC chromatogram of *Acacia catechu* water extract (Tak)

APPENDIX D

HPLC chromatogram of *Uncaria gambir* water extract

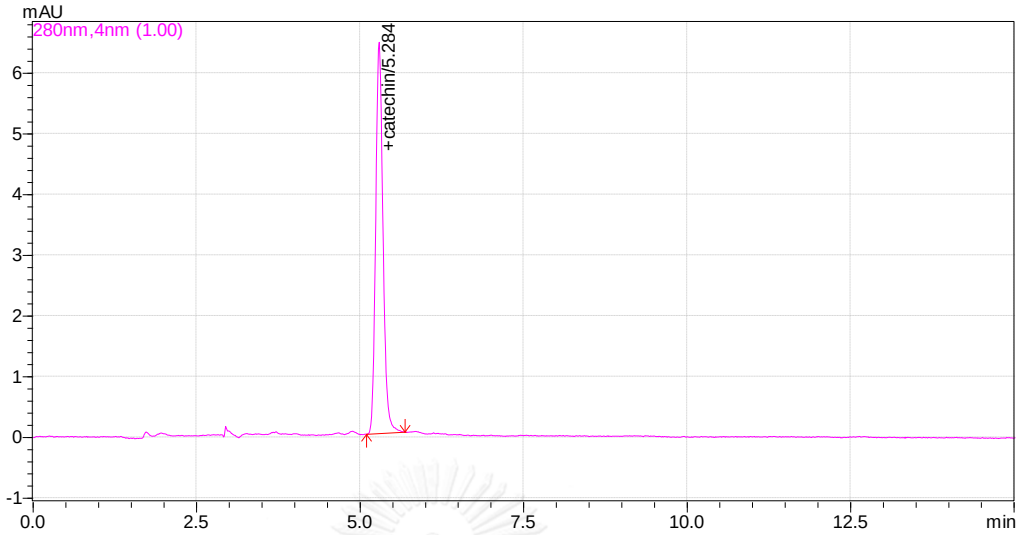

Figure 63 HPLC chromatogram of *Uncaria gambir* water extract (Bangkok Metropolis)

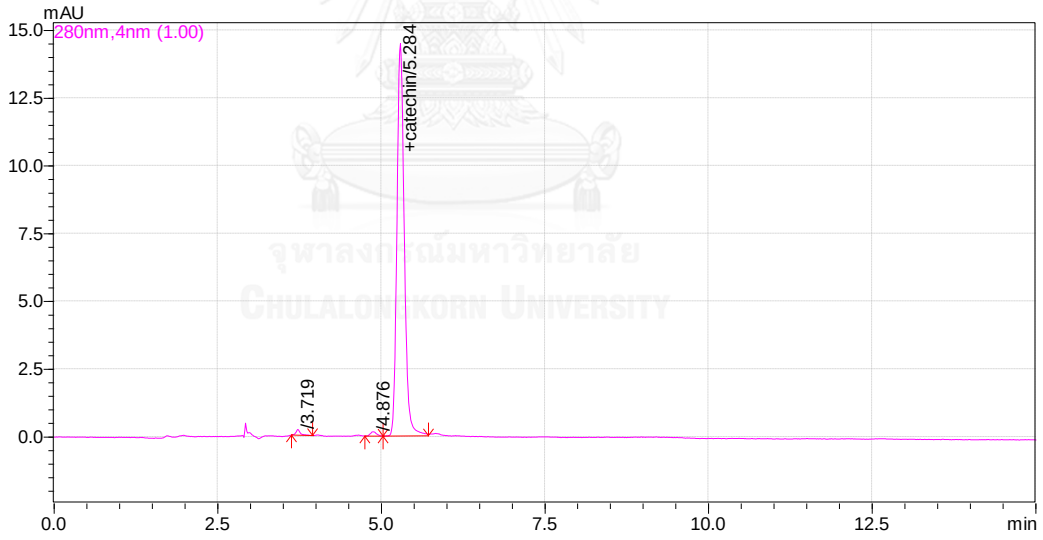

Figure 64 HPLC chromatogram of *Uncaria gambir* water extract (Chachoengsao)

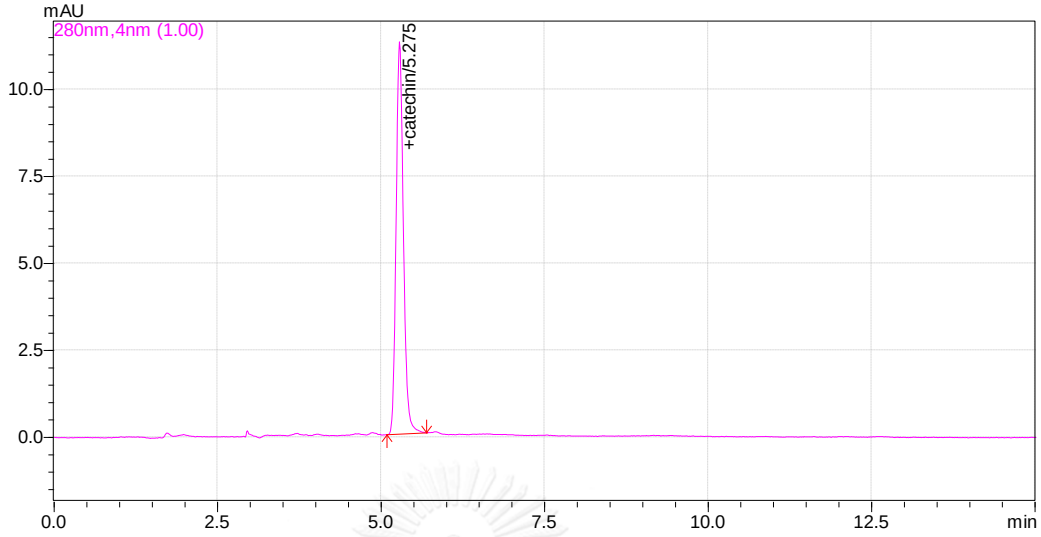

Figure 65 HPLC chromatogram of *Uncaria gambir* water extract (Chanthaburi)

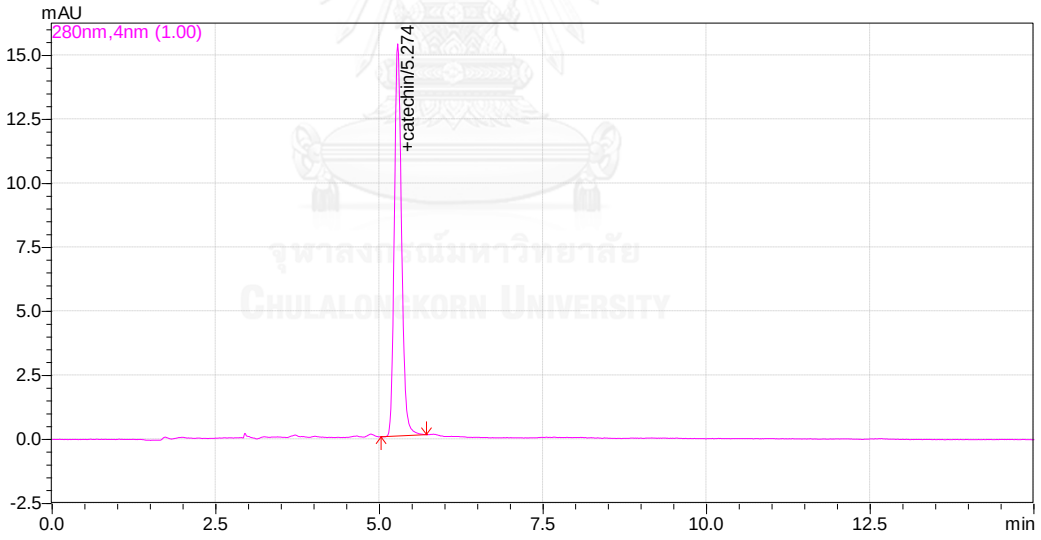

Figure 66 HPLC chromatogram of *Uncaria gambir* water extract (Chiang Mai)

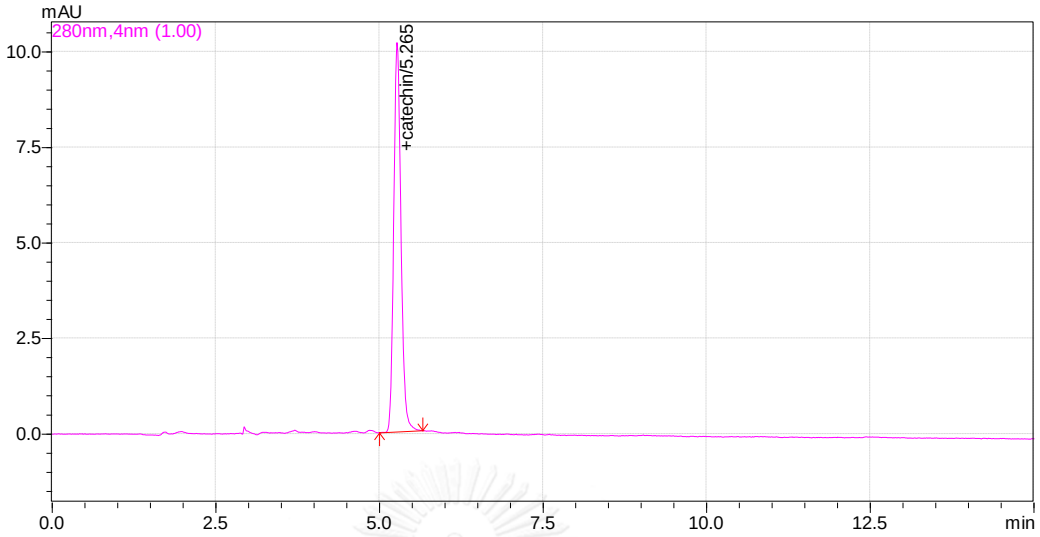

Figure 67 HPLC chromatogram of *Uncaria gambir* water extract (Chiang Mai 2)

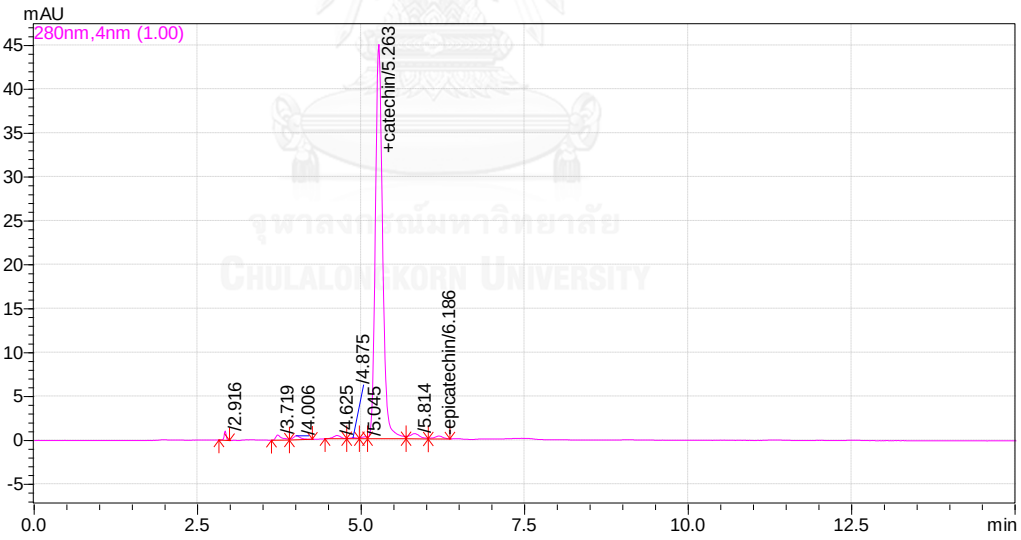

Figure 68 HPLC chromatogram of *Uncaria gambir* water extract (Kamphaeng Phet)

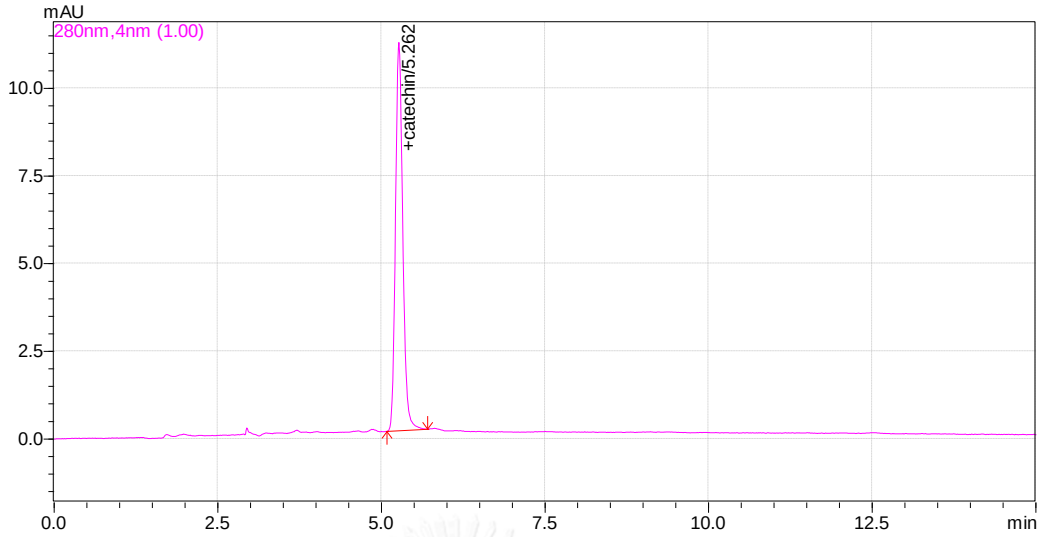

Figure 69 HPLC chromatogram of *Uncaria gambir* water extract (Kanchanaburi)

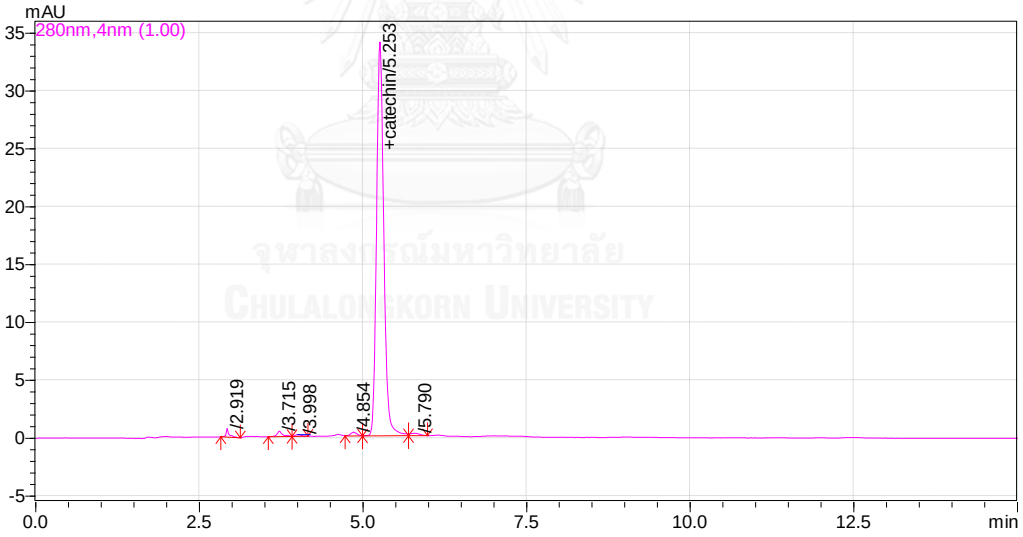

Figure 70 HPLC chromatogram of *Uncaria gambir* water extract (Khon Kaen)

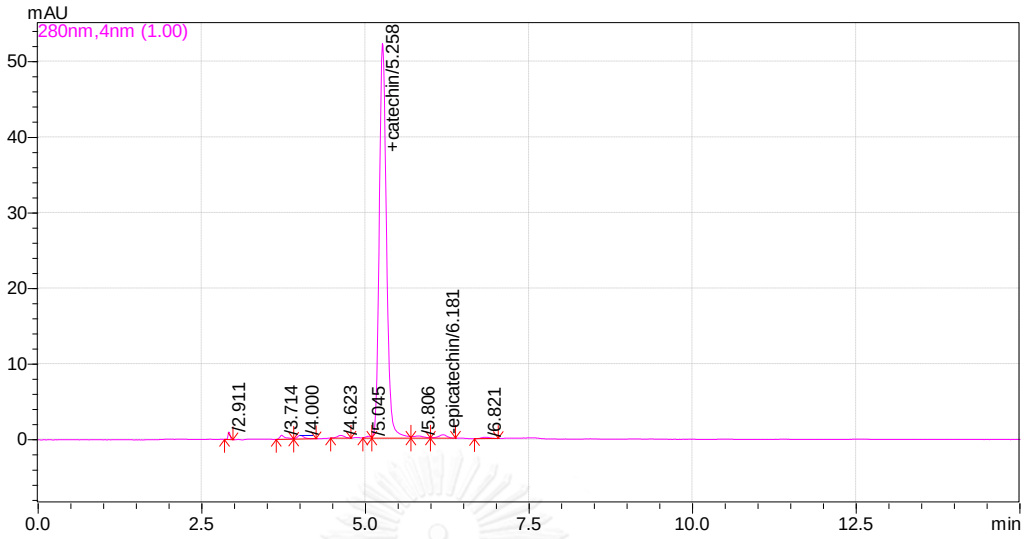

Figure 71 HPLC chromatogram of *Uncaria gambir* water extract (Krabi)

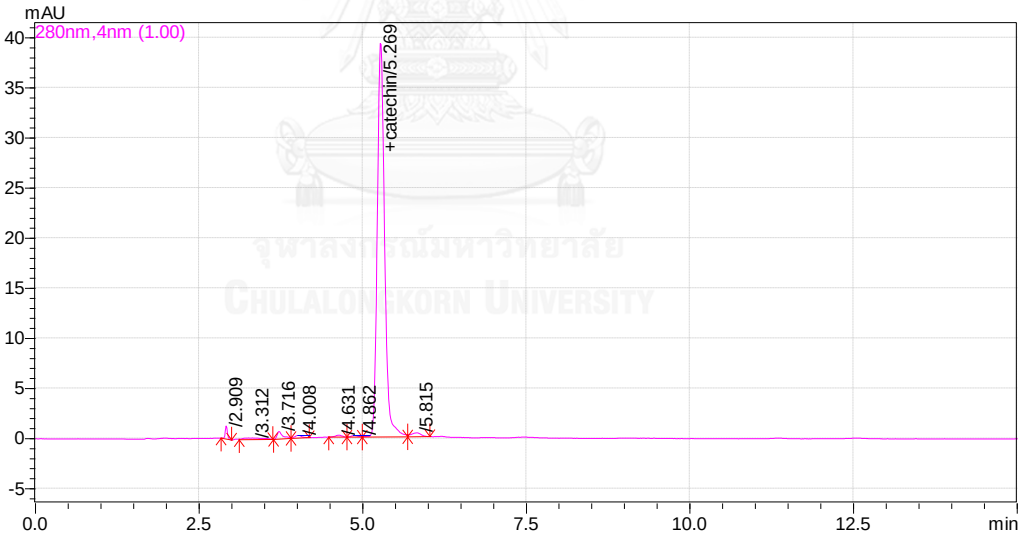

Figure 72 HPLC chromatogram of *Uncaria gambir* water extract (Nakhon Pathom)

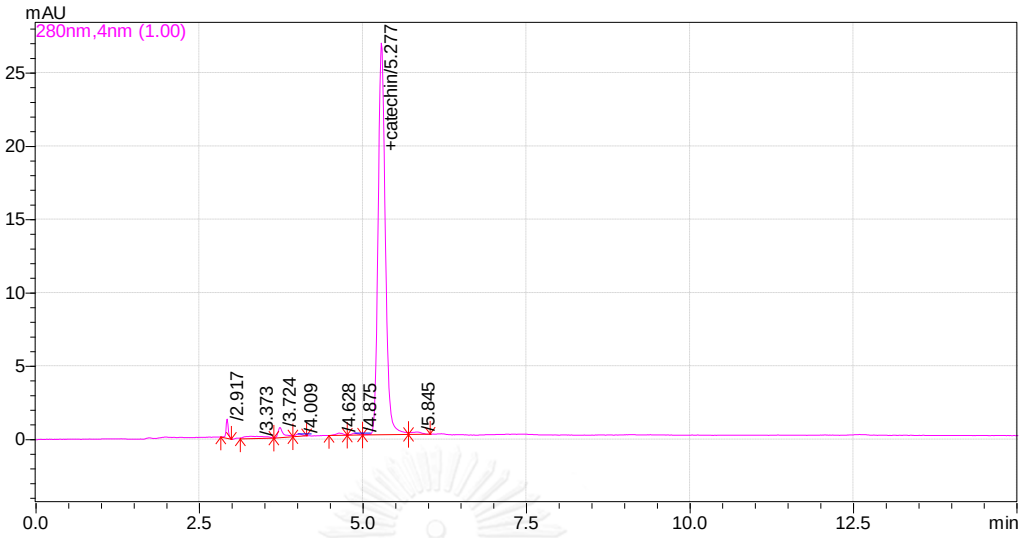

Figure 73 HPLC chromatogram of *Uncaria gambir* water extract (Nakhon Phanom)

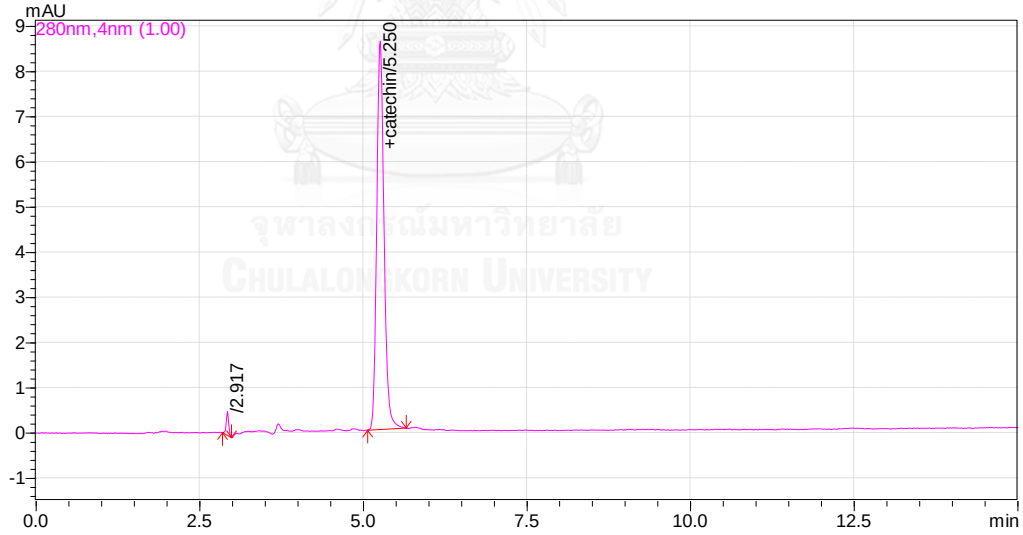

Figure 74 HPLC chromatogram of *Uncaria gambir* water extract (Nakhon Si Thammarat)

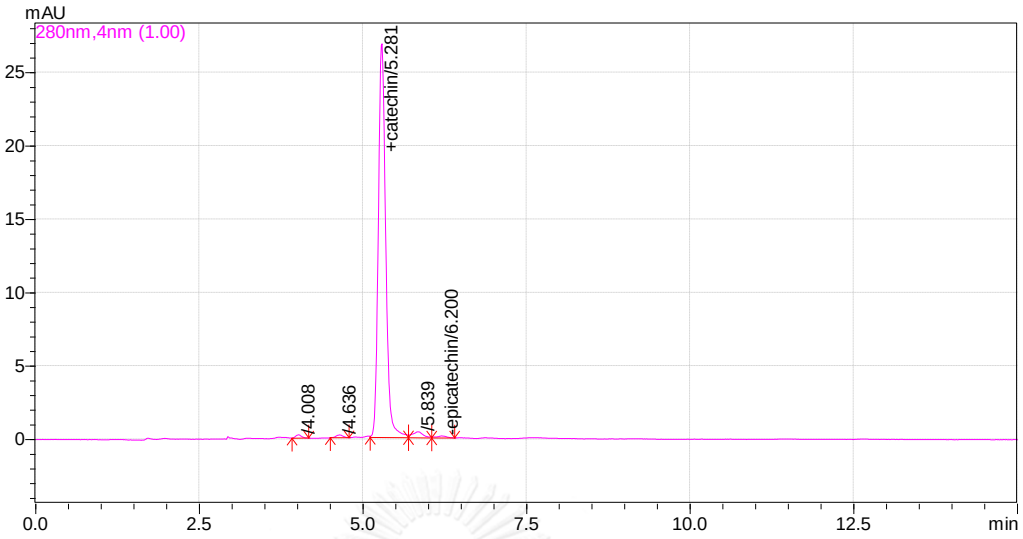

Figure 75 HPLC chromatogram of *Uncaria gambir* water extract (Phra Nakhon Si Ayutthaya)

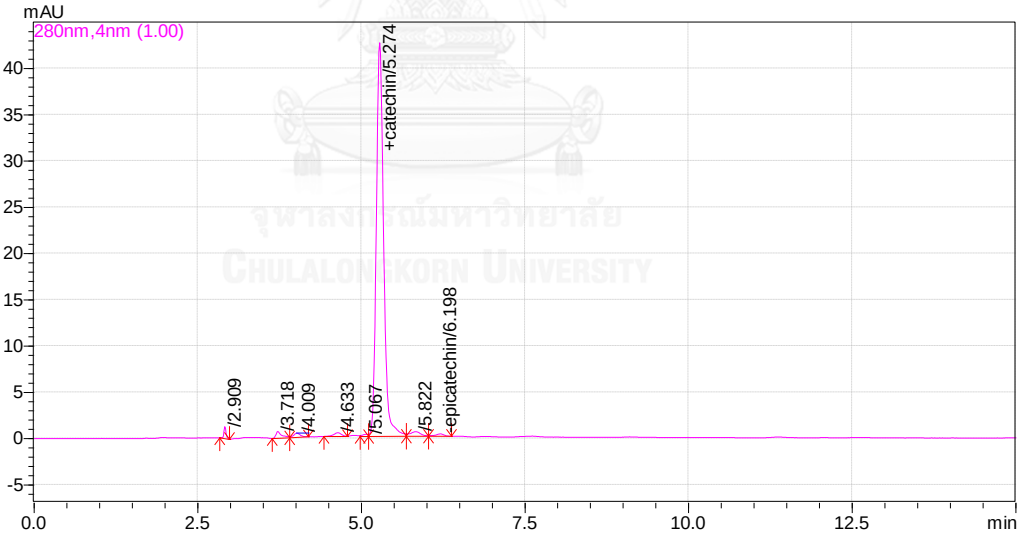

Figure 76 HPLC chromatogram of *Uncaria gambir* water extract (Ratchaburi)

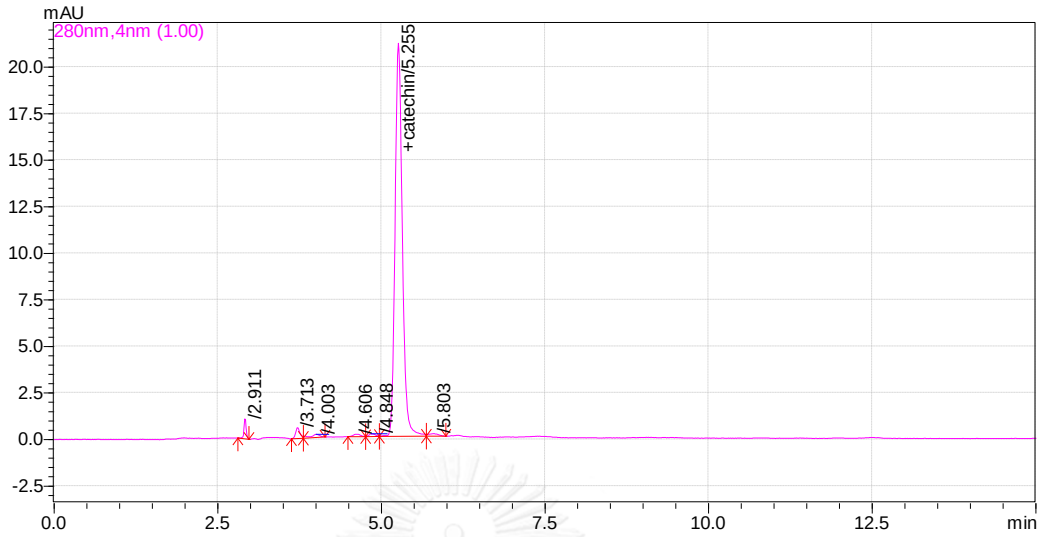

Figure 77 HPLC chromatogram of *Uncaria gambir* water extract (Rayong)

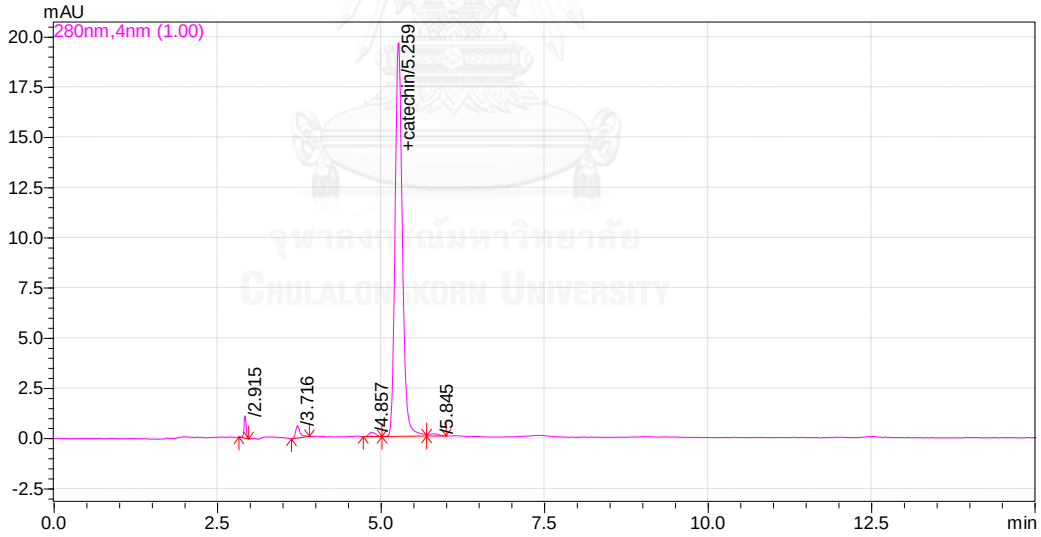

Figure 78 HPLC chromatogram of *Uncaria gambir* water extract (Samut Sakhon)

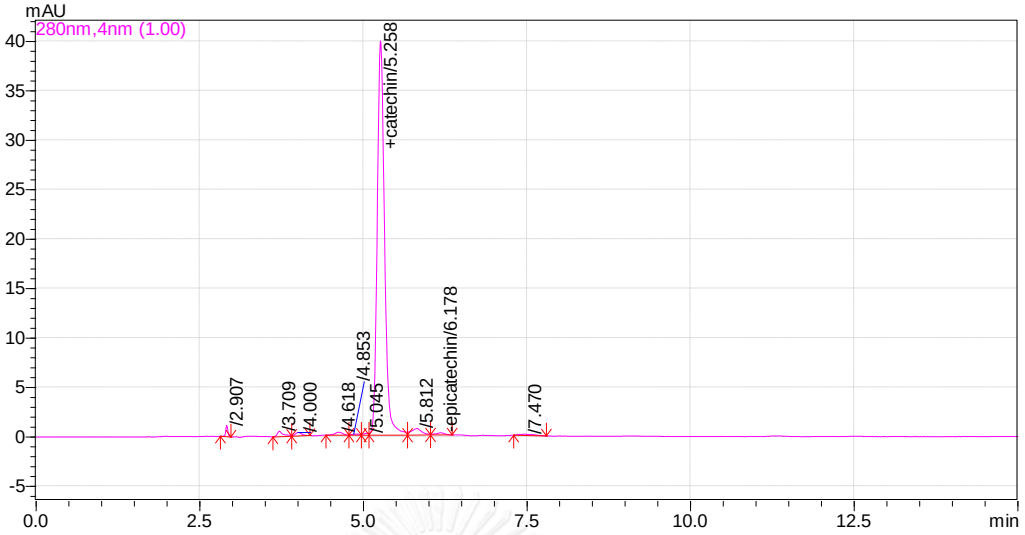

Figure 79 HPLC chromatogram of *Uncaria gambir* water extract (Samut Songkhram)

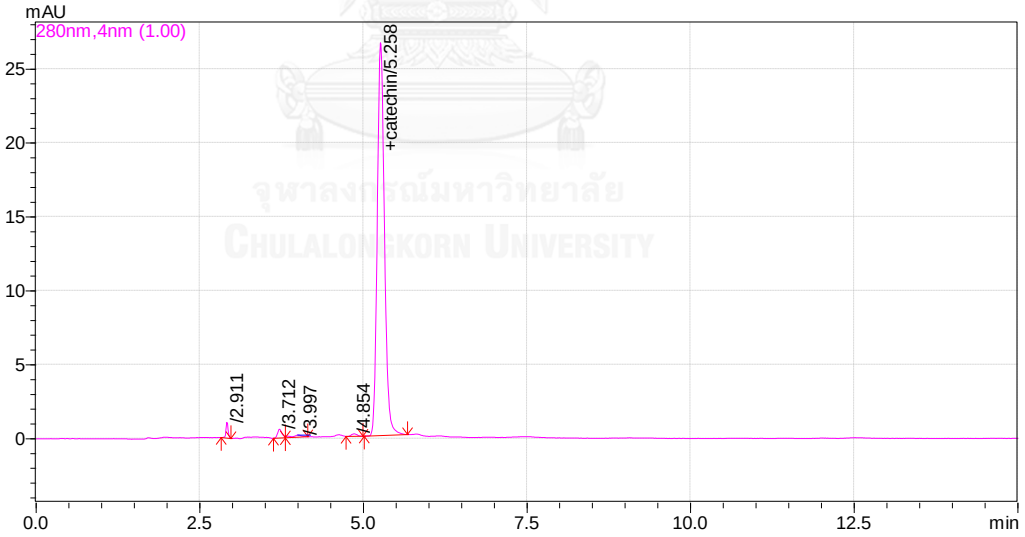

Figure 80 HPLC chromatogram of *Uncaria gambir* water extract (Satun)

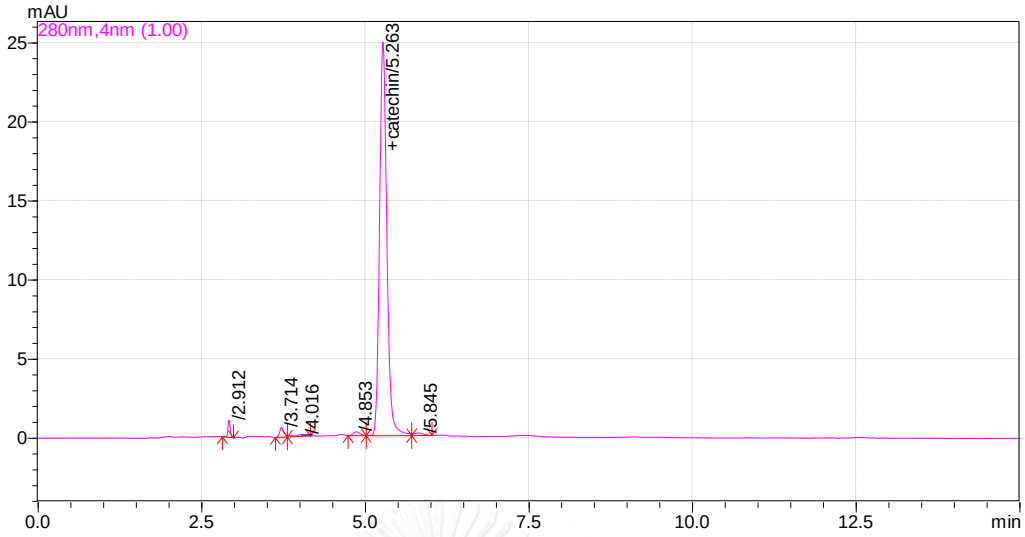

Figure 81 HPLC chromatogram of *Uncaria gambir* water extract (Songkhla)

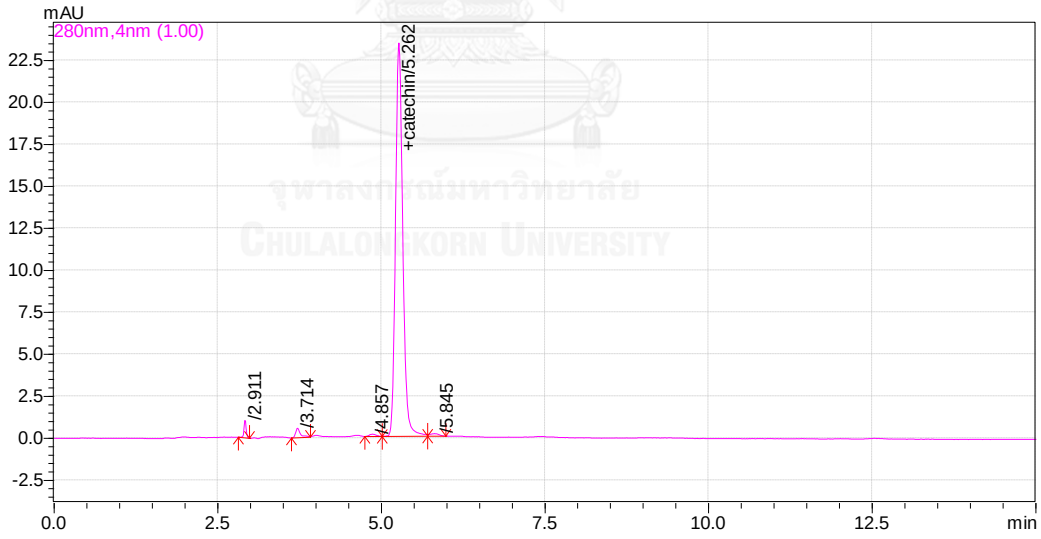

Figure 82 HPLC chromatogram of *Uncaria gambir* water extract (Songkhla 2)

APPENDIX E

HPLC chromatogram of *Pentace burmanica* stem bark extract

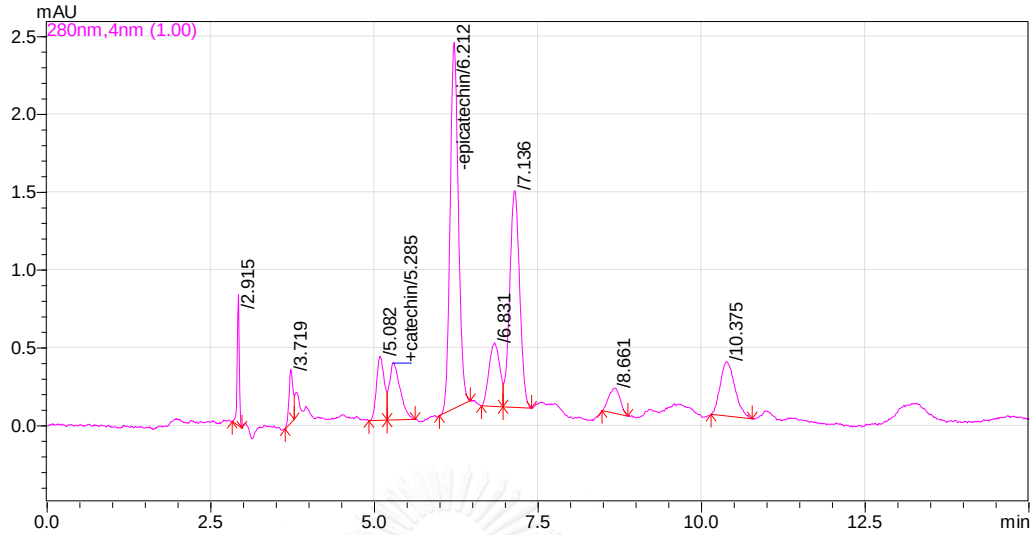

Figure 83 HPLC chromatogram of *Pentace burmanica* stem bark extract (Buri Ram)

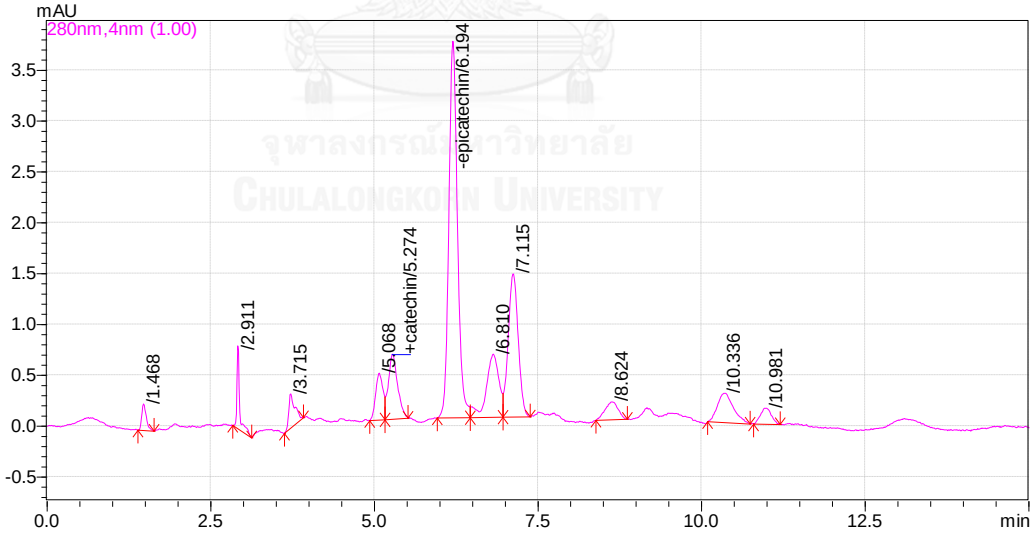

Figure 84 HPLC chromatogram of *Pentace burmanica* stem bark extract (Chaiyaphum)

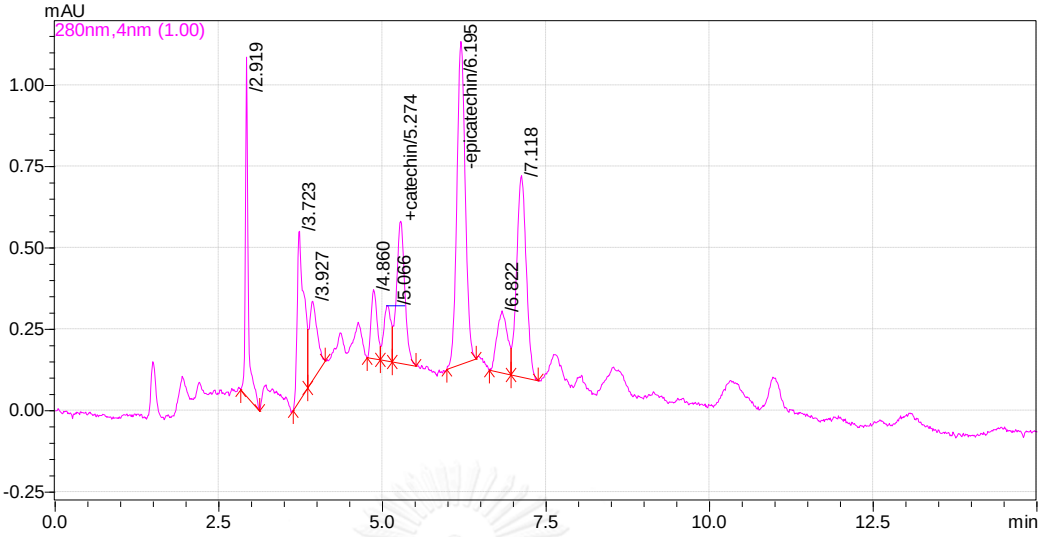

Figure 85 HPLC chromatogram of *Pentace burmanica* stem bark extract (Chiang Rai)

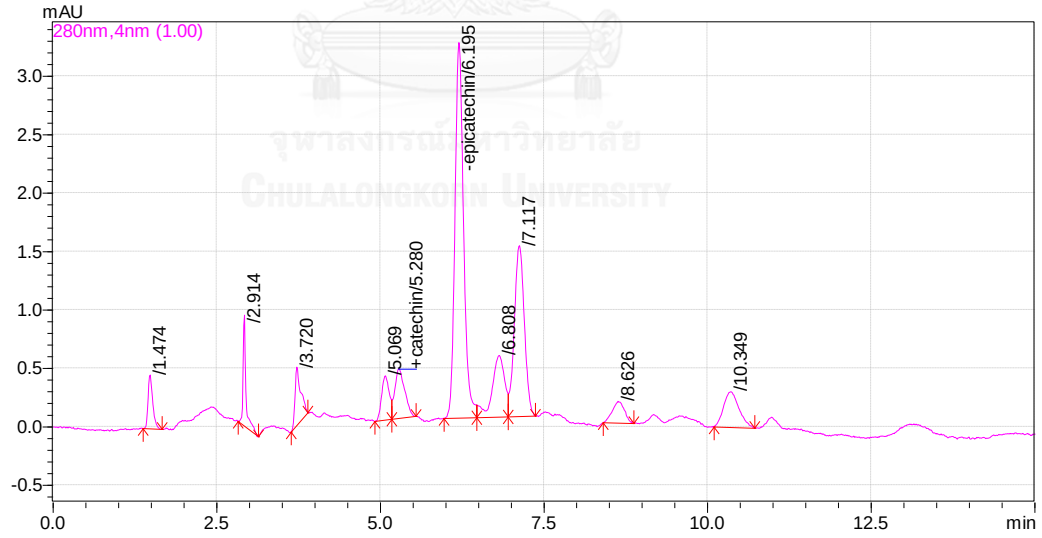

Figure 86 HPLC chromatogram of *Pentace burmanica* stem bark extract (Nakhon Nayok)

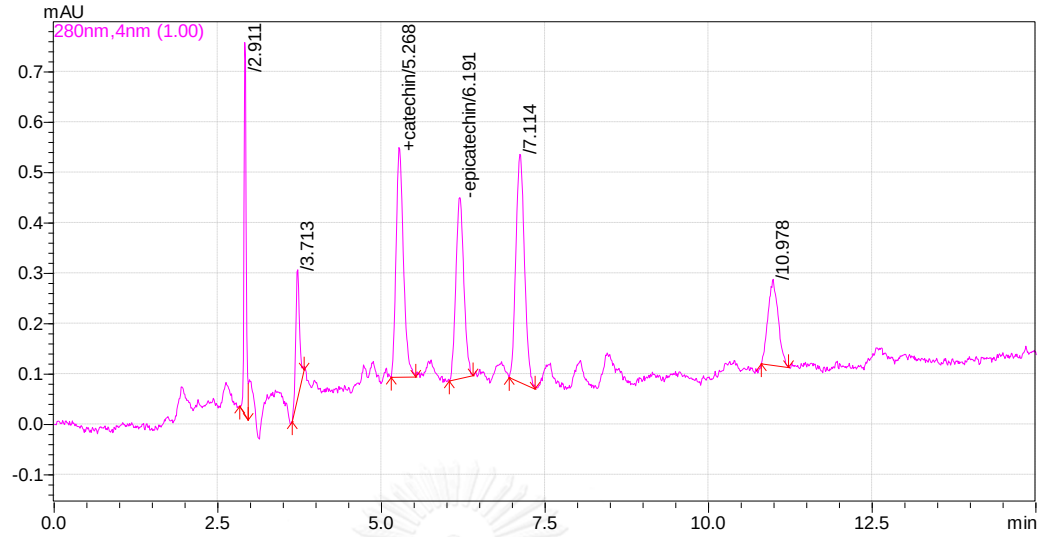

Figure 87 HPLC chromatogram of *Pentace burmanica* stem bark extract (Nakhon Nayok 2)

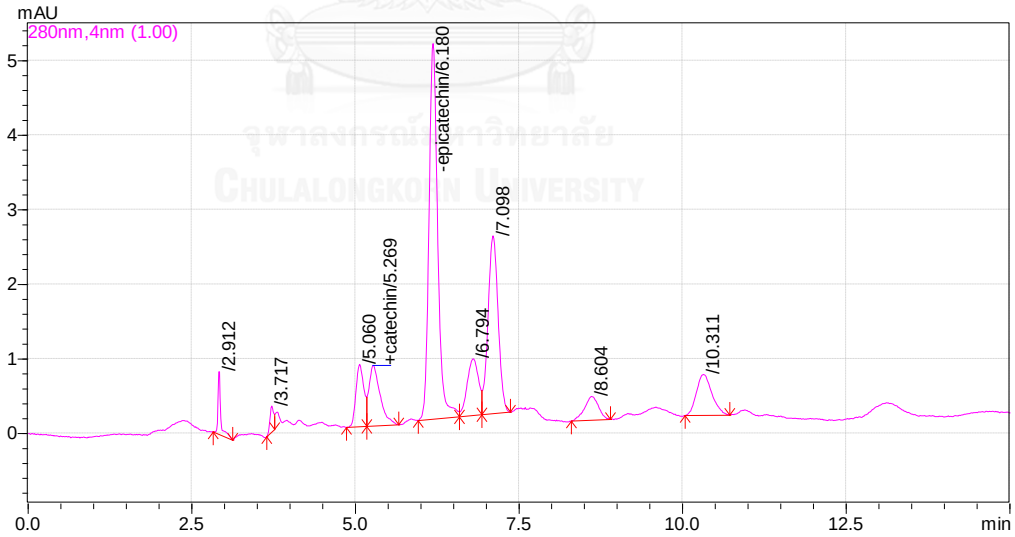

Figure 88 HPLC chromatogram of *Pentace burmanica* stem bark extract (Nakhon Pathom)

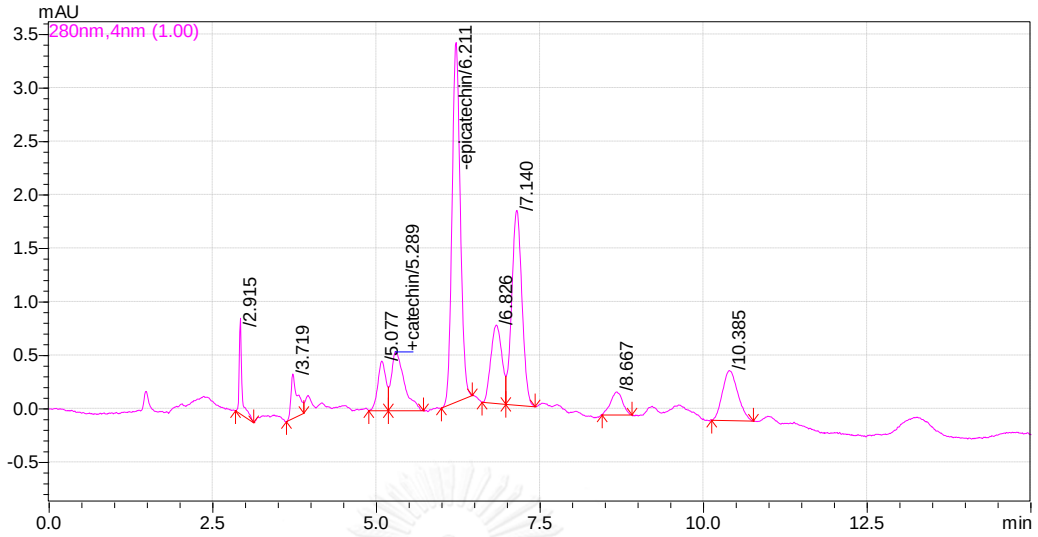

Figure 89 HPLC chromatogram of *Pentace burmanica* stem bark extract (Phetchabun)

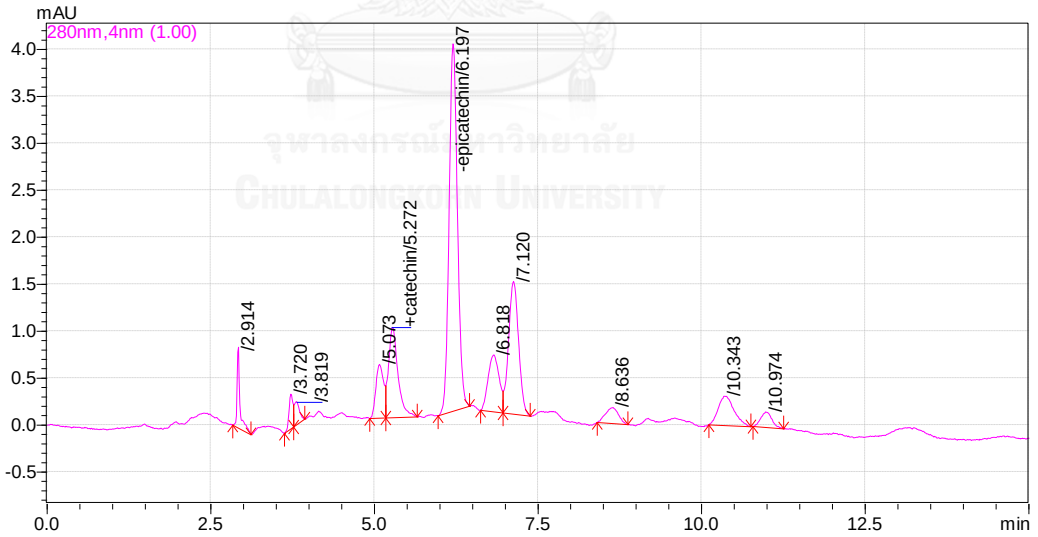

Figure 90 HPLC chromatogram of *Pentace burmanica* stem bark extract (Sa Keao)

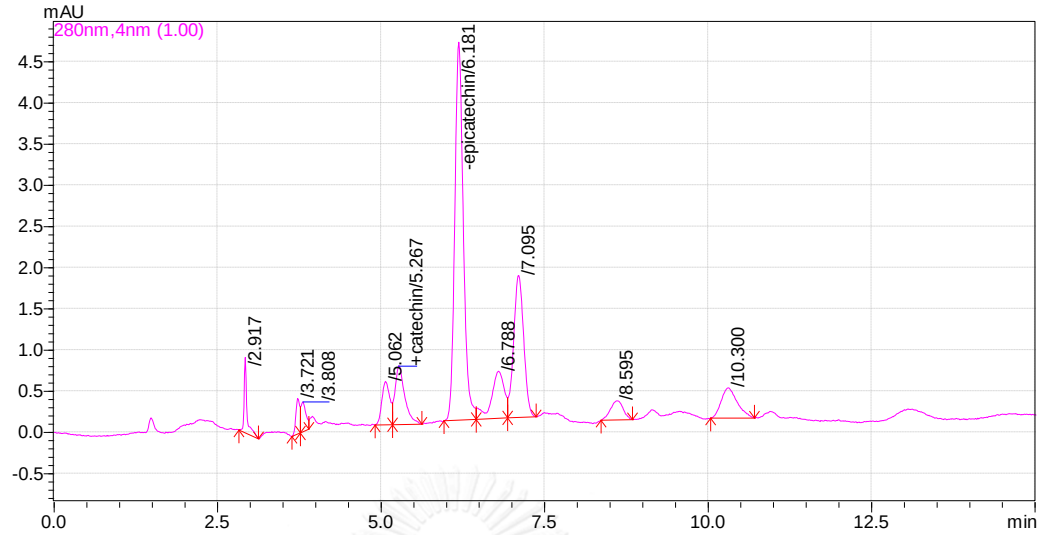

Figure 91 HPLC chromatogram of *Pentace burmanica* stem bark extract (Si Sa Ket)

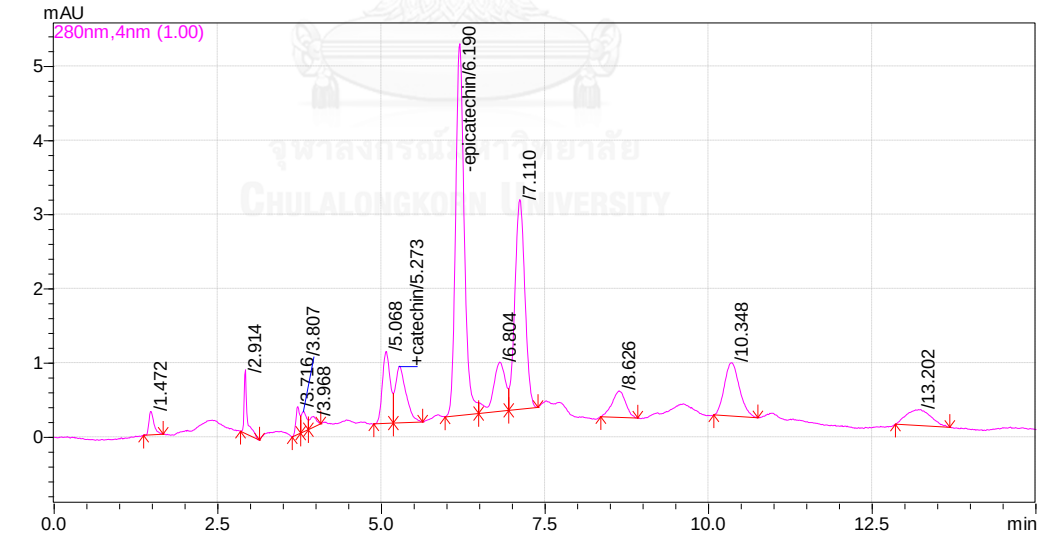

Figure 92 HPLC chromatogram of *Pentace burmanica* stem bark extract (Surin)

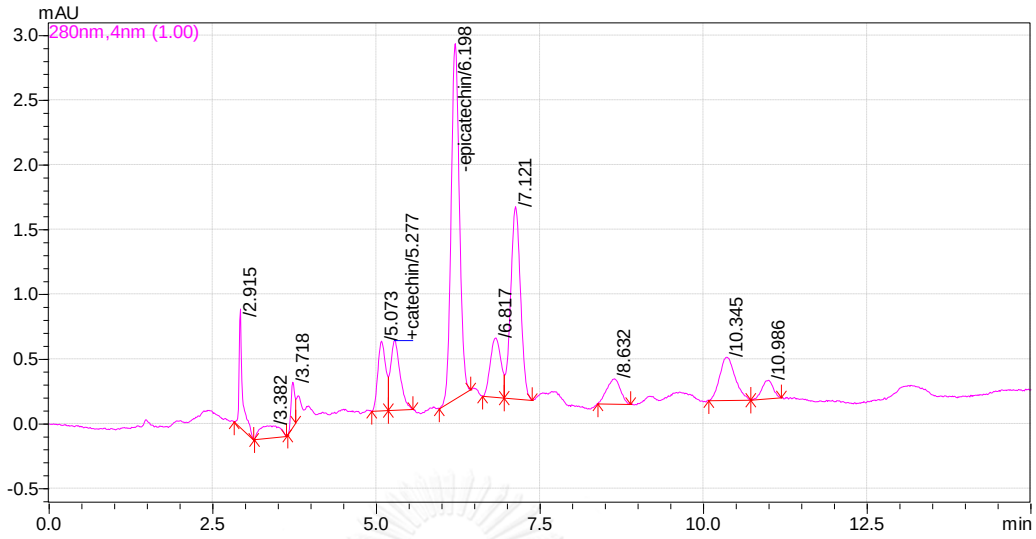

Figure 93 HPLC chromatogram of *Pentace burmanica* stem bark extract (Ubon Ratchathani)

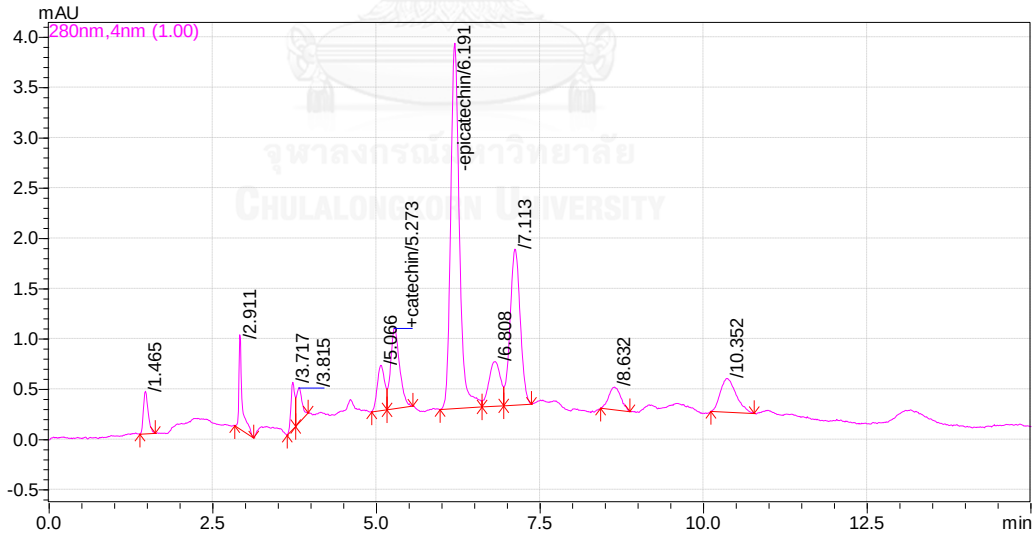

Figure 94 HPLC chromatogram of *Pentace burmanica* stem bark extract (Ubon Ratchathani 2)

VITA

Miss Thidarat Duangyod was born on January 30, 1987 in Songkhla, Thailand. She got a Bachelor's degree of Applied Thai Traditional Medicine with first class honor from School of Health Sciences, Mae Fah Luang University, Thailand in 2009 and got a Master of Science in 2011 from College of Public Health Sciences, Chulalongkorn University.

Publications

1. Duangyod, T., Palanuvej, C., and Ruangrungsi, R. Pharmacognostic specifications and quantitation of (+)-catechin and (-)-epicatechin in Pentace burmanica stem bark. Pharmacognosy Research 6 (2014): 251-256.

2. Duangyod, T., Palanuvej, C., and Ruangrungsi, R. (+)-Catechin and (-)-epicatechin contents and antioxidant activity of commercial black catechu and pale catechu. Journal of Chemical and Pharmaceutical Research 6 (2014): 2225-2232.