# Chemical composition profile and antioxidant activity of Thai medicinal plant-derived galactogogues extracts

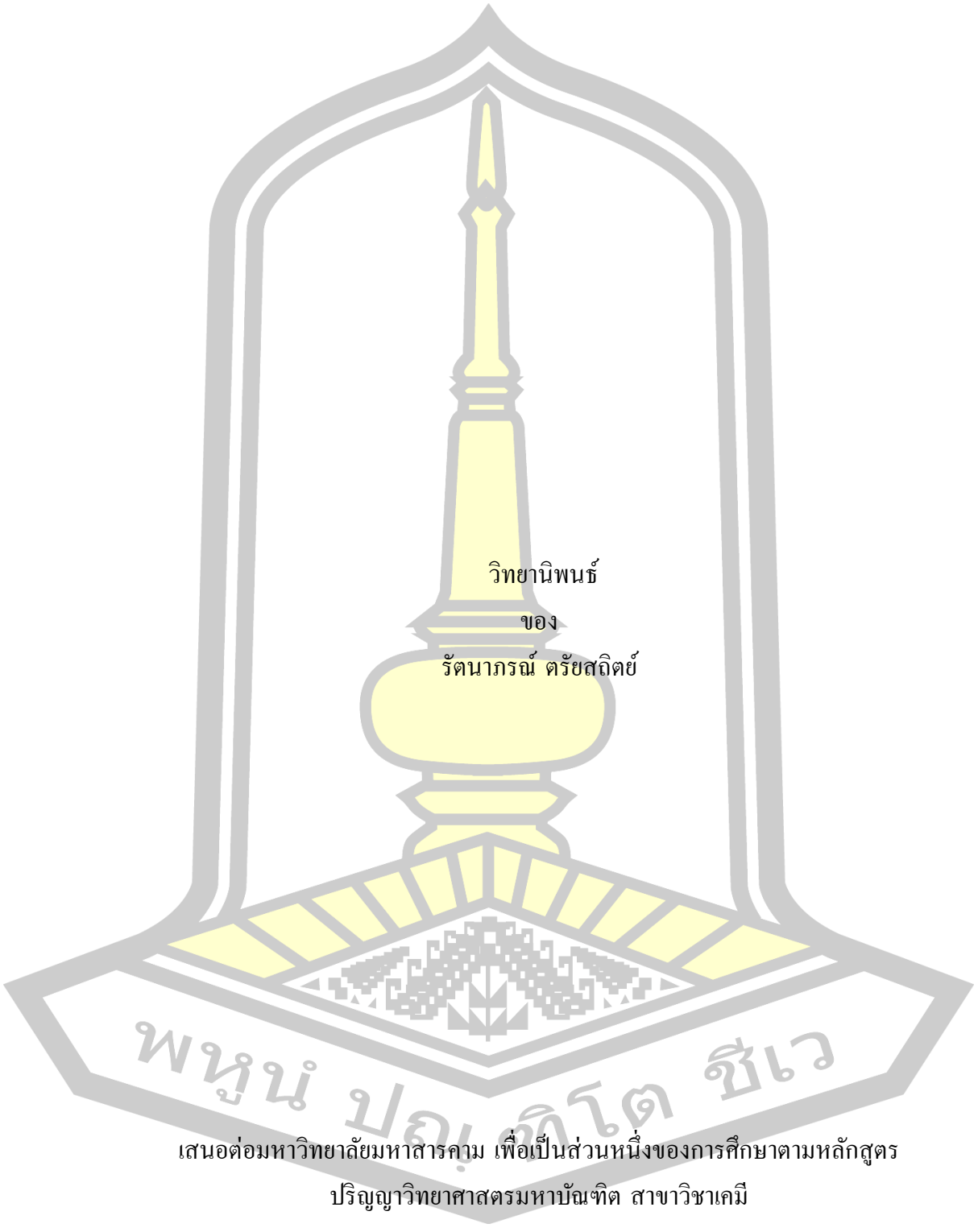
Rattanaporn Traisathit

A Thesis Submitted in Partial Fulfillment of Requirements for

degree of Master of Science in Chemistry

January 2021

# ข้อมูลองค์ประกอบทางเคมีและฤทธิ์ต้านออกซิเดชันของสารสกัดบำรุงน้ำนมจากพืชสมุนไพรไทย



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ปริญญาวิทยาศาสตรมหาบัณฑิต สาขาวิชาเคมี

มกราคม 2564

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for Master of Science (Chemistry)

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The examining committee has unanimously approved this Thesis, submitted by Miss Rattanaporn Traisathit , as a partial fulfillment of the requirements for the Master of Science Chemistry at Mahasarakham University

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**TITLE** Chemical composition profile and antioxidant activity of Thai medicinal plant-derived galactogogues extracts

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**DEGREE** Master of Science **MAJOR** Chemistry

**UNIVERSITY** Mahasarakham University **YEAR** 2021

## ABSTRACT

The phytochemicals or secondary metabolites of 16 Thai medicinal plant-derived galactogogue extracts were screened by TLC, $^{1}$H NMR and HPLC techniques. Based on TLC analysis and reagent derivatization, phenolic and antioxidant compounds were detected in both of methanol and ethyl acetate extracts of all plant while flavonoids and alkaloids were detected in some of the plant extracts. Most of the ethyl acetate extracts which were partitioned from their methanol extract revealed signals of aromatic protons ($d_H \geq 7$ ppm). The HPLC chromatograms of methanol and ethyl acetate extracts showed poor separation system. The HPLC separation of active extracts need to be optimized further.

The stem barks of *Caesalpinia sappan* (CS) and *Ochna integerrima* (OI) from methanol and ethyl acetate extracts showed potent antioxidant activity based on the DPPH, ABTS and FRAP test assays compared to the standard ascorbic acid (except for the ethyl acetate extract of OI tested the ABTS method). The scavenging activity ranging from $IC_{50}$ 5.26 ± 0.04 - 22.47 ± 0.46 mg/mL toward $DPPH^{\bullet}$, 28.61 ± 0.04 - 46.35 ± 0.18 mg/mL toward $ABTS^{\bullet+}$, and the ferric reducing activity power of 2,812.70 ± 1.22 - 950.21 ± 4.94 µM Fe (II)/g extract of CS and OI extracts were evaluated. These two plant extracts also possessed the two highest TPC of 463.61 ± 0.73 - 979.33 ± 6.93 mg GAE/g extract and moderate TFC which correlated well to their antioxidant capacities.

Keyword : Antioxidant activity, Galactogogue, Chemical composition, Antioxidant, DPPH, ABTS, FRAP, Total phenolic content, Total flavonoid content

# ACKNOWLEDGEMENTS

I would like to thank Center of Excellence for Innovation in Chemistry (PERCH-CIC), Ministry of Higher Education Science, Research and Innovation, for financial support. The council of Thailand (NRCT) and the Department of Chemistry, Faculty of Science, Mahasarakham University (MSU), are acknowledged.

Most importantly, I would like to gratefully acknowledge Asst. Prof. Dr. Prapairat Seephonkai, my supervisor, who gave me guidance, support and encouragement throughout my study.

Finally, I would like to thank my family and friends for their support, care and encouragement throughout my studies.

Rattanaporn Traisathit

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# CHAPTER 1

# INTRODUCTION

## 1.1 Background

Plants are a rich source of bioactive compounds with large variety of biological activities. Numerous of drugs or potential candidate drugs are produced by plants [1],[2]. Although research on biologically active compounds from plants and medicinal plant properties have been studying extensively a large number of plants including their biological activities are still unexplored [3].

In our continuous search for bioactive natural products from local medicinal plants, we are interested in biological property of galactogogue medicinal plants due to the wide distribution and utilization of these plants in northeast Thailand. This type of plants is used to induce, maintain and increase milk production [4],[5]. There are numbers of indigenous galactogogue plants available in Thailand as recorded in traditional recipes but there is limited scientific evidence supporting their uses [6]. Similarly to other medicinal plants, many of the galactogogue plants are also traditionally used for other treatments as well. From literature survey, research study related the galactogoge medicinal plants to their antioxidant of some plants is limited.

Therefore, in this study, sixteen of the galactogogue Thai medicinal plants from six plant families collected in northeastern Thailand were selected to evaluate for their chemical compositions and antioxidant activity in terms of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activities, ferric reducing activity power, total phenolic content and total flavonoid content.

## 1.2 Objective of research

The objectives of this research were study the chemical compositions and evaluation of antioxidant activity of sixteen galactogogue Thai medicinal plants collected in northeastern Thailand.

## 1.3 Expected result

This research aims to search potential local plants which might be utilized as antioxidant source in food and healthy product applications.

## 1.4 Scope of the research

The scopes of this research were analysis of determination of chemical compositions (thin layer chromatography, proton nuclear magnetic spectroscopy and high performance liquid chromatography) and was tested antioxidant activity ($DPPH^{\bullet}$ and $ABTS^{\bullet+}$ scavenging activity, ferric reducing activity power, total phenolic and total flavonoid contents) of Thai medicinal plant-derived galactogogue extracts.

# CHAPTER 2

# LITERATURE REVIEW

## 2.1 Bioactive compound

Chemical investigation of the bioactive compounds of sixteen galactogogue Thai medicinal plants in this research has been reported herein.

### 2.1.1 *Hubera cerasoides*

In 2007, the air-dried roots of *H. cerasoides* were collected in Khon Kaen province, Thailand, and studied by Kanokmedhakul et al., [7]. The extract was purified and tested for biological activity. Caryophyllene oxide (**1**) and octadeca-9,11,13-triynoic acid (**2**) were isolated from the hexane extract. α-Humulene (**3**), (-)-α-cadinol (**4**) and bidebiline E (**5**) were isolated from the ethyl acetate extract. Bidebiline E (**5**), laudanosine (**6**), codamine (**7**), luadanidine (**8**) and reticuline (**9**) were isolated from the methanol extract. Among these isolates, **1**, **2**, **4**, **7** and **8** exhibited antimalarial activity against *Plasmodium falciparum*, while **1**–**3** showed antimycobacterial activity against *Mycobacterium tuberculosis* in vitro assays.

In 2011, the stem barks of *H. cerasoides* were collected from Saraburi province, Thailand, and studied by Treeratanapiboon et al., [8]. The dichloromethane extract displayed the highest activity against *Corynebacterium diphtheriae* NCTC 10356 with MIC of 32 µg/mL, as well as, the highest SOD activity with an $IC_{50}$ of 4.51 µg/mL and showed antioxidation activity by DPPH method at $IC_{50}$ of 100.76 µg/mL. Purification of the hexane and chloroform extracts led to the isolation of stigmasterol (**10**). *N*-(4-Hydroxy-β-phenethyl)-4-hydroxycinnamide or paprazine (**11**) and β-sitosterol (**12**), were obtained from the ethyl acetate extract.

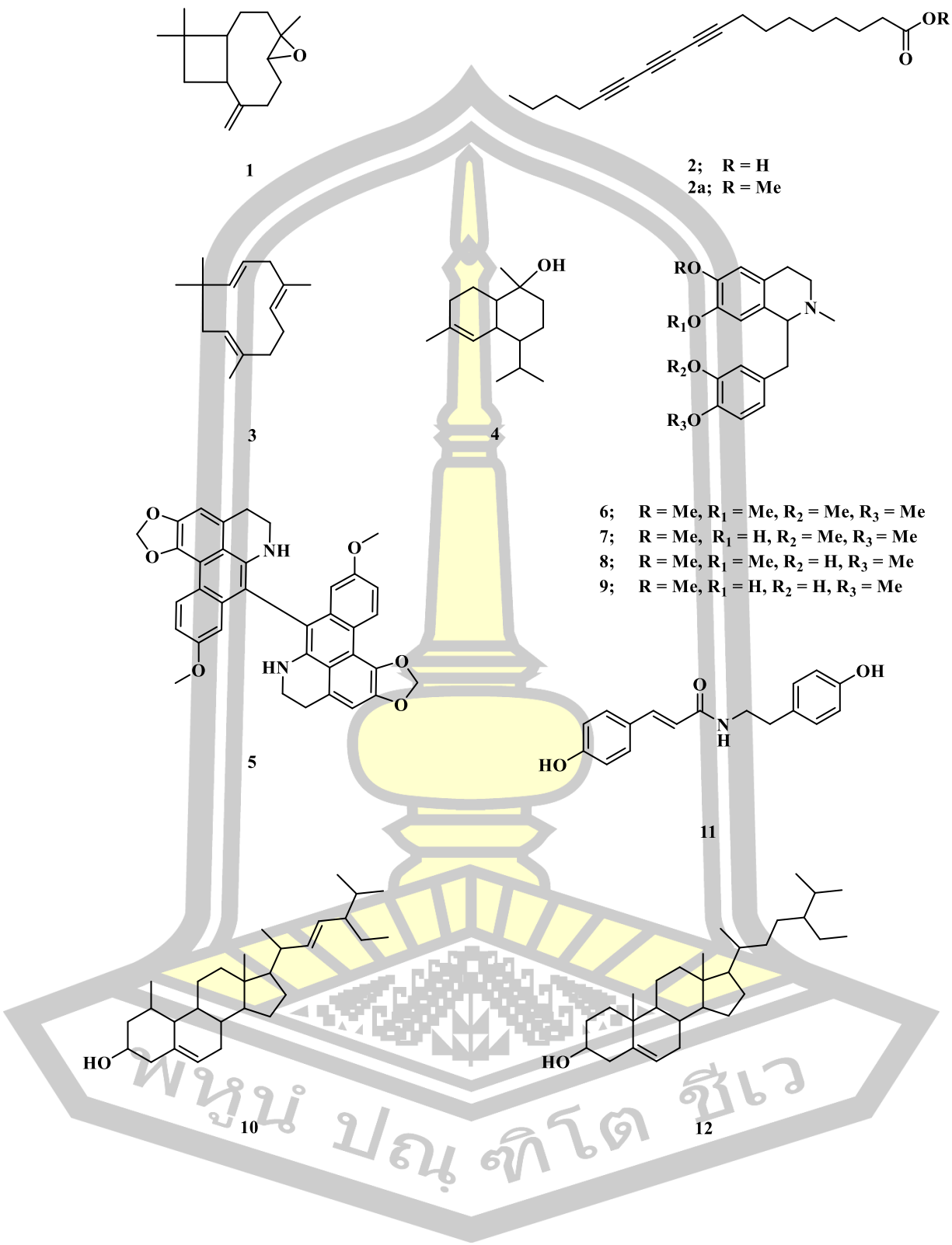



**Figure 1.** The structure of compounds **1-12** isolated from *H. cerasoides*

### 2.1.2 *Polyalthia debilis*

In 2009, the roots of *P. debilis* were collected from Ubon Ratchathani province, Thailand, and studied by Prachayasittikul et al., [9]. Purification of the chloroform extract led to the isolation of 1-methyl-4-azafluoren-9-one or onychine (**13**) and 7-methoxy-1-methyl-4-azafluoren-9-one (**14**), β-sitosterol (**12**) and stigmasterol (**10**) and fractionation of ethyl acetate extract led to the isolation of β-sitosterol (**12**), stigmasterol (**10**). Purification of the hexane extract led to the isolation of 3β-*O*-acetyltaraxer-14-en-28-oic acid or 3-acetylaleuritolic acid (**15**) and stigmasterol (**10**). Purification of the methanol extract led to the isolation of 24-methylenelanosta-7,9(11)-dien3 β,15α–diol or suberosol (**16**), β-sitosterol (**12**) and stigmasterol (**10**).

In 2003, the roots of *P. debilis* were collected from Khon Kaen province, Thailand, and studied by Kanokmedhakul et al., [10]. Fractionation of the dichloromethane extract led to the isolation of bis-7,7′-dehydroanonaine or bidebiline A (**17**), 7-dehydroanonaine-7′-dehydro-8′-methoxyanonaine or bidebiline B (**18**), bis-7,7′-dehydro-8,8′-dimethoxy anonaine or bidebiline C (**19**) and bis7,7′-dehydro-10,10′ dimethoxyanonaine or bidebiline D (**20**). The isolated compounds **19** and **20** showed moderate antimalarial activity with $IC_{50}$ values of 5.4 and 4.1 μg/mL, respectively.

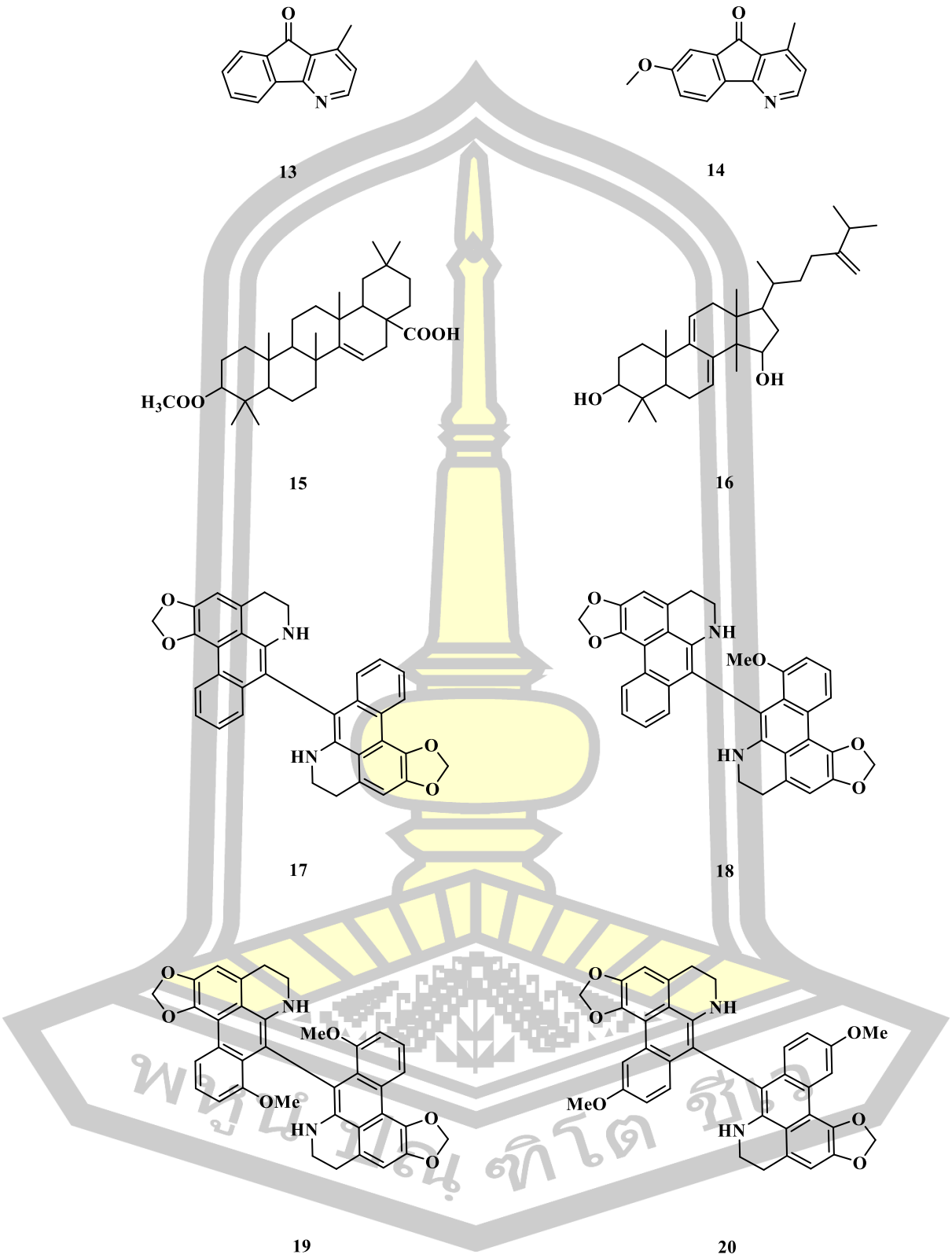



**Figure 2.** The structure of compounds **13-20** isolated from *P. debilis*

### 2.1.3 *Polyalthia evecta*

In 2015, the aerial parts dried-powder of *P. evecta* were collected from Sakon Nakhon province, Thailand, and studied by Nantapap et al., [11]. Purification of hexane extract led to the isolation of stigmasterol (**10**) and β-sitosterol (**11**). Fractionation of the ethyl acetate extract led to the isolation of oxostephanine (**21**) and goniothalamin (**22**). Purification of the methanol extract led to the isolation of oxostephanine (**23**), dicentrinone (**24**) and allantoin (**25**).

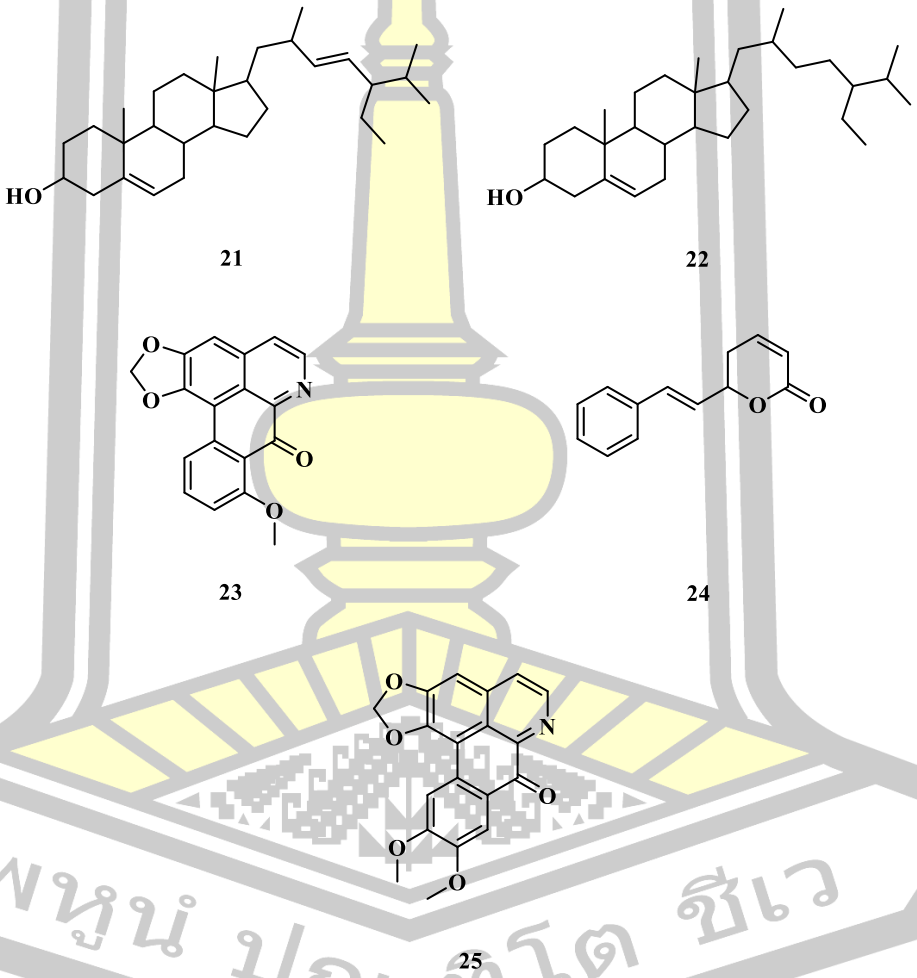
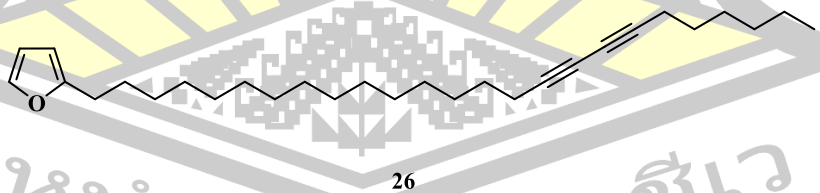



**Figure 3.**The structure of compounds **21**-**25** isolated from *P. evecta*

### 2.1.4 *Polyalthia suberosa*

In 1993, the stems and leaves of *P. suberosa* were collected from Yunnan province, China, and studied by Li et al., [12]. Fractionation of the ethyl acetate extract led to the isolation of 24-methylenelanosta-7,9(11)-dien3 β,15,α–diol or suberosol (**16**). The isolated compound **16** was tested for inhibition HIV replication in H9 lymphocyte cells with $EC_{50}$ value of 3 µg/ml and inhibition of uninfected H9 cell growth with $IC_{50}$ value of 20 µg/ml.

In 2001, the leaves, stems and barks of *P. suberosa* were collected from Kalasin province, Thailand, and studied by Tuchinda et al., [13]. Purification of the acetone extract led to the isolation of 2-substituted furans are 1-(2-furyl)pentacosa-16,18-diyne (**26**) and 23-(2-furyl)tricosa-5,7-diynoic acid (**27**). The isolated compound **26** and **27** were tested by HIV-1 reverse transcriptase and syncytium assay using MC99 virus and 1A2 cell line system and co-reported with previously reported HIV inhibitors are kalasinamide, *N-trans*-feruloyltyramine and *N-trans*-coumaroyltyramine. Compounds **26** and *N-trans*-coumaroyltyramine were inactive, whereas **27**, kalasinamide and *N-trans*-feruloyltyramine showed significant inhibitory activities ($IC_{50}$ 39.9 µg/ml, 153.9 µg/ml and 9.6 µg/ml, respectively) with HIV-1 reverse transcriptase. The isolation of **26**, **27**, kalasinamide, *N-trans*-feruloyltyramine and *N-trans*-coumaroyltyramine were active in the MC99 syncytium assay with $EC_{50}$ values of 43.3, 8.9, 7.3, 3.2 and < 3.9 µg/ml, respectively.



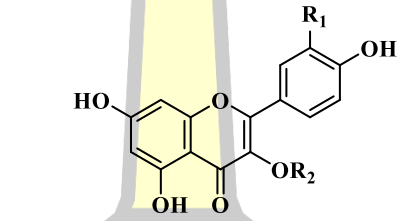

26


27

**Figure 4.** The structure of compounds **26** and **27** isolated from *P. suberosa*

### 2.1.5 *Uvaria rufa*

In 2009, leaves of *U. rufa* were collected from Chachoengsao province, Thailand and studied by Deeppralard et al., [14]. Fractionation of the ethyl acetate extract led to the isolation of rutin (**28**), isoquercitrin (**29**), kaempferol 3-O-β-D-galactopyranoside (**30**), astragalin (**31**) and isoquercitrin-6-acetate (**32**). All the isolated compounds were tested advanced glycation end products or AGEs inhibitory activity that cause diabetes. Compound **29** and **32** showed inhibitory activity of AGEs at $IC_{50}$ of 8.4 and 9.6 μM, and were compared to quercetin with inhibitory activity of AGEs at $IC_{50}$ of 10.9 μM.




**28;** $R_1$ = OH, $R_2$ = *β*-D-glucose-*α*-rhamnose
**29;** $R_1$ = OH, $R_2$ = *β*-D-glucose
**30;** $R_1$ = H, $R_2$ = *β*-D-galectose
**31;** $R_1$ = H, $R_2$ = *β*-D-glucose
**32;** $R_1$ = OH, $R_2$ = *β*-D-glucose-6-acetate

**Figure 5.** *The structure of compounds **28-32** isolated from U. rufa*

### 2.1.6 *Caesalpinia sappan*

In 1987, the heartwoods of *C. sappan* were studied by Namikoshi et al., [15]. Purification of the methanol extract led to the isolation of 3'-*O*-methylsappanol (**33**), 3'-*O*-methylepisappanol (**34**), 3'-*O*-methylbrazilin (**35**), sappanol (**36**), episappanol (**37**), 3'-deoxysappanol (**38**), **33**, **34** and brazilin (**39**).

In 2014, the heartwoods of *C. sappan* were collected from Bozhou, China, and studied by Wang et al., [16]. The ceasalpinia A (**40**) and ceasalpinia B (**41**),

protosappanin A (**42**), 3,9-dihydroxy-8-methoxy-dibenzo[b,d]pyran-6-one (**43**) and 3,8,9-thihydroxy-6H-methoxy-benzo[c]chromem-6-one (**44**) were obtained from the ethyl acetate extract. Compound **44** was discovered for the first time of this genus. All isolated compounds were tested for cytotoxicity against MCF7 cells, A549 cells and LN229 cells by MTT assay. Compound **40** showed moderate toxicity to A549 cells at $IC_{50}$ of 54.2 μM, while there is not present inhibition of MCF7 cells and LN229 cells. Compounds **43** and **44** showed inhibitory activity on MCF7 cells and A549 cells at $IC_{50}$ 79.6 μM and 68.3 μM, respectively. Compounds **41** and **42** did not present any inhibitory activity.

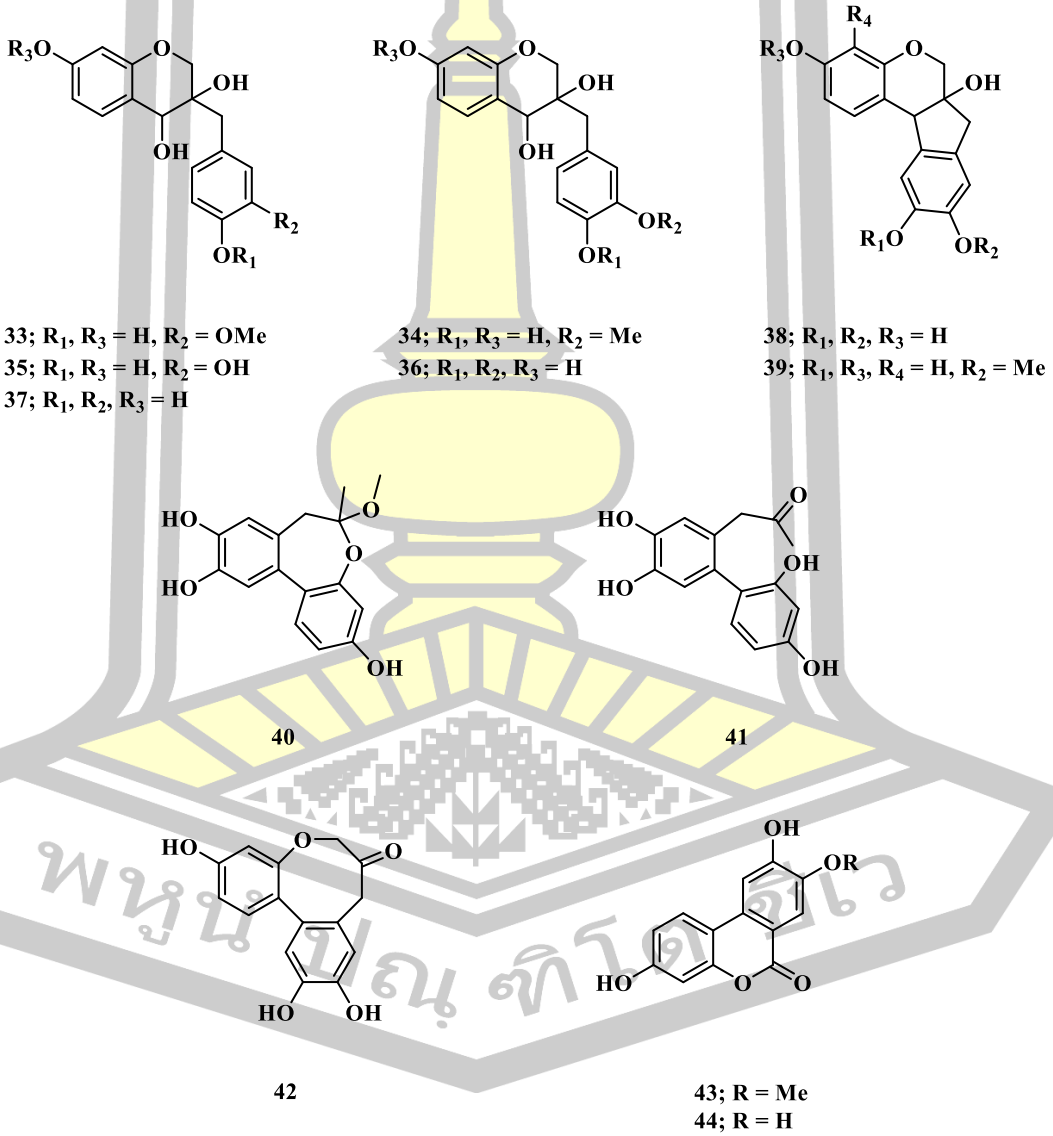



**Figure 6.** The structure of compounds **33-44** isolated from *C. sappan*

### 2.1.7 *Celastrus paniculatus*

In 1989, the root barks of *C. paniculatus* were collected from Bangkok, Thailand, and studied by Pavanand et al., [17]. The chloroform extract was tested for antimalarial activity and showed the height of antimalarial activity at MIC of 6 μg/ml. The purification of chloroform extract led to isolation of pristimerin (**45**). The isolated compound **45** was tested for *P. falciparum* with antimalarial drugs. This compound **45** are showed to be less effective against malaria than conventional antimalarial drugs.

In 2004, the seeds of *C. paniculatus* were collected from Mumbai, India, and studied by Borrelli et al., [18]. Purification of methanol extract led to isolation of 1α,8β,14-triacetoxy-9β-furoyloxydihydro-β-agarofuran (**46**),1α,6β,8β,14-triacetoxy-9β-benzyloxy dihydro-β-agarofuran (**47**) and 1α, 8β-diacetoxy-9β-benzyloxydihydro-β-agarofuran (**48**).

In 2013, the whole plants of *C. paniculatus* were collected from Pingtung, Taiwan, and studied by Weng et al., [19]. Fractionation of methanol extract led to the isolation of (1α,2α,8β,9β)-1,8-bis(acetyloxy)-9-(benzoyloxy)-2-hydroxy-β-dihydro agarofuran (**49**), (1α,2α,8β,9β)-1,8,14-tris(acetyloxy)-2,9-bis(benzoyloxy)-β-dihydro agarofuran (**50**) and ( 1α,2α,8β,9β)-1,8-bis(acetyloxy)-2,9-(benzoyloxy)-14-hydroxy-β-dihydro agarofuran (**51**). All isolated compounds were tested for anticancer activity (MCF-7). Compound **51** showed anti-cancer activity at $IC_{50}$ of 17 ± 1 μM.

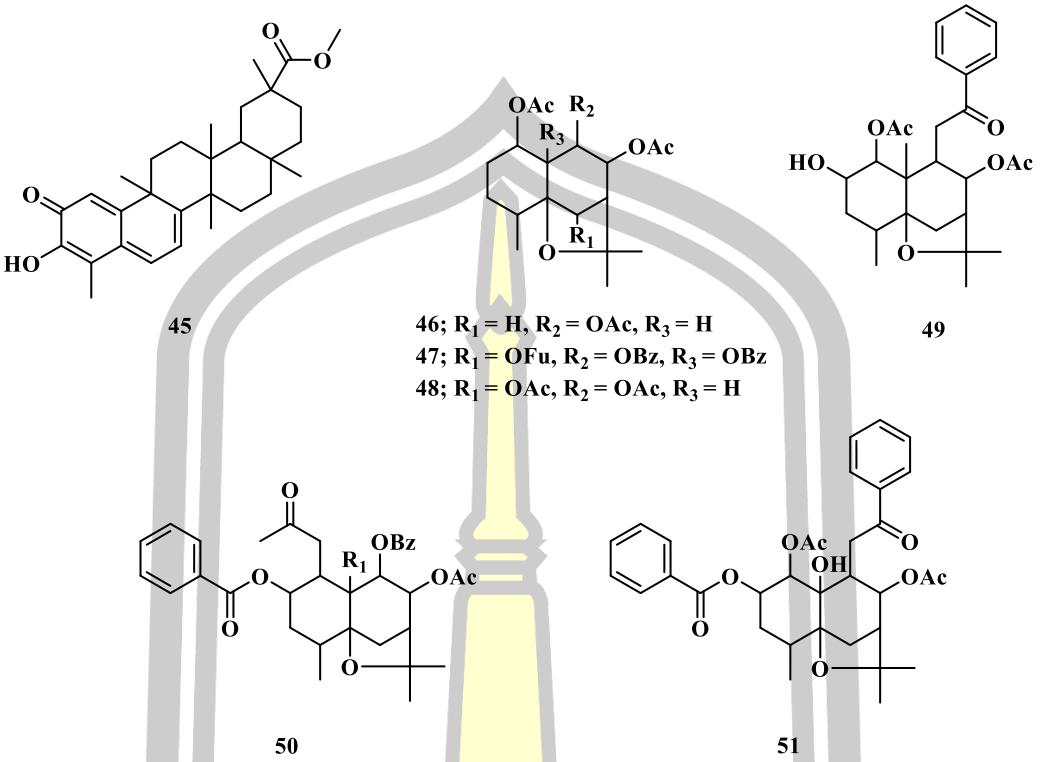



**Figure 7.** The structure of compounds **45**-**51** isolated from *C. paniculatus*

### 2.1.8 *Salacia chinensis*

In 2003, the stems of *S. chinensis* were collected from Thailand, and investigated by Kishi et al., [20]. Purification of the methanol extract led to the isolation of salasone D (**52**), salasone E (**53**), salaquinone B (**54**) and salasone B (**55**).

In 2008, the leaves of *S. chinensis* were collected from Nakhon Si Thammarat province, Thailand and studied by Zhang et al., [21]. Fractionation of the methanol extract led to the isolation of fuliasalacin D1 (**56**), fuliasalacin D2 (**57**) and fuliasalacin D3 (**58**).

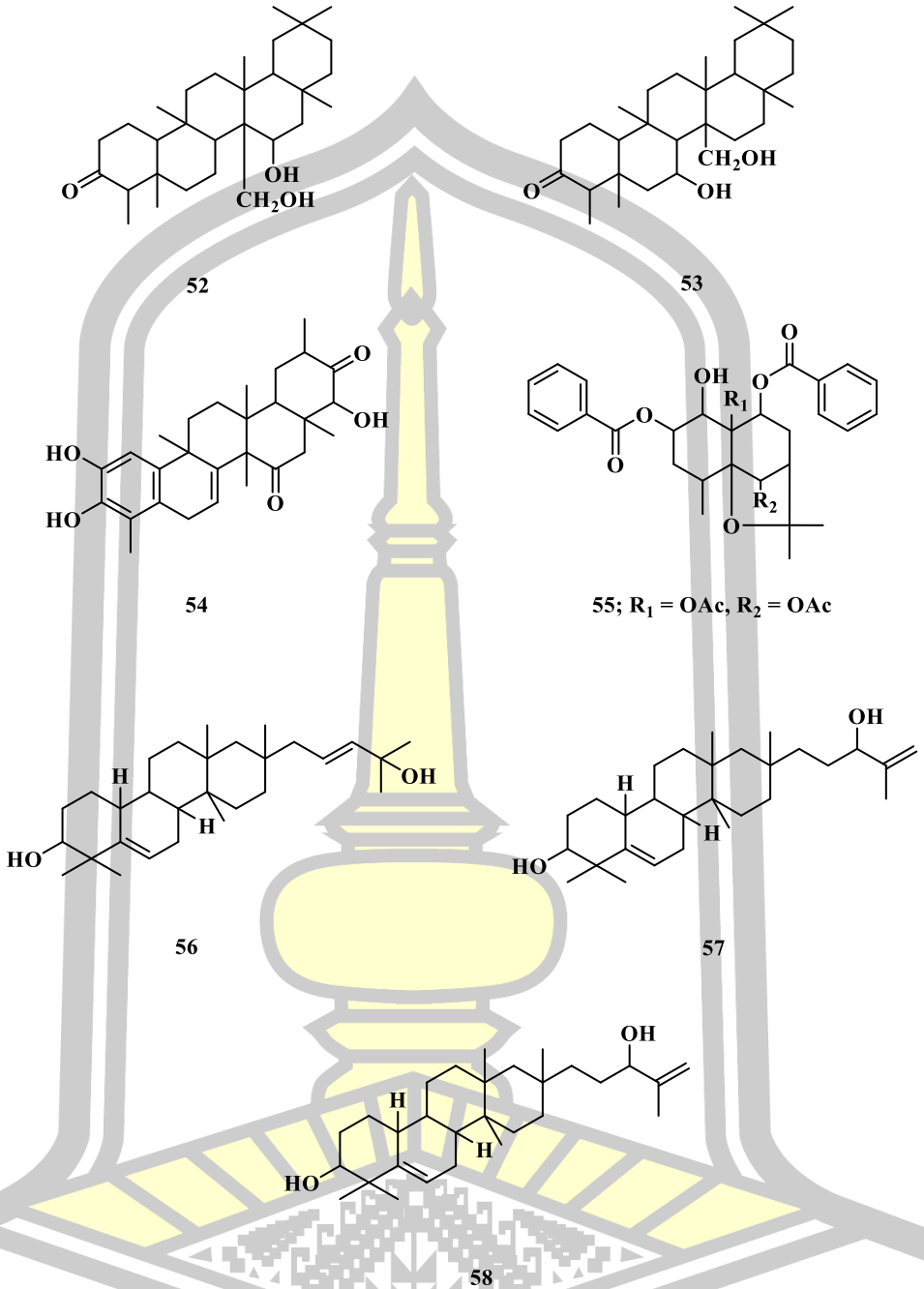



**Figure 8.** The structure of compounds **52-58** isolated from *S. chinensis*

### 2.1.9 *Salacia verrucose*

In 2011, the barks of *S. verrucose* were collected from Bueng Kan province, Thailand, and investigated by Somwong et al., [22]. Purification of the hexane extract led to the isolation of friedelane-1,3-dione (**59**) and friedelin (**60**). Fractionation of the ethyl acetate extract led to the isolation of 1,3-diketofriedelane triterpene, 21-α-hydroxyfriedelane-1,3-dione (**61**), 30-hydroxyfriedelane-1,3-dione (**62**), 26-hydroxy friedelane-1,3-dione (**63**), 21α-hydroxy-D:A-friedo-olean-3-one (**64**) and kokoonol (**65**). All isolated compounds were tested for cytotoxicity of SW620, HepG2 and KATO-II cell line. Compound **59** showed good cytotoxicity of SW620 cell line at $IC_{50}$ of 2.02 μM. Compounds **64** and **65** showed moderate cytotoxicity of SW620, HepG2 and KATO-II cell lines.

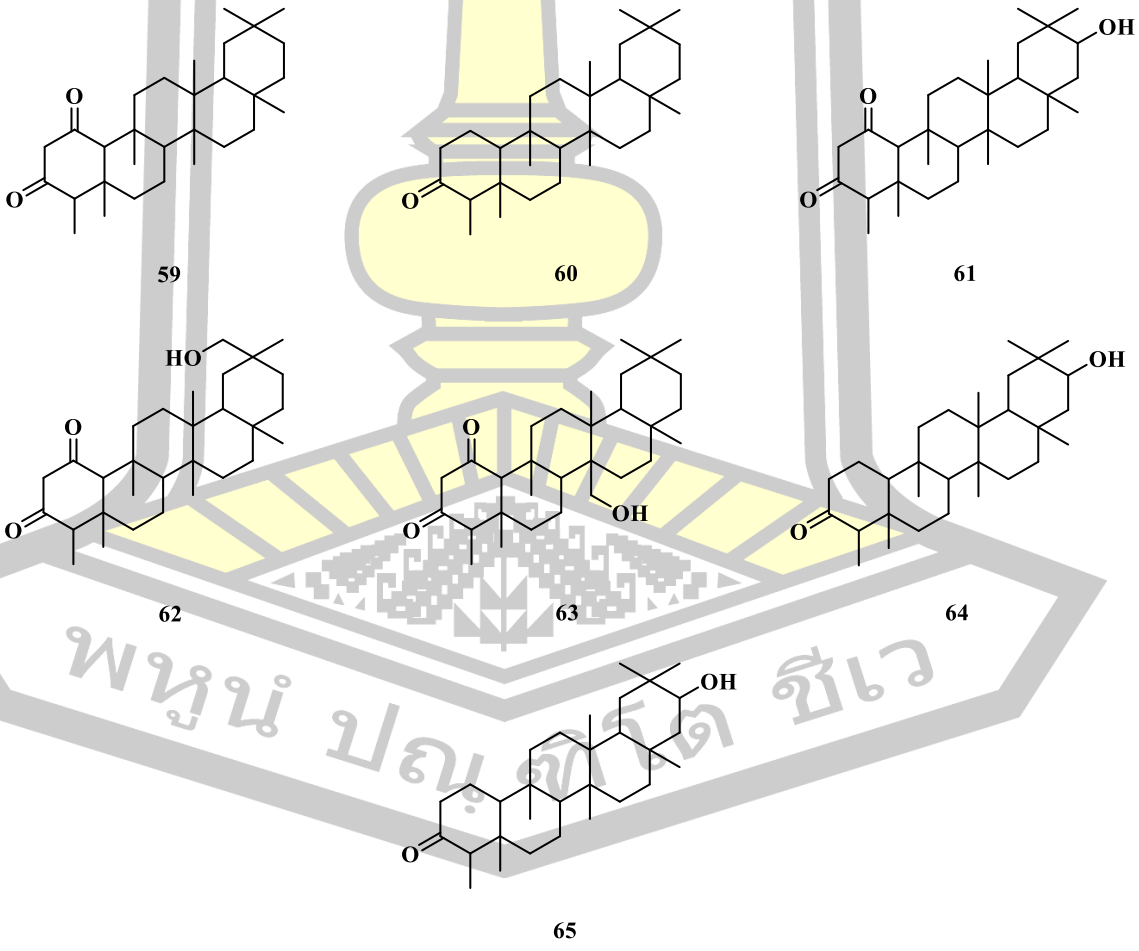



**Figure 9.** The structure of compounds **59-65** isolated from *S. verrucose*

### 2.1.10 *Siphonodon celastrineus*

In 2005, the root barks of *S. celastrineus* were collected from Phitsanulok province, Thailand, and investigated by Niampoka et al., [23]. Purification of the hexane extract led to the isolation of 3β-acetoxy-11α-benzoyloxy-13βhydroxyolean-12-one (**66**) and pristimerin (**45**).

In 2013, the barks of *S. celastrineus* were collected from Nakhon Ratchasima province, Thailand, and investigated by Kaweetripob et al., [24]. Fractionation of the hexane extract led to the isolation of 3β-25-epoxy-25-methoxylup-20(29)-ene (**67**), 25-hydroxy friedelane-3,21-dione (**68**), 25-benzoyloxyfriedelane-3,21-dione (**69**), 3β-12-dihydroxyolean-12-en-11-one (**70**), 3β,28-epoxyurs-11-ene-3β,15α-diol (**71**), 3β,12,15α-trihydroxyurs-12-en-11-one (**72**), 11α-methoxyurs-12-ene-3β,12,15α-triol (**73**), Usr-12-ene-3β,11,15α-triol (**74**), 12,15α, 20β- trihydroxyurs-12-en-3,11-dione (**75**), 11α-methoxyurs-12-ene-3β,15α-diol (**76**), 3β,12,24-trihydroxyurs-12-en-11-one (**77**), 15α-benzoyloxyurs-12-ene-3β,11α-triol (**78**), 11α-methoxyurs-12-ene-3β,12-diol (**79**), 3β,11α,12,15α-tetrahydroxyurs-12-en-24-al (**80**), 3β,11α,12,15α-tetrahydroxyurs-12-en-24-oic acid methylester (**81**), usr-12-ene-3β,11α,12,15α-tetrol (**82**), 12,15α,24-trihydroxyurs-12-en-3,11-dione (**83**), 15α-benzoyloxyurs-3β,11α,12-trihydroxyurs-12-en-24-al (**84**), 21β-hydroxy-3-oxo-2,3-secours-12-en-2-oic acid (**85**), 11α,12-[2-(hydroxymethyl)-3-(4-hydroxy-3-methoxyphenyl)ethane-1,2-dioxy]-use-12-ene3β,15α-diol (**86**) and 11α,12-[3-(hydroxymethyl)-2-(4-hydroxy-3-methoxy phenyl) ethane-1,2-dioxy]-use-12-ene-3β,15α-diol (**87**).

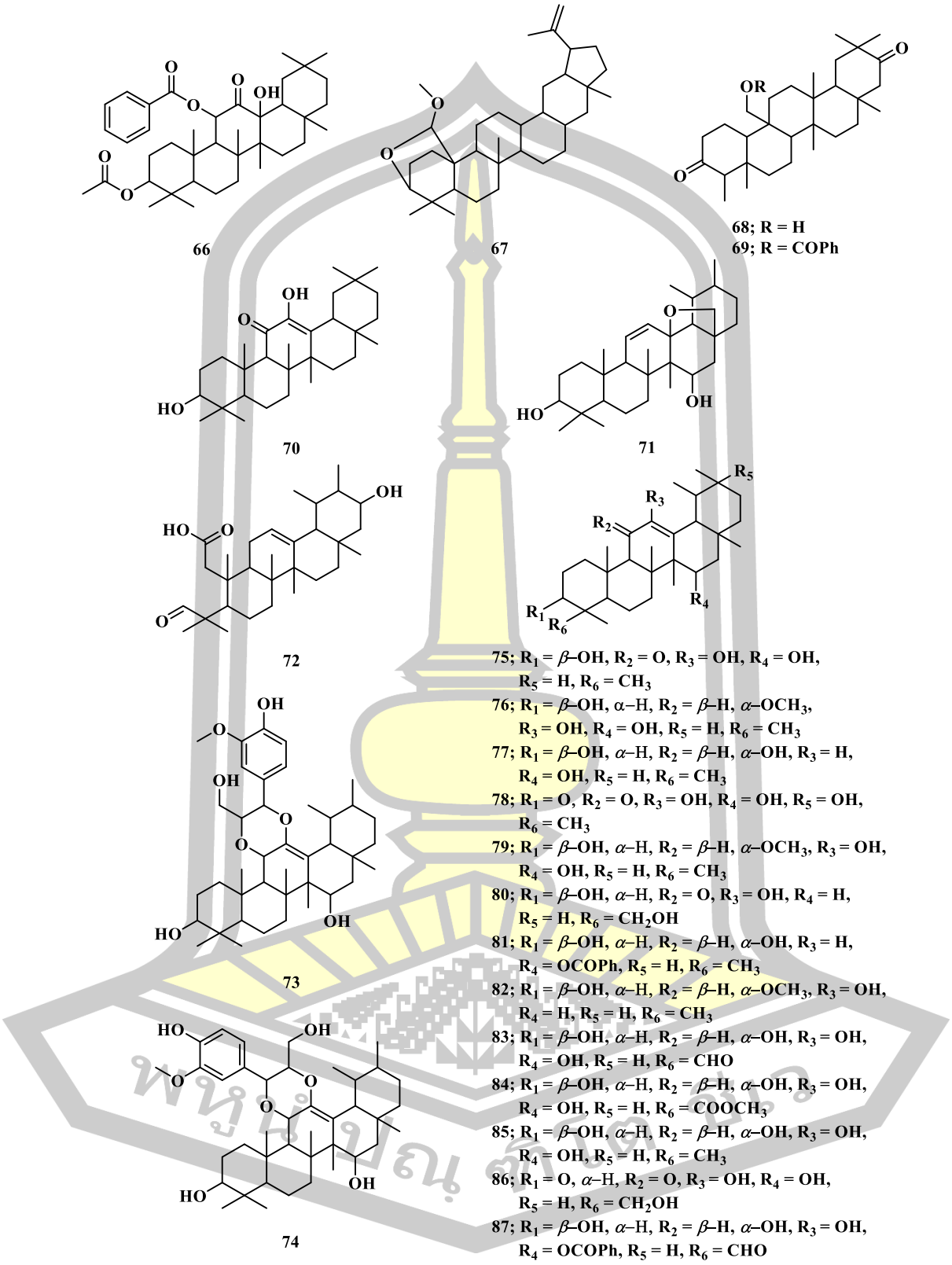



**Figure 10.** The structure of compounds **66-87** isolated from *S. celastrineus*

### 2.1.11 *Clausena harmandiana*

In 2010, the roots of *C. harmandiana* were collected from Khon Khan province, Thailand, and investigated by Thongthoom et al., [25]. Purification of the ethyl acetate extract led to the isolation of osthol (**88**), decosyl ferulate (**89**), methyl carbazole-3-carboxylate (**90**), mukonal (**91**), heptazoline (**92**), 7-methoxymukonal (**93**), glycosinine (**94**), clausine-C (**95**), clauszoline-K (**96**) and clausine-V (**97**). All isolated compounds were tested for cytotoxicity of NCI-H187, MCF-7 and KB cell line. Compound **93** showed cytotoxicity to NCI-H187 cell line at $IC_{50}$ 1.63 µg/mL. Compound **94** showed cytotoxicity to MCF-7 and KB cell lines at $IC_{50}$ 2.21 and 1.74 µg/mL, respectively. Compounds **91** and **93** showed moderate cytotoxicity to MCF-7 cell line, and **96** showed moderate cytotoxicity to NCI-H18 and KB cell lines, whereas **92** and **94** showed antimalarial activity $IC_{50}$ 3.27 and 2.94 µg/mL, respectively.

In 2012, the limbs of *C. harmandiana* were collected from Chiang Rai province, Thailand and investigated by Maneerat et al., [26]. Fractionation of the mixed hexane and acetone extracts led to the isolation of harmandianamine A (**98**), harmandianamine B (**99**) and harmandianamine C (**100**) which is new alkaloids, and clausevatine D (**101**), clausamine A (**102**), clausamine B (**103**), clausine S (**104**), girinimbine (**105**), *O*-demethylmurrayanine (**106**), clauszoline I (**107**), clausine Z (**108**), clauszoline N (**109**), clausine D (**110**), clausine F (**111**), clausemine D (**112**), heptaphylline (**113**), dectamine (**114**) and γ-fagarine (**115**). All compounds were tested for anti-bacterial activity. Compound **103** showed anti-bacterial activity to MRSA SK1 at MIC 0.25 µg/mL. Compounds **111** and **102** exhibited anti-bacterial activity to MRSA SK1 at MIC 4 and 8 µg/mL, respectively. Only compound **111** was active against *S. aureus* TISTR 1466 at MIC 4 µg/mL.

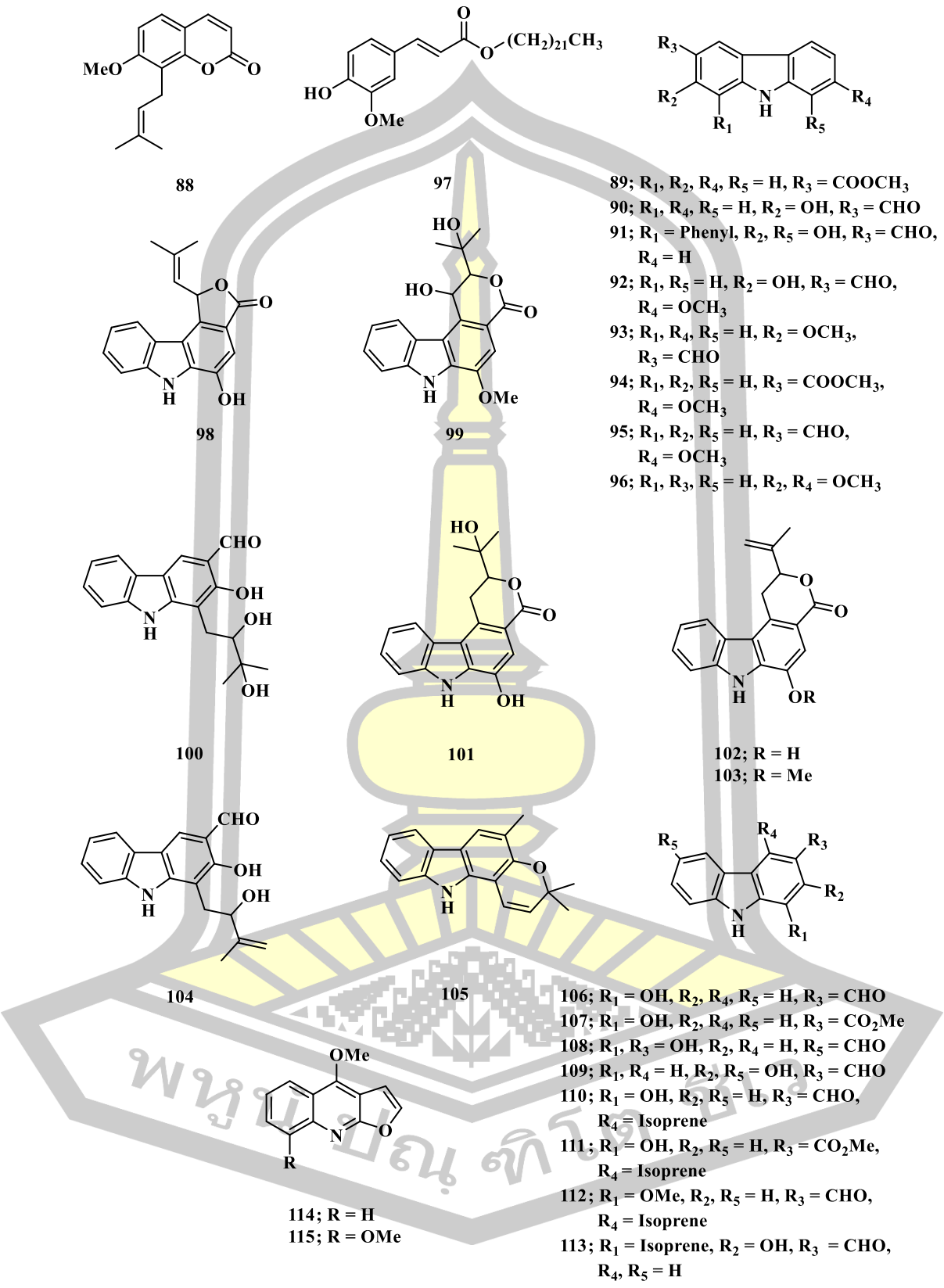



**Figure 11.** The structure of compounds **88-115** isolated from *C. harmandiana*

### 2.1.12 *Glycosmis pentaphylla*

In 1999, the barks of *G. pentaphylla* were collected from Papua New Guinea, and investigated by Ito et al., [27]. Purification of the mixed methanol and dichloromethane extracts led to the isolation of glyciquinone (**116**) and glycocitrine-III (**117**).

In 2012, studied the barks of *G. pentaphylla* were collected from Yunnan province, China, and extracted by Yang et al., [28]. Purification of the ethyl acetate extract led to the isolation of 4-(7-hydroxy-3-methoxy-6methyl-9H-carbazol-4-yl) but-3-en-2-one (**118**), bisglybomine B (**119**), biscarbalexine A (**120**), glybomine B (**121**), glycoborinine (**122**), carbalexine A (**123**), 4,8-dimethoxy-1-methyl-3-(3-methylbut-2-en-1-yl) quinolin-2(1H)-one (**124**), 4,8-dimethoxyfuro [2,3-b]quinolone (**115**), skimmianine (**125**) and arborinine (**126**).

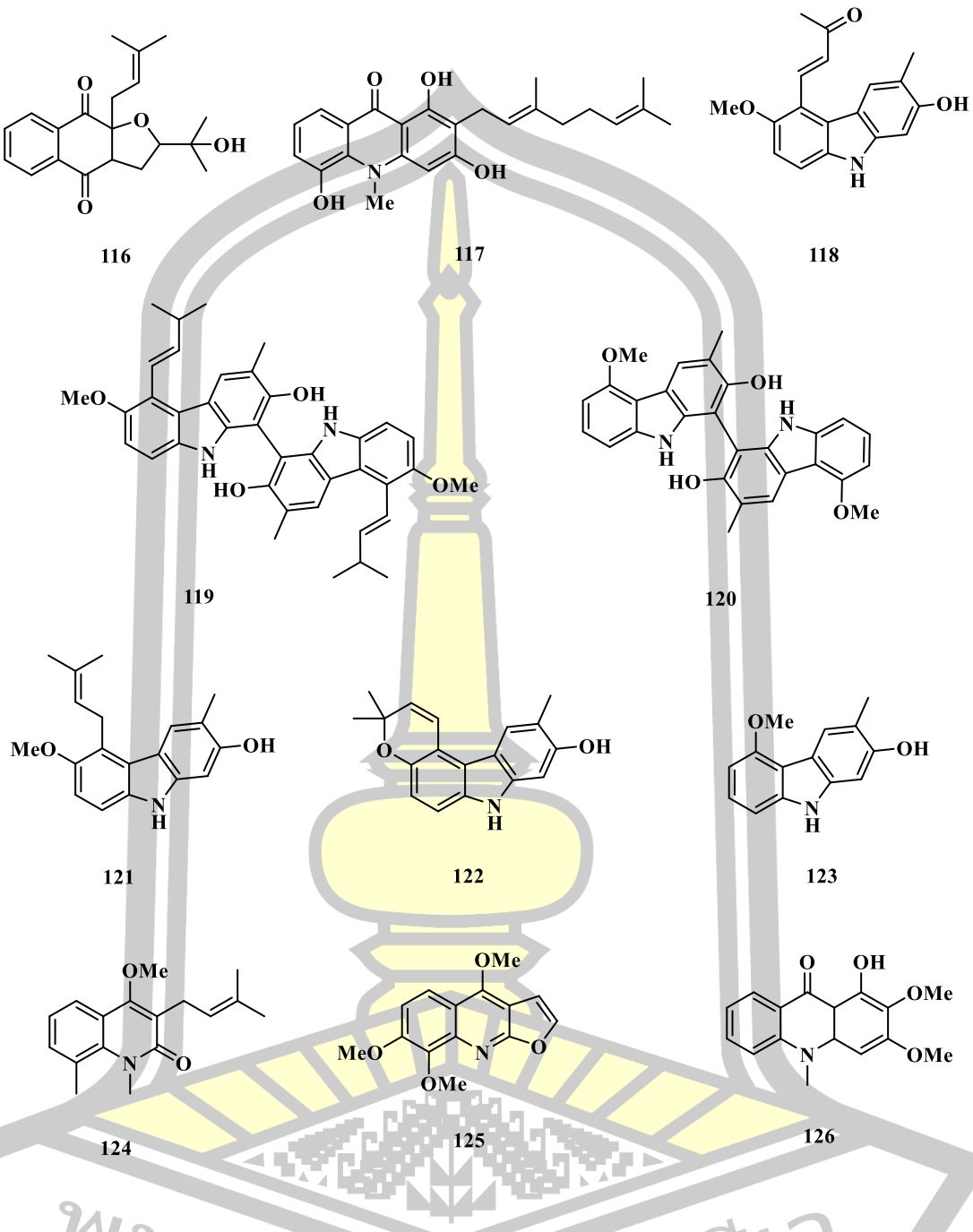



**Figure 12.** The structure of compounds **116-126** isolated from *G. pentaphylla*

### 2.1.13 *Micromelum minutum*

In 2000, the barks of *M. minutum* were collected from Nakhon Ratchasima province, Thailand, and studied by Ito et la., [29]. Purification of the acetone extract led to the isolation of coumarin A (**127**), coumarin B (**128**), coumarin C (**129**), coumarin F (**130**), coumarin G (**131**) and coumarin H (**132**).

In 2011, fruits of *M. minutum* were collected from Khon Khan province, Thailand, and investigated by Lekphrom et al., [30]. Fractionation of the ethyl acetate extract led to the isolation of 7-demethylmurralonginol isovalerate (**133**), murralonginol (**134**), murralonginol isovalerate (**135**), murralongin (**136**), micromelin (**137**), scopoletin (**138**), microminutin (**139**), murrangatin (**140**) and minumicrolin (**141**). Compound of **134** is a new natural product from *M. minutum*.

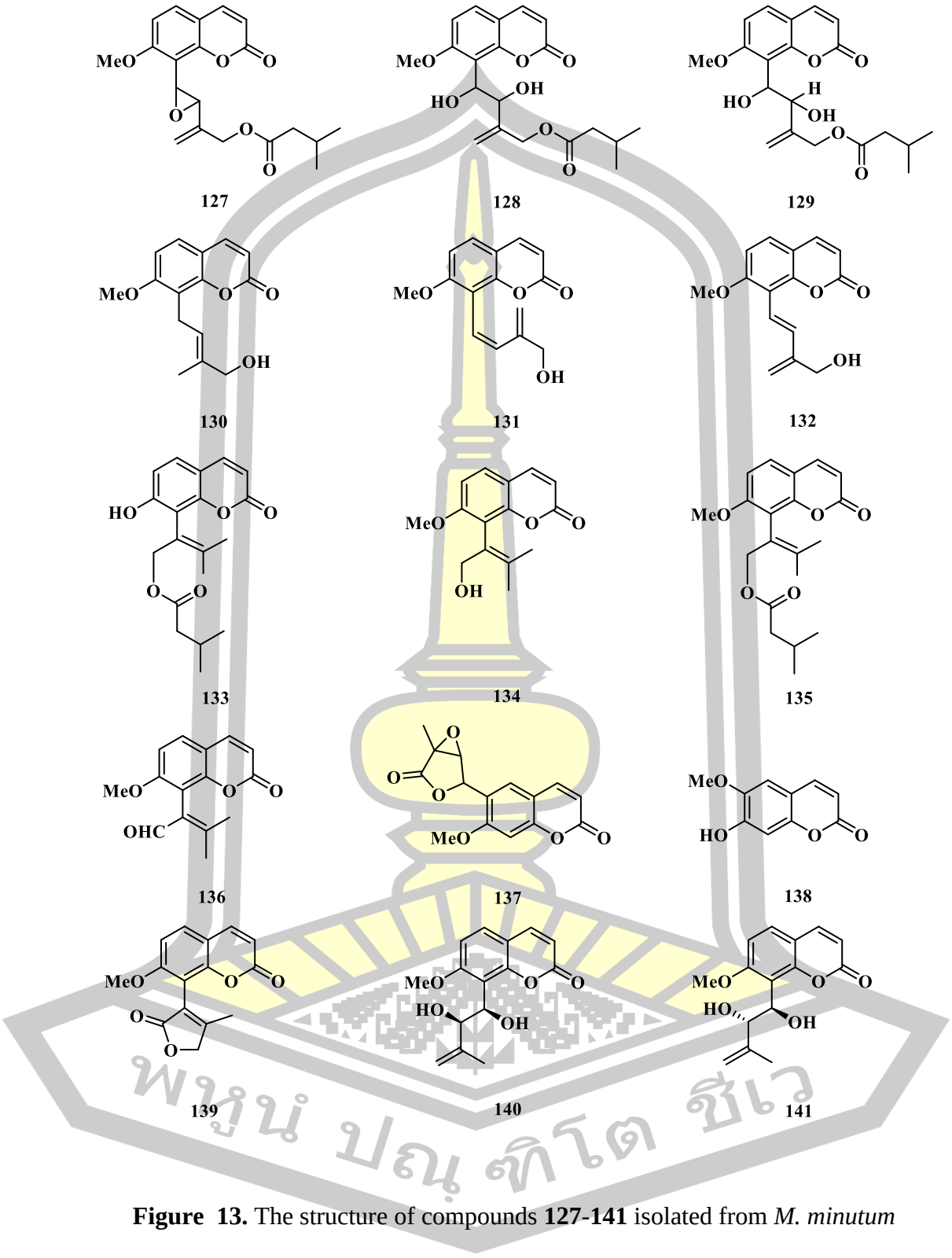
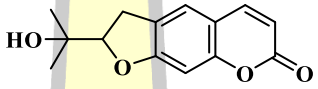



**Figure 13.** The structure of compounds **127-141** isolated from *M. minutum*

### 2.1.14 *Naringi crenulate*

In 2004, the bark of *N. Crenulata* was studied by Joo et al., [31]. Purification of the mixed methanol and chloroform extracts (ratio 1:1) led to the isolation of 2,3-dihydro-2 (1-hydroxy.1 -methylethyl)-furanocoumarin or marmesin (**142**). Compound **142** showed protection effect of UV rays.

In 2016, the fresh leaves of *N. Crenulata* were collected from Tamil Nadu, India, and investigated by Allayie et al., [32]. The aqueous, acetone and chloroform were used as extraction solvents of sample. The yield percentage (% yield) of the aqueous, acetone and chloroform extracts was 11.34%, 4.24% and 6.06%, respectively. Moreover, the three extracts had been confirmed of alkaloids, saponins, flavonoids and phenolic compounds.




**142**

**Figure 14.** The structure of compound **142** isolated from *N. Crenulata*

### 2.1.15 *Diospyros ehretioides*

In 2005, fruits of *D. ehretioides* were collected from Ubon Ratchathani Province, Thailand, and investigated by Prajoubklang et al., [33]. Fractionation of the dichloromethane extract led to the isolation of palmarumycins JC1 (**143**), palmarumycin JC2 (**144**), isodiospyrin (**145**) and isodiospyrol A (**146**). All isolated compounds was tested for biological activity. Compound **143** showed exhibition antimalarial activity at $IC_{50}$ 4.5 µg/mL, antifungal activity at $IC_{50}$ 12.5 µg/mL, antimicrobial activity at MIC 6.25 µg/mL and anti-cytotoxic activity at $IC_{50}$ 11.0 µg/mL. Compound **145** showed antimalarial activity at $IC_{50}$ 2.7 µg/mL, antimicrobial activity at MIC 50 µg/mL and antitoxic effect against BC cell line at $IC_{50}$ 12.3 µg/mL.

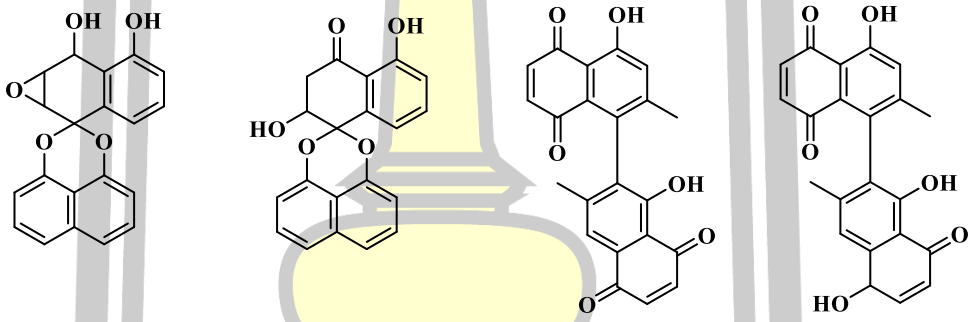



**Figure 15.** The structure of compounds **143-146** isolated from *D. ehretioides*

### 2.1.16 *Ochna integerrima*

In 2002, the stem barks of *O. integerrima* were collected from Nakhon Ratchasima province, Thailand, and studied by Kaewamatawong et al., [34]. Purification of the methanol extract led to the isolation of 6‴-hydroxylophirone B (**147**) and 6‴-hydroxyl ophirone B 4‴-*O*-β-glucoside (**148**) which is a new flavonoids.

In 2006, the barks of *O. integerrima* were collected from Ubon Ratchathani province, Thailand, and studied by Ichino et al., [35]. Fractionation of the ethyl acetate extract led to the isolation of biflavanone (**149**) and biflavanone (**150**). Compounds **148** and **149** were tested for antimalarial activity and showed inhibition antimalarial activity at $IC_{50}$ of 6.5 μg/mL, 80 ng/mL and 5.2 μg/mL, respectively.

In 2007, the leaves and limbs of *O. integerrima* were collected from Uthai Thani province, Thailand, and investigated by Ruetraku et al., [36]. Purification of the ethyl acetate extract led to the isolation of 6-*γ,γ*-dimethylallyldihydrokeampferol 7-*O*-*β*-D-glucoside **(151)**, 6-*γ,γ*-dimethyl allylkeampferol 7-*O*-*β*-D-glucoside **(152)** 6-*γ,γ*-dimethylallyl querectin 7-*O*-*β*-D-glucoside **(153)**, 6-(3-hydroxy-3-methylbutyl) taxifolin 7-*O*-*β*-D-glucoside **(154)** and 6-(3-hydroxy-3-methylbutyl) quercetin 7-*O*-*β*-D-glucoside **(155)**. Compounds **151**, **152**, **153** and **154** were tested for cytotoxic activity and showed cytotoxicity at $EC_{50}$ of 29.1, 14.0, 102.4 and 45.8 μg/mL, respectively.

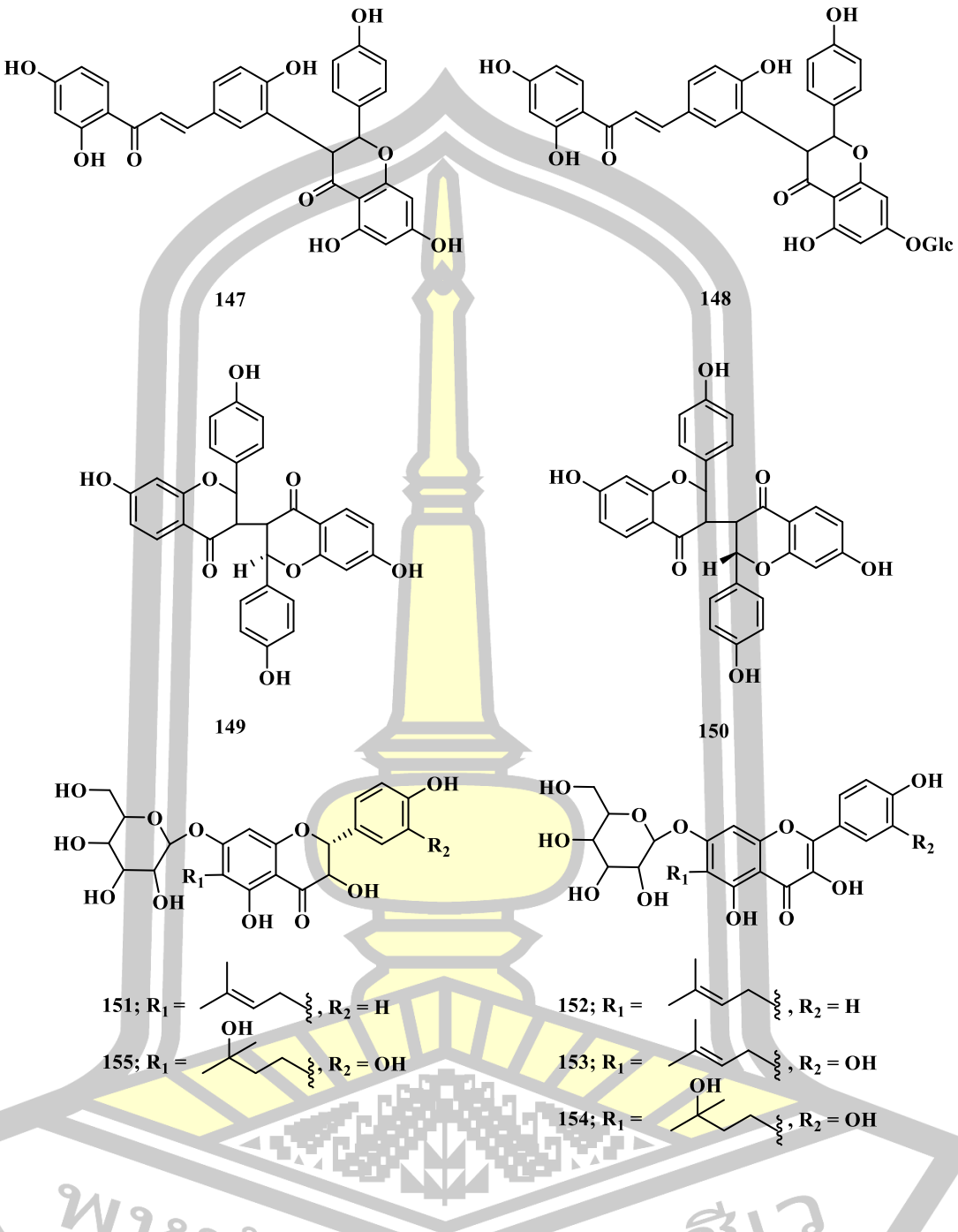



**Figure 16.** The structure of compounds **147-154** isolated from *O. integerrima*

# CHAPTER 3

# MATERIAL AND METHOD

## 3.1 Abbreviation

List of abbreviations used in this research is shown in **Table 1**

**Table 1.** List of abbreviations

| Abbreviation | Full word | Abbreviation | Full word |
|---|---|---|---|
| Abc | Absorbance of control | MW | Molecular weight |
| Abs | Absorbance of sample | NC | *Naringi crenulata* |
| $ABTS^{\bullet+}$ | 2,2´-Azino-bis(3-ethyl benzothiazoline-6-sulphonic acid radical cation | OI | *Ochna integerrima* |
| ACTs | Artemisinin-based combination therapies | PD | *Polyalthia debilis* |
| CH | *Clausena harmandiana* | PE | *Polyalthia evecta* |
| CP | *Celastrus paniculatus* | PS | *Polyalthia suberosa* |
| CS | *Caesalpinia sappan* | QE | Quercetin equivalent |
| DE | *Diospyros ehretioides* | %RSA | Percentage of radical scavenging activity |
| DMSO | Dimethyl sulfoxide | SCe | *Siphonodon celastrineus* |
| $DPPH^{\bullet}$ | 2,2-Diphenyl-1-picrylhydrazyl radical | SCh | *Salacia chinensis* |
| FC | Folin-Ciocalteu | SD | Standard deviation |
| FRAP | Ferric reducing activity power | SV | *Salacia verrucosa* |
| GAE | Gallic acid equivalent | TFC | Total flavonoid content |
| GP | *Glycosmis pentaphylla* | TLC | Thin layer chromatography |
| HC | *Hubera cerasoides* | TPC | Total phenolic content |
| $IC_{50}$ | 50% Inhibitory concentration | TPTZ | 2,4,6-Tripyridyl-*S*-triazine |
| IPNI | International plant names index | UR | *Uvaria rufa* |
| MM | *Micromelum minutum* | WHO | World health organization |

### 3.2 Chemical

List of chemicals in this research is shown in **Table 2**

**Table 2.** List of chemicals

| Chemical | Company | Country |
|---|---|---|
| Aluminium chloride | Global Chemie | India |
| Ascorbic acid | UNILAB | Australia |
| 2,2´-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid (ABTS) | Sigma-Aldrich | China |
| Chloroform (chloroform-d; $CDCl_3$) | Cambridge Isotope Laboratories | USA |
| 2,2-Diphenyl-1-picrydydrazyl radical (DPPH•) | Sigma-Aldrich | Germany |
| Iron (II) sulfate | Ajax FineChem Laboratory Chemicals | New Zealand |
| Iron (III) chloride hexahydrate solution | Global Chemie | India |
| Folin-Ciocalteu | Carlo Erba Reagents | France |
| Gallic acid | Fluka | Spain |
| Methanol (methanol-$d_4$; $CD_3OD$) | Cambridge Isotope Laboratories | USA |
| Potassium acetate | KemAus | Australia |
| Potassium persulfate | KemAus | Australia |
| Quercetin | Sigma-Aldrich | China |
| Sodium carbonate | KemAus | Australia |
| 2,4,6-Tripyridyl-*S*-triazine (TPTZ) | Sisco Research Laboratories | India |

### 3.3 Instrument

List of instruments used in this research is shown in **Table 3**

**Table 3.** List of instruments

| Instrument | Model |
|---|---|
| High performance liquid chromatography (HPLC) photodiode array detector | Shimadzu |
| Nuclear magnetic resonance spectroscopy ($^1$H NMR) | Varian and Bruker (400 MHz) |
| Rotary evaporator | Buchi R120 |
| Ultraviolet-visible spectrophotometer (UV-Vis) | Thermo Scientific GENESYS 20 |

### 3.4 Material

Sixteen plant species with assigned codes from six plant families (**Table 4** and **Figure 18**) were selected according to their traditional use as galactogogue plants in northeastern Thailand [37]. The plant samples with different used part were collected with permission in 2018 from arboretum and affiliate garden, Walai Rukhavej Botanical Research Institute (WRBRI), Mahasarakham University (MSU), Maha Sarakham province, except for *Salacia verrucosa* (SV) which was collected from Amnat Charoen province, by K. Wongpakam and identified by S. Sedlak, WRBRI, MSU, where the voucher specimens (Wongpakam 19-XX) are deposited. Fresh samples were washed, cut into small pieces and air dried. All dried samples were kept in zipped plastic bag and stored at room temperature until use. It should be noted that, the *Caesalpinia sappan* L. species (CS) (**Table 4**) was recently renamed to *Biancaea sappan* (L.) Tod. based on taxonomic revision and International Plant Names Index (IPNI) produced by the Royal Botanic Gardens, Kew.

**Table 4.** Information of sixteen Thai galactogogue medicinal plant materials.

| Plant species (Code) | Local Thai name (in English) | Family | Used part | Voucher no. |
|---|---|---|---|---|
| *Hubera cerasoides* (Roxb.) Chaowasku (HC) | กะเจียน (Ka chian) | Annonaceae | Stem bark | Wongpakam 19-08 |
| *Polyalthia debilis* (Pierre) Finet & Gagnep. (PD) | กล้วยเต่า (Kluai Tao) | Annonaceae | Stem bark | Wongpakam 19-09 |
| *Polyalthia evecta* (Pierre) Finet & Gagnep. var. *evecta* (PE) | นมน้อย (Nom Noi) | Annonaceae | Stem bark and root | Wongpakam 19-11 |
| *Polyalthia suberosa** (Roxb.) Thwaites (PS) | กลึงกล่อม (Klueng Klom) | Annonaceae | Stem bark | Wongpakam 19-14 |
| *Uvaria rufa* Blume (UR) | นมควาย (Nom Khwai) | Annonaceae | Stem bark | Wongpakam 19-07 |
| *Caesalpinia sappan*** L. (CS) | ฝาง (Fang) | Fabaceae | Stem bark | Wongpakam 19-01 |
| *Celastrus paniculatus* Willd. (CP) | กระทงลาย (Kra Thong Lai) | Celastraceae | Stem bark | Wongpakam 19-10 |
| *Salacia chinensis* L. (SCh) | กำแพงเจ็ดชั้น (Kam phaeng Chet Chan) | Celastraceae | Stem bark | Wongpakam 19-04 |
| *Salacia verrucosa* Wight (SV) | กำแพงเก้าชั้น (Kam phaeng Kao Chan) | Celastraceae | Stem bark | Wongpakam 19-03 |
| *Siphonodon celastrineus* Griff. (SCe) | มะดูก (Ma Duk) | Celastraceae | Stem bark | Wongpakam 19-13 |
| *Clausena harmandiana** (Pierre) Pierre ex Guillaumin (CH) | ส่องฟ้าคง (Song Fa Dong) | Rutaceae | Root | Wongpakam 19-15 |
| *Glycosmis pentaphylla** (Retz.) DC. (GP) | เขยตาย (Khoei Tai) | Rutaceae | Stem bark | Wongpakam 19-05 |
| *Micromelum minutum* (Forst.f.) Wight & Arn. (MM) | หัสคุณ (Hatsa Khun) | Rutaceae | Stem bark | Wongpakam 19-16 |
| *Naringi crenulata* (Roxb.) Nicolson (NC) | ตุมตัง (Tum Tang) | Rutaceae | Stem bark | Wongpakam 19-12 |
| *Diospyros ehretioides* Wall. ex G. Don (DE) | ลิ้นกวาง (Lin Kwang) | Ebenaceae | Stem bark | Wongpakam 19-06 |
| *Ochna integerrima* (Lour.) Merr. (OI) | ช้างน้าว (Chang Nao) | Ochnaceae | Stem bark | Wongpakam 19-02 |

*Samples were received as dried plant materials from Thai folk healers in Maha Sarakham province. ***Caesalpinia sappan* L. was recently renamed to *Biancaea sappn* (L.) Tod.


**Figure 17.** Sixteen Thai medicinal plant-derived galactogogue

### 3.5 Extraction

Each plant sample (50 g) was extracted by refluxing in distilled methanol (500 mL, 24 h). After filtration, the collected methanol layer was evaporated under reduced pressure using rotary evaporator at 40 ˚C. A small portion of the methanol extract was taken for experiments when the rest was suspended in deionized water (100 mL) and further partitioned with distilled hexane and ethyl acetate (150 mL), respectively. The hexane and methanol layers were evaporated to obatain hexane and ethyl acetate extracts.

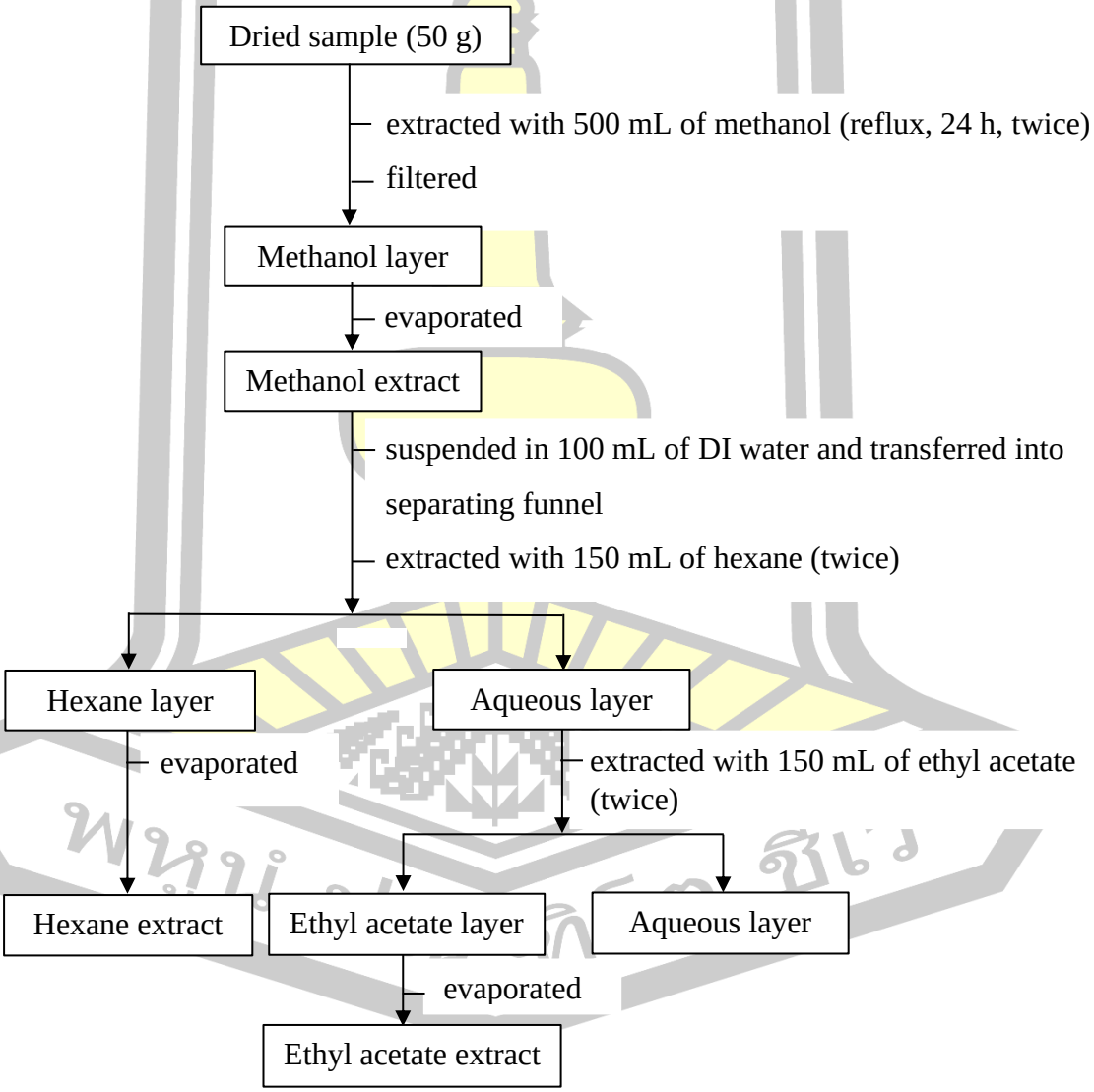



**Figure 18.** Extraction process

### 3.6 Chemical composition profile

Chemical composition profile of the extracts was analysed by TLC, NMR and HPLC.

#### 3.6.1 TLC

TLC is a technique used to isolate non-volatile mixtures. The experiment is conducted on a sheet of aluminium plastic or glass which is coated with a thin layer of adsorbent material. The material usually used is aluminium oxide, cellulose or silica gel. On completion of the separation, each component appears as spots separated vertically. Each spot has a retention factor ($R_f$) expressed as:

$R_f$ = Distance travelled by sample / Distance travelled by solvent

The factors affecting retardation factor are the solvent system, amount of material spotted, absorbent and temperature. The TLC is one of the fastest, least expensive, simplest and easiest chromatography technique.

The principle of TLC is the same as other chromatography techniques which is on the separation principle. The separation relies on the relative affinity of compounds towards two phases, stationary phase and mobile phase. The compounds in the mobile phase move over the surface of the stationary phase. The movement occurs in such a way that the compounds which have a higher affinity to the stationary phase move slowly while the other compounds travel fast. Therefore, the separation of the mixture is attained. On completion of the separation process, the individual components from the mixture appear as spots at respective levels on the plates. Their character and nature are identified by suitable detection techniques [38].

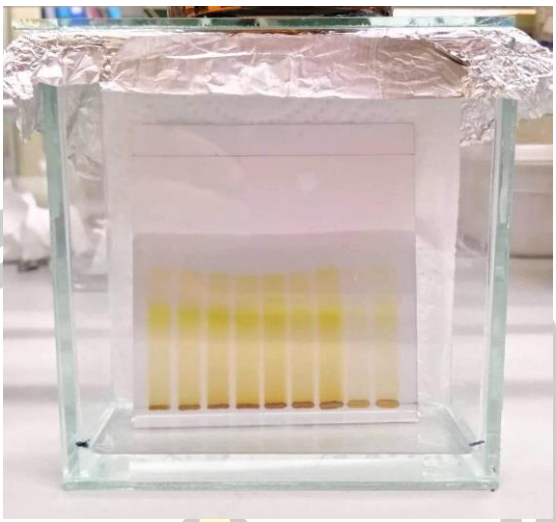


**Figure 19.** Development of TLC

In this study, the sample solution (1 mg/ 200 μL) was prepared in methanol and loaded (~20 μL) into TLC plate (9 x 20 cm; 7 cm of migration distance) with microcapillary tube followed by development with toluene:ethyl acetate:acetic acid (2:8:1, v/v/v) mobile phase system for the methanol extracts and toluene:ethyl acetate (3:7, v/v) for the ethyl acetate extracts. Visualization of the phytochemical bands on developed TLC chromatogram was performed under visible UV light 245 nm and with spray reagent indication; *p*-anisaldehyde-sufuric acid to detect (poly)phenols, 0.4 mM DPPH$^{\bullet}$ to determine antioxidant compounds and 2% (w/v) aluminium chloride to confirm flavonoids. The spray reagents were prepared as described in literatures with modifications in the used concentrations [39], [40], [41].

**3.6.1.1 Preparation of spray reagent**

Four of the sprayed reagent; *p*-anisaldehyde sulfuric acid, 0.4 mM 2,2-diphenyl-1-picrydydrazyl radical (DPPH$^{\bullet}$), 2% aluminium chloride and dragendorff reagents were used to detect chemical compositions of the extracts.

**A. *p*-Anisaldehyde sulfuric acid reagent**

*p*-Anisaldehyde sulfuric acid reagent was freshly prepared followed by reference method TLC visualization reagents [42]. The 0.5 mL of *p*-anisaldehyde was mixed with 50 mL of glacial acetic acid and 1 mL of sulfuric acid, respectively (**Figure 20A**).

The phenolic, sugar, steroid and terpene compounds were detected using by *p*-anisaldehyde sulfuric acid reagent. After spray *p*-anisaldehyde sulfuric acid reagent and heating TLC plate at temperature 110 ± 5 ºC in the oven, the color of spots phenolic, sugar, steroid and terpene compounds appeared as violet, blue, red, grey or green.

**B. 0.4 mM DPPH• reagent**

The DPPH• reagent was prepared followed by reference method by TLC visualization reagents [42]. The 0.4 mM of DPPH• solution was prepared by 15.77 mg of DPPH• dissolved in 100 mL of methanol (**Figure 20B**).

Antioxidant compounds in the extracts were detected using DPPH• reagent. After spray DPPH• reagent on TLC plate, purple color of DPPH• reagent will change to colorless once reacted with antioxidant compound and it can be seen with the naked eye.

**C. 2% Aluminium chloride reagent**

The Aluminium chloride reagent was prepared followed by reference method TLC visualization reagents [42]. The 2% aluminium chloride reagent was prepared by 1 g of aluminium chloride dissolved in 100 mL of 95% ethanol (**Figure 20C**).

Flavonoid compounds in the extracts were detected using by aluminium chloride reagent. The sample extracts on TLC plate was sprayed with 2% aluminium chloride an orange-yellow spot of flavonoids will be detected under UV-365 nm.

**D. Dragendorff reagent**

The dragendorff reagent was prepared by reference method TLC visualization reagents [42]. The stock solution of dregendorff reagent was prepared by 1.7 g of basic bismuth nitrate and 20 g of tartaric acid dissolved in 80 ml of DI water, this is solution 1. The solution 2 was prepared by 16 g of potassium iodide dissolved in 40 ml water. The stock solution was mixed equal volumes of solution 1 and 2. The dragendorff reagent was prepared by 10 g of tartaric acid dissolved in 50 ml of water, pipetted 5 mL of tartaric acid solution and mixed 5 mL of stock solution (**Figure 20D**).

Alkaloid compounds in the extracts were detected using dragendorff reagent. The orange spot of alkaloids in the extracts on the TLC plate after spraying with dragendorff reagent will be detected and can be seen with naked eyes.

**(A)** **(B)** **(C)** **(D)**

**Figure 20.** Prepared spray reagents of *p*-anisaldehyde sulfuric acid (**A**), 0.4 mM DPPH (**B**) and 2% aluminium chloride (**C**) and dragendorff reagents (**D**).

**3.6.2 NMR**

$^{1}$H NMR spectroscopy is an analytical chemistry technique used in quality control and research for determining the content and purity of a sample as well as its molecular structure. For example, NMR can quantitatively analyze mixtures containing known compounds. For unknown compounds, NMR can either be used to

match against spectral libraries or to infer the basic structure directly. Once the basic structure is known, NMR can be used to determine molecular conformation in solution as well as studying physical properties at the molecular level such as conformational exchange, phase changes, solubility, and diffusion. In order to achieve the desired results, a variety of NMR techniques are available. The basics of NMR are described here.

The principle behind NMR is that many nuclei have spin and all nuclei are electrically charged. If an external magnetic field is applied, an energy transfer is possible between the base energy to a higher energy level (generally a single energy gap). The energy transfer takes place at a wavelength that corresponds to radio frequencies and when the spin returns to its base level, energy is emitted at the same frequency. The signal that matches this transfer is measured in many ways and processed in order to yield an NMR spectrum for the nucleus concerned [43].

In this research, the methanol and ethyl acetate extracts were measured by $^{1}H$ NMR spectroscopy to analyze proton signals of their chemical compositions. The $^{1}H$ NMR spectrum of each extract was compared among sample families. The methanol extract is dissolved with methanol-$d_4$ and ethyl acetate extract was dissolved with chloroform-*d*.

### 3.6.3 HPLC

Liquid chromatography is a well-established technique for the separation of substances. The separation principle of HPLC is based on the distribution of the analyte (sample) between a mobile phase (eluent) and a stationary phase (packing material of the column). Depending on the chemical structure of the analyte, the molecules are retarded while passing the stationary phase. The specific intermolecular interactions between the molecules of a sample and the packing material define their time “on-column”. Hence, different constituents of a sample are eluted at different times. Thereby, the separation of the sample ingredients is achieved.

A detection unit (e.g. UV detector) recognizes the analytes after leaving the column. The signals are converted and recorded by a data management system

(computer software) and then shown in a chromatogram. After passing the detector unit, the mobile phase can be subjected to additional detector units, a fraction collection unit or to the waste. In general, a HPLC system contains the following modules: a solvent reservoir, a pump, an injection valve, a column, a detector unit and a data processing unit. The solvent (eluent) is delivered by the pump at high pressure and constant speed through the system. To keep the drift and noise of the detector signal as low as possible, a constant and pulseless flow from the pump is crucial. The analyte (sample) is provided to the eluent by the injection valve [44].

In this research, the methanol and ethyl acetate extracts were analysed by HPLC with photodiode array detector. The extract solution was filtered through a 0.45 µm nylon membrane, injected and 20 µL of sample to C18 column (250 mm x 4.6 mm, 5 µm, 40 ˚C), with a flow rate of 0.8 mL/min. Compounds were evaluated with 0.3% acetic acid solution (phase A) and acetonitrile (phase B) as a mobile phase. The gradient in ratio (v/v) of solvent A and B was as follow; 1-10 min, 10-20% solvent B; 10-60 min, 20-28% solvent B. The UV absorbance was set at 254 nm.

### 3.7 Antioxidant activity

#### 3.7.1 DPPH assay

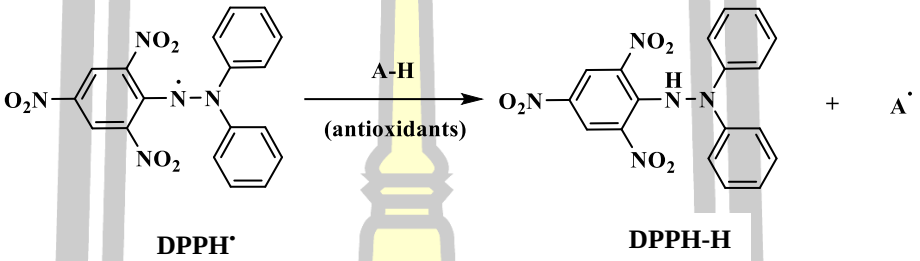
DPPH$^{\bullet}$ is characterized as a stable free radical by virtue of the delocalisation of the spare electron over the molecule as a whole equation 1, so that the molecules do not dimerise, like most other free radicals. The delocalisation also gives rise to the deep violet color, with an absorption in ethanol solution at around 517 nm. On mixing DPPH$^{\bullet}$ solution with a substance that can donate a hydrogen atom, it gives rise to the reduced form with the loss of violet color (**Figure 22**). Representing the DPPH$^{\bullet}$ and the donor molecule by AH, the primary reaction is **Figure 21.** Where DPPH-H is the reduced form and A$^{\bullet}$ is free radical produced in the first step. The latter radical will then undergo further reactions which control the overall stoichiometry. The reaction (1) is therefore intended to provide the link with the reactions taking place in an oxidising system, such as the autoxidation of a lipid or other unsaturated substance;

the DPPH• is thus intended to represent the free radicals formed in the system whose activity is to be suppressed by the substance AH [45].

Equation 1

DPPH• + AH----------DPPH-H + A•

DPPH• + R•---------- DPPH-R




DPPH• DPPH-H

**Figure 21.** The reaction of DPPH• with antioxidant compounds

The DPPH• scavenging activity was determined by modify method described by Seephonkai [46]. Briefly, a 2 mL of 0.2 mM methanolic DPPH• solution was added to 1 mL of the sample methanolic extract solution at different concentrations (5 – 300 µg/mL). The absorbance of the mixture (Abs of sample) was recorded at 517 nm by UV-Vis Spectrophotometer after incubation for 30 min in the dark at room temperature. The absorbance of control (Abs of control) was measured in the same manner of preparation using methanol without sample. Percentage of radical scavenging activity (%RSA) of each concentration was calculated based on the absorbance of sample and control solutions. Graph plotted between the %RSA and used concentrations led to the calculation of inhibitory concentration at 50% ($IC_{50}$ value; µg/mL) value of each extract.

$$\% \text{ RSA } = \frac{\text{Abs of control} - \text{Abs of sample}}{\text{Abs of control}} * 100$$

(Blank = 1 mL of methanol mixed with 20 mL of DPPH solution)

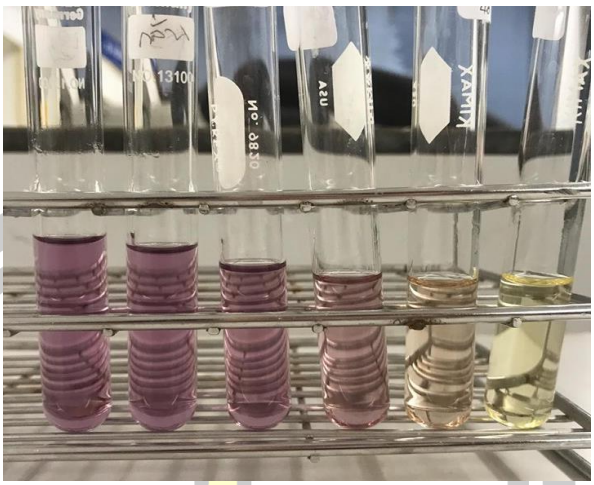


**Figure 22.** Decoloration of DPPH$^{\bullet}$ solution with antioxidant compounds.

### 3.7.2 ABTS assay

The ABTS assay measures the relative ability of antioxidants to scavenge the ABTS$^{\bullet+}$ generated in aqueous phase, as compared with a Trolox (water soluble vitamin E analogue) standard. The ABTS$^{\bullet+}$ is generated by reacting with a strong oxidizing agent (eg, potassium permanganate or potassium persulfate) with the ABTS salt. The reduction of the blue-green ABTS$^{\bullet+}$ radical by hydrogen-donating antioxidants is measured by the suppression of its characteristic long wave absorption spectrum [47].

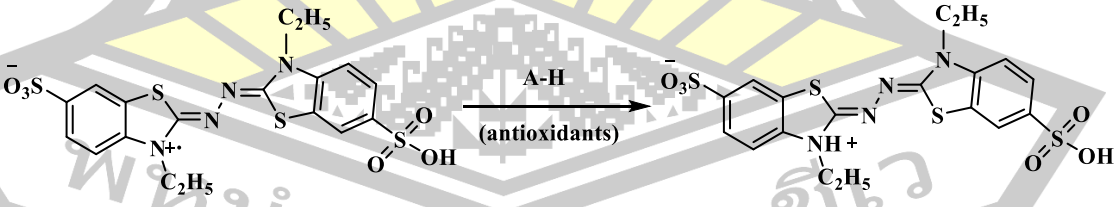



**Figure 23.** Reaction of ABTS$^{\bullet+}$ with antioxidant compounds

The $ABTS^{\bullet+}$ scavenging activity was tested according to the procedure reported by Re [48] The $ABTS^{\bullet+}$ was produced by reacting a 7 mM ABTS (Sigma-Aldrich, China) with 2.45 mM potassium persulfate (KemAus, Australia). The resulting mixture was stand in the dark at room temperature for 16 h before use. A working $ABTS^{\bullet+}$ solution was diluted with ethanol to an absorbance of 0.70 ± 0.020 nm at 734 nm. For the analysis of the antioxidant activity, 900 µL of the working $ABTS^{\bullet+}$ solution was mixed with 100 µL of the sample (5 – 300 µg/mL) dilution to 1:100. After 6 min, the absorbance at 734 nm was measured next to a blank with methanol. %RSA at 734 nm absorption was calculated and then converted to $IC_{50}$ value as the same manner described for the DPPH method above.

$$\% \text{ RSA } = \frac{\text{Abs of control} - \text{ Abs of sample}}{\text{Abs of control}} * 100$$

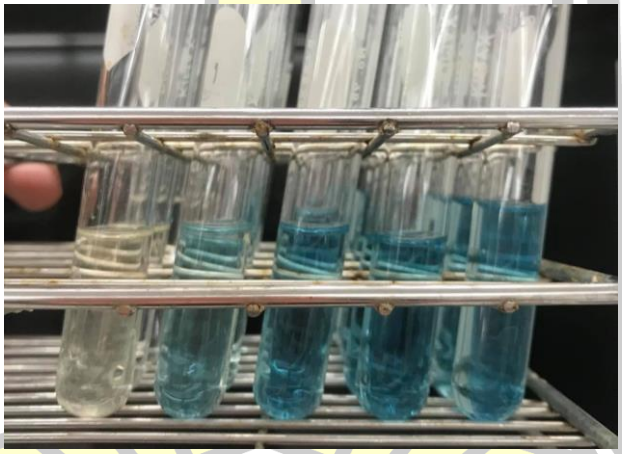


**Figure 24.** Decoloration of $ABTS^{\bullet+}$ solution with antioxidant compounds

### 3.7.3 FRAP assay

The total antioxidant activity can be measured by the FRAP assay. The flavonoids and phenolic acids are present in the medicinal plant exhibit strong antioxidant activity which is depending on their potential to form the complex with metal atoms, particularly iron and copper. This method is based on the principle of

increase in the absorbance of the reaction mixtures, the absorbance increases the antioxidant activity increases. The antioxidant compound present in the samples forms a colored complex with potassium ferricyanide, trichloroacetic acid and ferric chloride, which is measured at 700 nm by UV-Vis Spectrophotometer.

The ferric iron ($Fe^{3+}$) is initially reduced by electron-donating antioxidants present within the sample to its ferrous form ($Fe^{2+}$). The iron colorimetric probe complex develops a dark blue color product upon reduction which can be measured at 540-600 nm (**Figure 25**) [49].

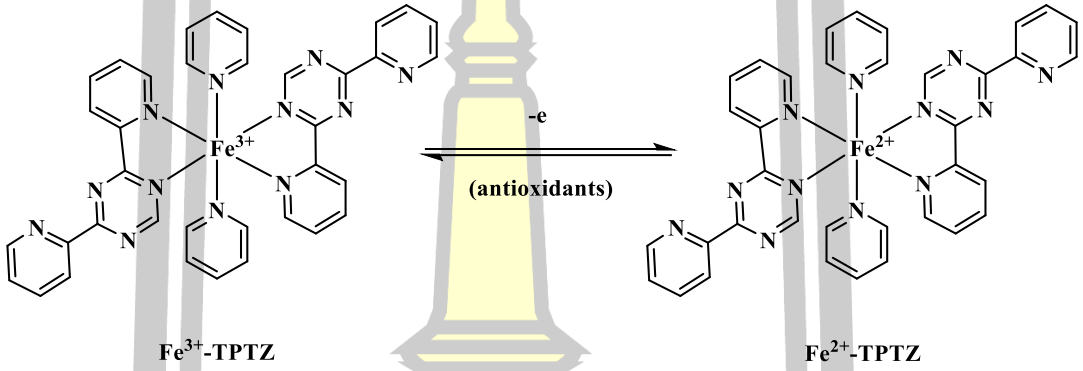



**Figure 25.** Reduction reaction of Fe (III)-TPTZ complex

FRAP assay was performed according to the method described by Benzie and Strain [50] with slight modification. The FRAP reagent was freshly prepared from 300 mM acetate buffer, pH 3.6, 10 mM 2,4,6-tripyridyl-*S*-triazine (TPTZ) made up in 40 mM HCl and 20 mM Fe (III) chloride hexahydrate solution. The mixture was made in the ratio of 10:1:1(v/v/v). Methanolic sample solution (50 µg/mL) of 1.2 mL was added to 3.6 mL of FRAP reagent, freshly prepared. The absorbance was measured at 593 nm after 2 min of incubation at 37 °C. Blank was done in the same ratio using methanol without samples. Fresh working solutions of reference Fe (II) sulfate (concentrations (0.360 – 7.194 µM) were used for calibration curve in our study. FRAP was reported as µM Fe (II)/g extract.

$$\text{Fe (II) content} = \left[\frac{X\ (\text{mg Fe (II)/L}) * \text{ Dilution factor} * V}{\text{Sample weight (mg)}}\right] * \text{MW of Fe (II) sulfate} * 1{,}000$$

X was calculated from linear equation of Fe (II) sulfate.

V was a total volume of the extract.

MW stands for molecular weight.

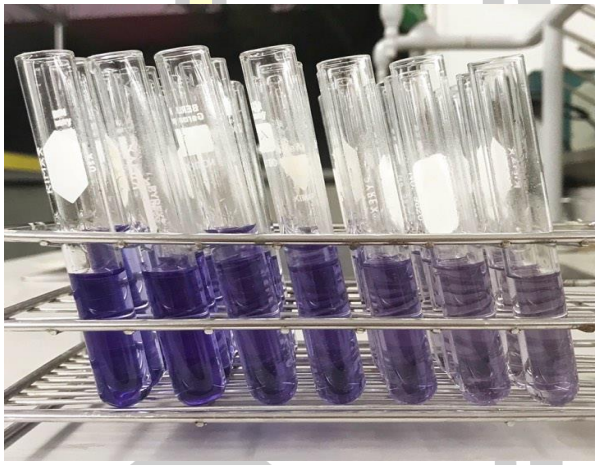


**Figure 26.** Decoloration reaction of Fe (III)-TPTZ complex solution with antioxidant compounds

### 3.8 TPC

TPC activity is the process to determine the amount of phenolic content in the samples. Phenolic compounds that contained in the plants have redox properties, and the properties allow them acting as antioxidants [51]. Reagent used in the determination of TPC is Folin-ciocalteu.

Folin-ciocalteu's phenol reagent is most commonly used in the Lowry method for determining protein concentration. It has also been used for the quantification of total phenolics [52]. In testing for TPC, the reagent is initially yellow-colored but turns blue when it is reduced. The blue color is then measured with a spectrophotometer (500-750 nm). Under alkaline conditions, phenolic compounds reduce the Folin reagent to form the blue color.

TPC was determine using Folin-Ciocalteu modify method reported by Seephonkai et al. [46]. Briefly, a 1 mL of methanolic sample solution (50 µg/mL) was mixed with 3 mL of 7.5% sodium carbonate solution and 2 mL of 10% Folin-Ciocalteu reagent. The mixture was vigorously mixed on a vortex and the absorbance was recorded at 756 nm after keeping in dark of 30 min at room temperature. Blank was done in the same ratio using methanol without samples. Results of the TPC were expressed as milligram of gallic acid equivalent per gram of dry extract (mg GAE/ g extract).

$$\text{TPC} = \frac{X\ (\text{mg GAE/L}) * \text{ Dilution factor} * V}{\text{Sample weight (mg)}}$$

*X* was calculated from linear equation of standard gallic acid.

*V* was a total volume of the extract.

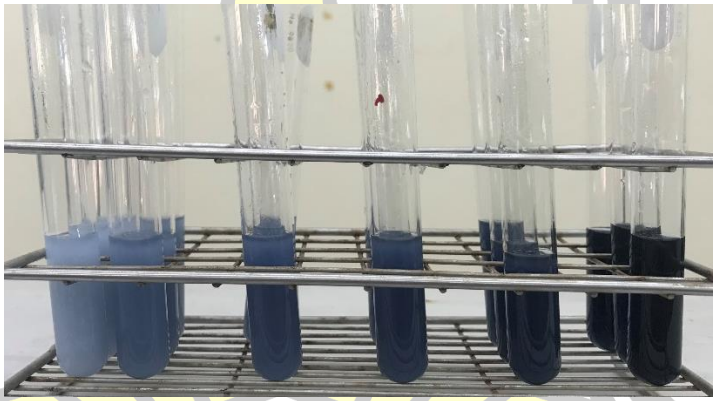


**Figure 27.** Decoloretion of phenolic compounds tested by Folin-Ciocalteu method

### 3.9 TFC

The principle involved in aluminium chloride ($AlCl_3$) colorimetric method is that $AlCl_3$ forms acid stable complexes with the C-4 keto groups and either the C-3 or C-5 hydroxyl group of flavones and flavonols. In addition it also forms acid labile complexes with the orthodihydroxyl groups in the A- or B-ring of flavonoids (**Figure 28**).

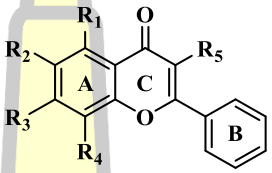



**Figure 28.** Basic structure of flavonoids

Studies have reported quercetin to be suitable reference for determination of total flavonoid content in plant sample extract. Therefore, quercetin solutions of various concentrations were used to make the standard calibration curve [53].

TFC was measured following the procedure described by Chang et al. [54] with slight modification. Briefly, a 1 mL of methanolic extracts (50 µg/mL) was mixed with 1.5 mL of methanol, 2.8 mL of distilled water, 1 mL of 1 M potassium acetate solution, and 0.1 mL of 10% aluminium chloride solution. The absorbance was measured at 415 nm after 30 min of reaction. Blank was done in the same ratio using methanol without samples. Results of the TFC were expressed as milligram of quercetin equivalent per gram of dry extract (mg QE/ g extract).

$$\text{TFC} = \frac{X\ (\text{mg QE/L}) * \text{Dilution factor} * V}{\text{Sample weight (mg)}}$$

*X* was calculated from linear equation of standard quercetin.

*V* was a total volume of the extract.

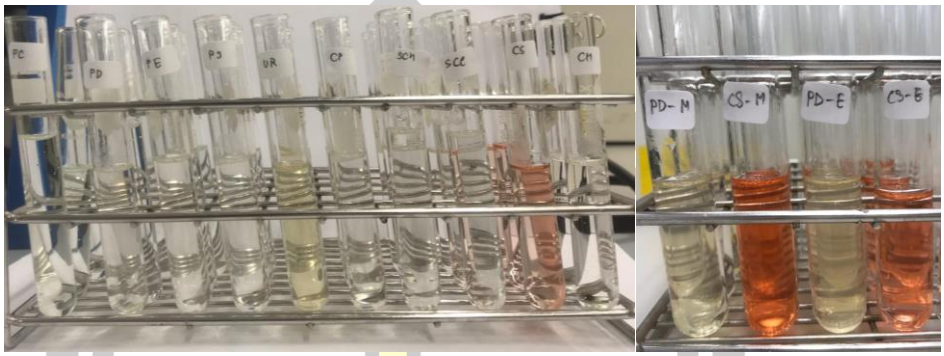



**Figure 29.** Decoloretion of flavonoid compounds tested by aluminiam chloride colorimetric method

### 3.10 Statistical analysis

The DPPH$^{\bullet}$ and ABTS$^{\bullet+}$ scavenging activities, FRAP, TPC and TFC assays were carried out in triplicate. The results were reported as mean ± standard deviation (SD). Data was analyzed for statistical significant ($p < 0.05$) by ANOVA post-hoc test (IBM SPSS statistics software, version 22, USA). The *p* values less than 0.05 were considered statistically significant.

# CHAPTER 4

# RESULT AND DISCUSSION

## 4.1 Results

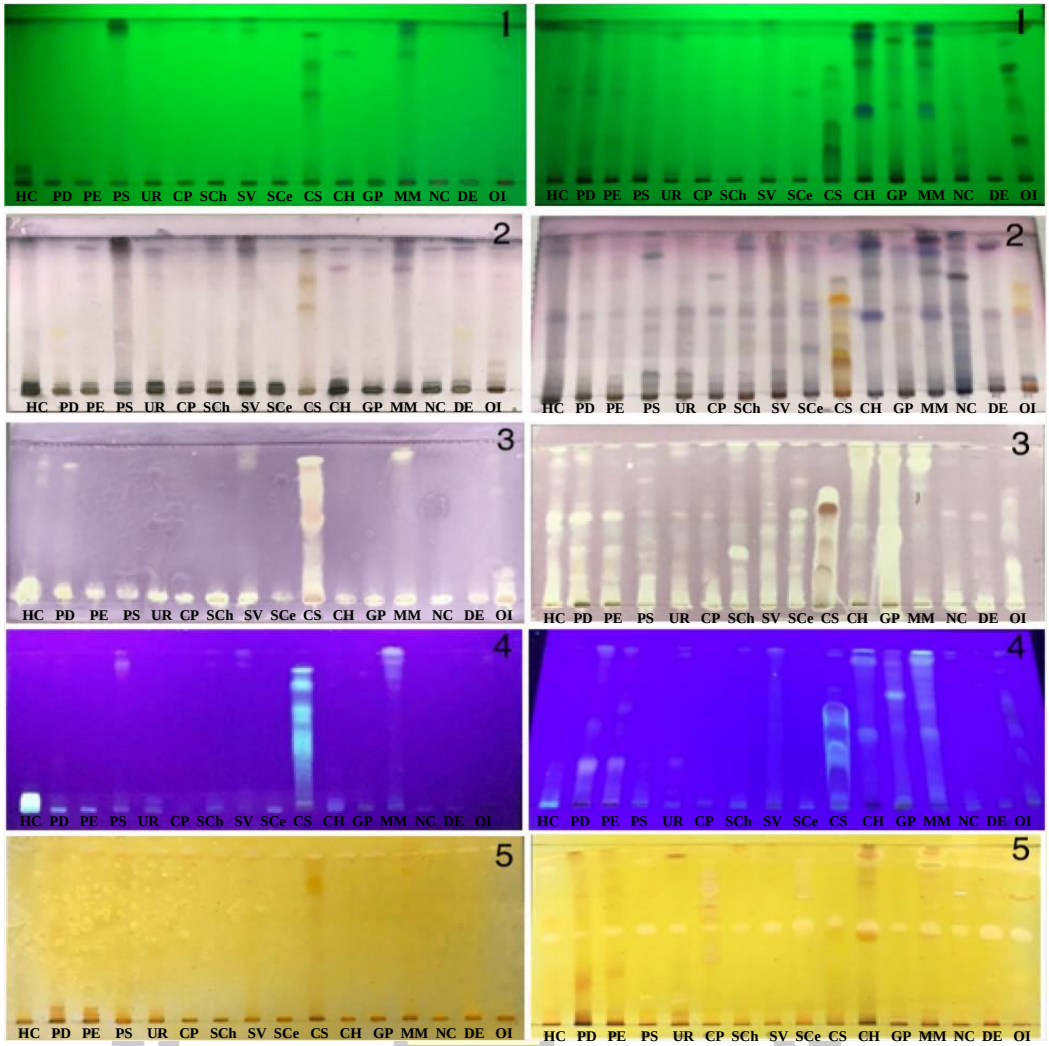
### 4.1.1 Chemical composition profile

(Poly)phenol, antioxidant and flavonoid compounds in the extracts were phytochemical screened by thin layer chromatography (TLC) with spray reagents; *p*-anisaldehyde-sulfuric acid, 0.4 mM DPPH$^{\bullet}$ and 2% aluminium chloride. Protons signals of phytochemical secondary metabolites in the extracts were measured by $^{1}$H NMR (nuclear magnetic resonance) spectroscopy technique.

#### 4.1.1.1 TLC chromatogram

Bands belong to phytochemical secondary metabolites of the extracts were detected on the TLC chromatograms under the visible UV 254 nm with the dark bands represented aromatic compounds (**Figure 30A1** and **B1**). Different types of natural product compounds in the extracts on the chromatograms were visualized after derivatization with *p*-anisaldehyde-sulfuric acid. Purple bands indicated (poly)phenols (and/or phytosterols and triterpenes) type of compounds [55] which appeared in most of the extracts (**Figure 30A2** and **B2**). All of the extracts revealed the antioxidant compounds by decolourizing of purple DPPH$^{\bullet}$ reagent to colorless reduced form (**Figure 30A3** and **B3**). Bands of the flavonoids were detected in the methanol extracts of PD, PE, UR, CS, CH, GP, MM and OI (**Figure 30A4**) and the ethyl acetate extracts of PD, (PE) PS, (UR) CS, GP, MM, NC, DE and OI (**Figure 30B4**) by observing orange fluorescence spots under the UV 365 nm of flavonoid-aluminium

complexes after derivatization with aluminium chloride reagent. Bands of the alkaloids were detected in the methanol extracts of HC, PD, PE, PS, UR, SCe, CH, GP, MM and DE (**Figure 30A5**) and the ethyl acetate extracts of HC, PD, PE, PS, UR, CP, SCh, SV, SCe, CS, CH, MM, DE and OI (**Figure 30B5)** by observing orange brow spots of alkaloids based on lone pair electron of nitrogen atom are examined to formed covalent coordinate bonding with metal ion in dragendorff reagents.



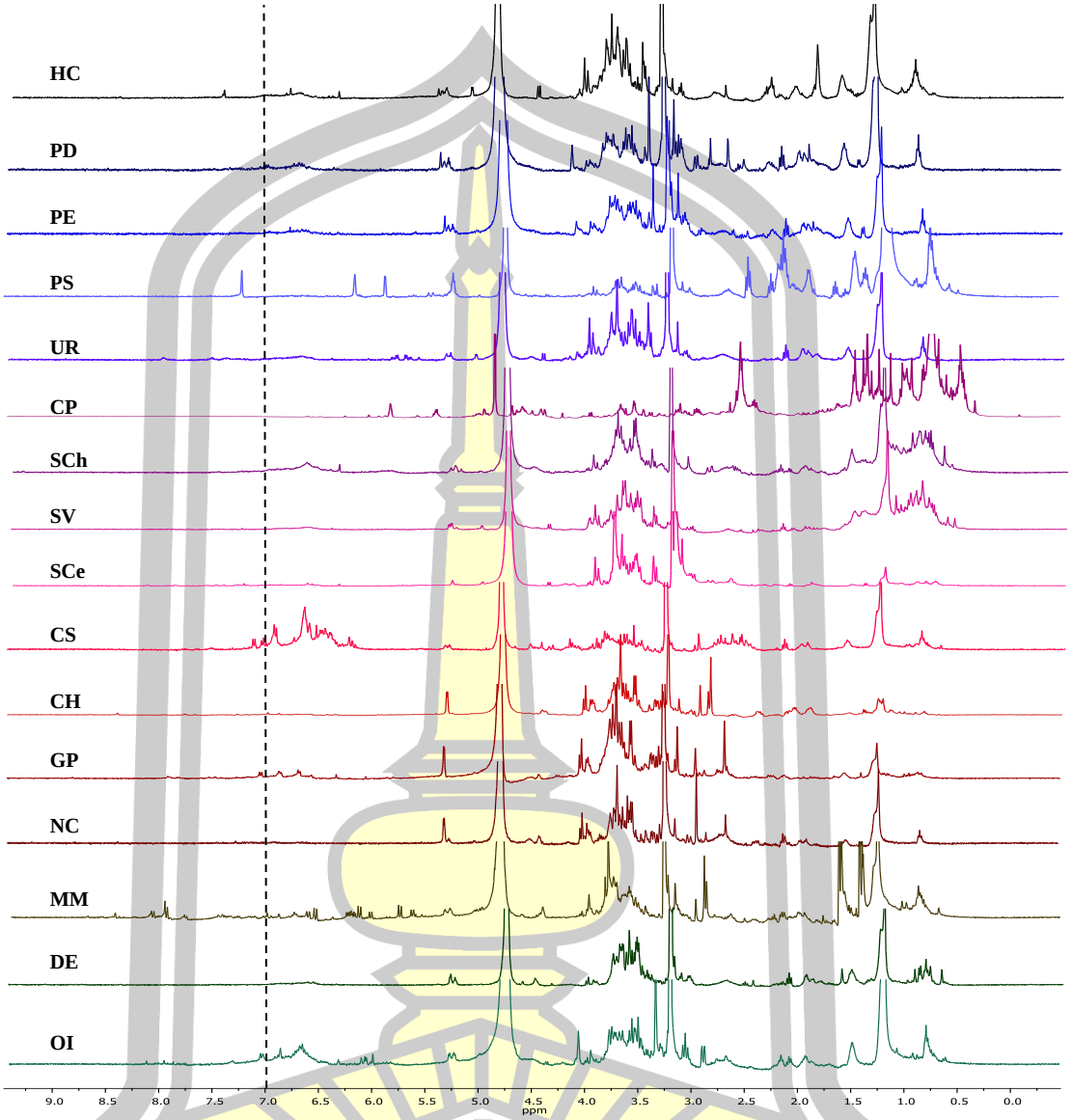

**(A)** **(B)**

**Figure 30.** TLC chromatograms of the methanol (**A**) and ethyl acetate (**B**) extracts when observed under UV light 254 nm (**1**) and then sprayed with *p*-anisaldehyde-sufuric acid reagent and heated at 100 – 105 °C in the oven (**2**), 0.4 mM DPPH reagent (**3**), 2% aluminium chloride reagent and observed under UV light 365 nm (**4**) and dragendorff reagent (**5**) (HC*; Hubera cerasoides*, PD; *Polyalthia debilis*, PE; *Polyalthia evecta*, PS; *Polyalthia suberosa*, UR; *Uvaria rufa*, CP; *Celastrus paniculatus*, SCh; *Salacia chinensis*, SV; *Salacia verrucose*, SCe; *Siphonodon celastrineus*, CS; *Caesalpinia sappan*, CH; *Clausena harmandiana*, GP; *Glycosmis pentaphylla*, NC; *Naringi crenulate*, MM; *Micromelum minutum*, DE; *Diospyros ehretioides and* OI; *Ochna integerrima*).

Numbers of the phytochemical constituents from the extracts were detected on the TLC chromatograms. Bands of the phytochemicals in most of the ethyl acetate extracts were well separated by mobile phase system used in the experiment and clearly see. These are because of more purity, less polarity and less complexity of compounds contained in the ethyl acetate extracts when compared with the methanol. Strong absorption dark bands detected in CS, CH, GP, MM and OI extracts (**Figure 30B**) which were characterized to bands of the aromatic compounds correlated well with signals of aromatic compounds appeared in their $^{1}$H NMR spectra (**Figure 32**). From literature survey, several classes of isolated constituents including phenolic compounds, phenolic acids, flavonoids, tannins, coumarins, lignans, quinones and stilbenes from CS [56],[57] coumarins, flavonoids, alkaloids, terpenes from GP [58], coumarins, triterpenes, alkaloids and phenylpropanoids from MM [59] have been reviewed. The isolation of carbazole alkaloids and coumarins from CH [25-25,[60], [61], [62] and flavonoids from OI [34-36] have also been reported.

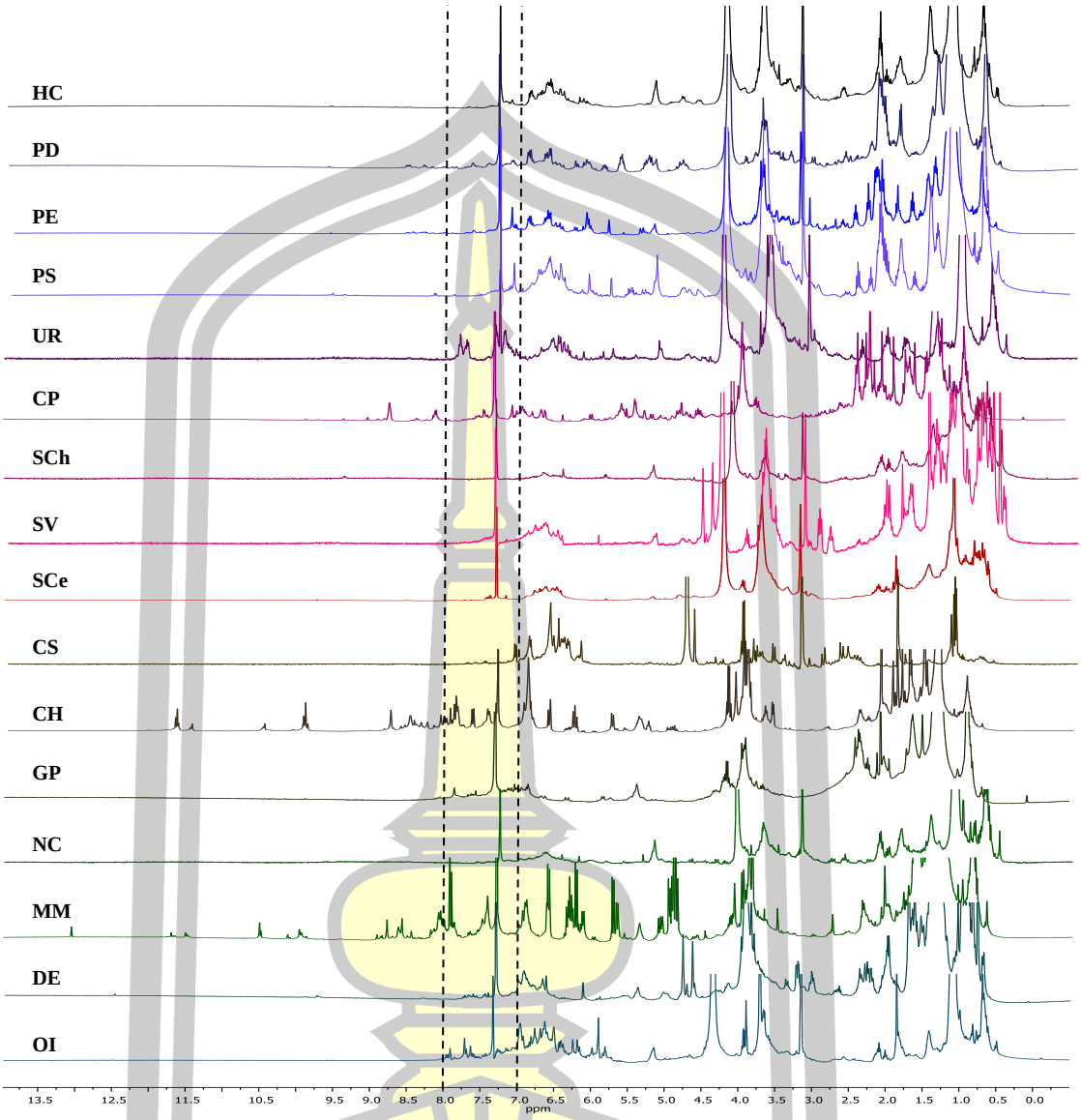
#### 4.1.1.2 $^{1}$H NMR spectrum

Signals of the aromatic protons were marked due to common phenolic compounds which including phenolic acids and flavonoids comprise of an aromatic ring bearing one or more of hydroxyl group in the structures. $^{1}$H NMR spectra of the methanol and ethyl acetate extracts are shown in **Figure 31A** and **B**. For the methanol extracts, the aromatic proton signals in region $\delta_H$ 7 – 8 ppm were detected for HC, PS, CS, GP, MM and OI (**Figure 31A**). All of the ethyl acetate extracts obviously revealed the aromatic proton signals of their phytochemicals except for SCh and NC (**Figure 31B**). In addition, CP, CH and MM ethyl acetate extracts appeared low field proton signals at $\delta_H > 8$ ppm of their phytochemical compounds (**Figure 31B**).




**(A)**

**Figure 31.** $^{1}$H NMR spectra of the methanol extracts (**A**) in methanol-$d_4$ (400 MHz) (HC; *Hubera cerasoides*, PD; *Polyalthia debilis*, PE; *Polyalthia evecta*, PS; *Polyalthia suberosa*, UR; *Uvaria rufa*, CP; *Celastrus paniculatus*, SCh; *Salacia chinensis*, SV; *Salacia verrucose*, SCe; *Siphonodon celastrineus*, CS; *Caesalpinia sappan*, CH; *Clausena harmandiana*, GP; *Glycosmis pentaphylla*, NC; *Naringi crenulate*, MM; *Micromelum minutum*, DE; *Diospyros ehretioides* and OI; *Ochna integerrima*).




**(B)**

**Figure 31.** $^{1}$H NMR spectra of the ethyl acetate extracts (**B**) in chloroform-d plused 2–3 drops of methanol-$d_4$ (400 MHz) (HC; *Hubera cerasoides*, PD; *Polyalthia debilis*, PE; *Polyalthia evecta*, PS; *Polyalthia suberosa*, UR; *Uvaria rufa*, CP; *Celastrus paniculatus*, SCh; *Salacia chinensis*, SV; *Salacia verrucose*, SCe; *Siphonodon celastrineus*, CS; *Caesalpinia sappan*, CH; *Clausena harmandiana*, GP; *Glycosmis pentaphylla*, NC; *Naringi crenulate*, MM; *Micromelum minutum*, DE; *Diospyros ehretioides* and OI; *Ochna integerrima*)**.**

#### 4.1.1.3 HPLC chromatogram

HPLC chromatograms of methanol and ethyl acetate extracts were shown in **Figure 32** and **33**. With the used system, separation of the methanol extract of CS and the ethyl acetate extracts of CS and OI was better than other extracts. The separation of the ethyl acetate seemed slightly better than the methanol extracts. It may be because height purity and less polarity of the compounds when compared to the compounds of the CS and OI extracts were not enough in good separation. The HPLC separation system need to be optimized.

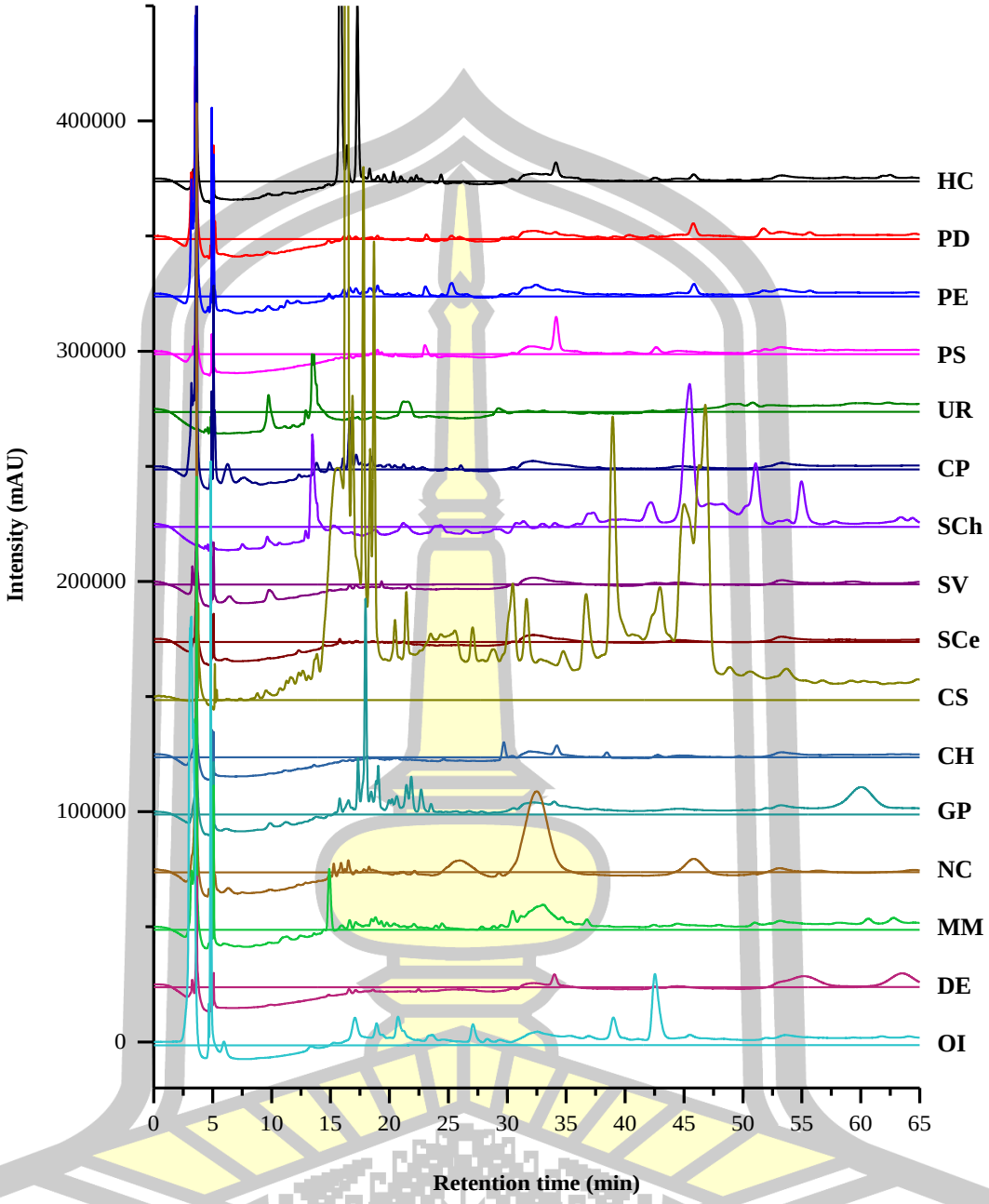



**Figure 32.** HPLC chromatogram of the methanol extracts (2 mg/500 µL) (**A**) (HC; *Hubera cerasoides*, PD; *Polyalthia debilis*, PE; *Polyalthia evecta*, PS; *Polyalthia suberosa*, UR; *Uvaria rufa*, CP; *Celastrus paniculatus*, SCh; *Salacia chinensis*, SV; *Salacia verrucose*, SCe; *Siphonodon celastrineus*, CS; *Caesalpinia sappan*, CH; *Clausena harmandiana*, GP; *Glycosmis pentaphylla*, NC; *Naringi crenulate*, MM; *Micromelum minutum*, DE; *Diospyros ehretioides* and OI; *Ochna integerrima*)**.**

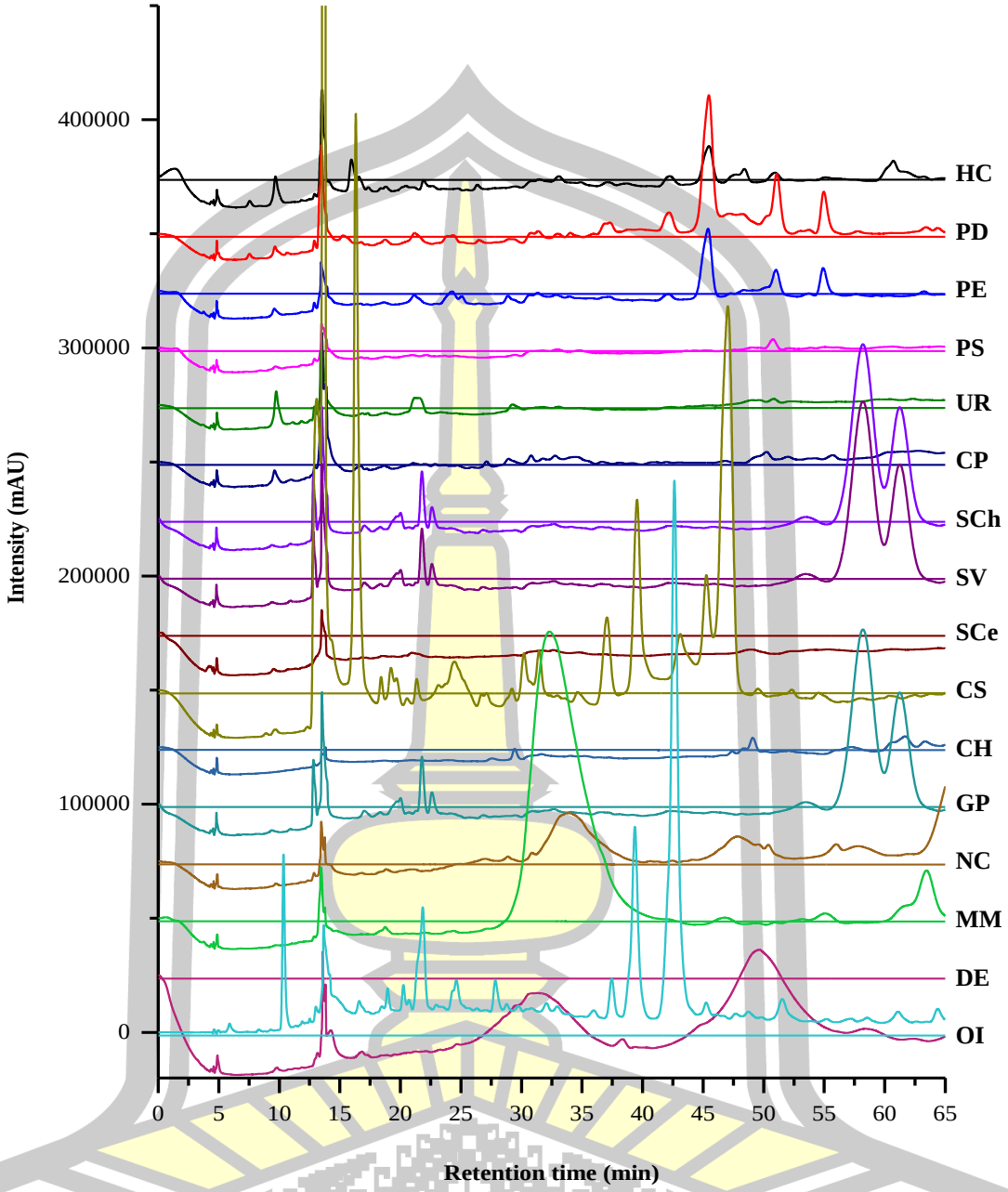



**Figure 33.** HPLC chromatogram of the ethyl acetate extracts (2 mg/500 µL) (**B**) (HC; *Hubera cerasoides*, PD; *Polyalthia debilis*, PE; *Polyalthia evecta*, PS; *Polyalthia suberosa*, UR; *Uvaria rufa*, CP; *Celastrus paniculatus*, SCh; *Salacia chinensis*, SV; *Salacia verrucose*, SCe; *Siphonodon celastrineus*, CS; *Caesalpinia sappan*, CH; *Clausena harmandiana*, GP; *Glycosmis pentaphylla*, NC; *Naringi crenulate*, MM; *Micromelum minutum*, DE; *Diospyros ehretioides* and OI; *Ochna integerrima*)**.**

### 4.1.2 Antioxidant activity

#### 4.1.2.1 DPPH•

The DPPH• scavenging activity results are shown in **Table 5**. The least of $IC_{50}$ value indicates good DPPH• scavenging property. The ethyl acetate extracts of each plant showed significantly higher scavenging activity than their methanol extracts in most cases, except for CS, SCh and OI. For both of the methanol and ethyl acetate extracts of CS exhibited the scavenging capacity toward the DPPH• in the same potency level and the strongest activity when compared other extracts. These abilities were as good as ascorbic acid ($IC_{50}$ 5.39 ± 0.32 μg/mL). The methanol extracts of SCh and OI possessed slightly better scavenging activity than their ethyl acetate extracts. As results, among sixteen extracts, the DPPH• scavenging effect decreased in the order of CS > OI > SCh ($IC_{50}$ 6.70 ± 0.02, 16.86 ± 0.27 and 19.23 ± 0.11 μg/mL) for the methanol and CS > GP = MM = OI ($IC_{50}$ 5.26 ± 0.04, 21.49 ± 0.14, 22.11 ± 0.17 and 22.47 ± 0.46 μg/mL) for the ethyl acetate extracts.

**Table 5.** DPPH• scavenging activity of the methanol and ethyl acetate extracts.

| No. | Botanical name (Code) | DPPH• scavenging activity ($IC_{50}$; μg/mL) | |
|---|---|---|---|
| | | **Methanol extract** | **Ethyl acetate extract** |
| 1 | *Hubera cerasoides* (HC) | $45.81 \pm 0.15^{d,G}$ | $25.67 \pm 0.23^{e,E}$ |
| 2 | *Polyalthia debilis* (PD) | $103.10 \pm 1.10^{j,P}$ | $55.83 \pm 0.82^{h,H}$ |
| 3 | *Polyalthia evacta* (PE) | $61.80 \pm 0.29^{f,J}$ | $59.54 \pm 0.58^{i,I}$ |
| 4 | *Polyalthia suberosa* (PS) | $110.45 \pm 0.98^{k,Q}$ | $61.79 \pm 0.15^{j,J}$ |
| 5 | *Uvaria rufa* (UR) | $94.73 \pm 1.55^{i,O}$ | $61.63 \pm 0.29^{j,J}$ |
| 6 | *Caesalpinia sappan* (CS) | $6.70 \pm 0.02^{a,A}$ | $5.26 \pm 0.04^{a,A}$ |
| 7 | *Celastrus paniculatus* (CP) | $61.06 \pm 0.77^{f,J}$ | $25.66 \pm 0.29^{e,E}$ |
| 8 | *Salacia chinensis* (SCh) | $19.23 \pm 0.11^{c,C}$ | $24.61 \pm 0.32^{d,E}$ |
| 9 | *Salacia verrucosa* (SV) | $56.74 \pm 0.15^{e,H}$ | $45.98 \pm 0.99^{g,G}$ |
| 10 | *Siphonodon celastrineus* (SCe) | $193.71 \pm 0.43^{n,T}$ | $67.65 \pm 0.74^{k,K}$ |
| 11 | *Clausena harmandiana* (CH) | $203.84 \pm 1.31^{o,U}$ | $39.76 \pm 0.24^{f,F}$ |
| 12 | *Glycosmis pentaphylla* (GP) | $75.71 \pm 0.16^{g,M}$ | $21.49 \pm 0.14^{b,D}$ |
| 13 | *Micromelum minutum* (MM) | $83.70 \pm 0.05^{h,N}$ | $22.11 \pm 0.17^{b,c,D}$ |
| 14 | *Naringi crenulata* (NC) | $112.38 \pm 0.36^{l,R}$ | $40.13 \pm 0.68^{f,F}$ |
| 15 | *Diospyros ehretioides* (DE) | $120.89 \pm 3.43^{m,S}$ | $71.36 \pm 0.86^{l,L}$ |
| 16 | *Ochna integerrima* (OI) | $16.86 \pm 0.27^{b,B}$ | $22.47 \pm 0.46^{c,D}$ |

Different superscript lowercase and uppercase letters indicate statistically significant difference within the same column and between two columns respectively (ANOVA, post-hoc; $p < 0.05$). The $IC_{50}$ of ascorbic acid was $5.39 \pm 0.32$ μg/mL.

#### 4.1.2.2 $ABTS^{\bullet+}$ scavenging activity

Results of the $ABTS^{\bullet+}$ scavenging activity are presented in **Table 6**. Most of the $ABTS^{\bullet+}$ scavenging activity of the ethyl acetate extracts was significantly stronger than their methanol extracts by showing least number of $IC_{50}$ except for CS, SV and OI. This observation was similar to the DPPH results. For CS, both of the methanol and ethyl acetate extracts displayed the strongest scavenging property among the tested samples and this property was slightly lower (~1.6 fold) than ascorbic acid ($IC_{50}$ 18.78 ± 0.06 μg/mL). Among the methanol extract group, $ABTS^{\bullet+}$ scavenging capacity decreased in the series of CS > OI > SV ($IC_{50}$ 30.10 ± 0.29, 46.35 ± 0.18 and 53.58 ± 0.53 μg/mL) while decreasing activity in the sequence of CS > CP = GP > CH ($IC_{50}$ 28.61 ± 0.04, 50.09 ± 1.00, 50.00 ± 0.55 and 61.12 ± 0.24 μg/mL) was found in the ethyl acetate extract group.

**Table 6.** $ABTS^{\bullet+}$ activity of the methanol and ethyl acetate extracts

| No. | Botanical name (Code) | ABTS scavenging activity ($IC_{50}$; ppm) | |
|---|---|---|---|
| | | **Methanol extract** | **Ethyl acetate extract** |
| 1 | *Hubera cerasoides* (HC) | $323.16 \pm 0.70^{k,U}$ | $70.33 \pm 0.99^{d,F}$ |
| 2 | *Polyalthia debilis* (PD) | $226.76 \pm 5.58^{f,P}$ | $191.83 \pm 4.97^{m,O}$ |
| 3 | *Polyalthia evacta* (PE) | $353.42 \pm 1.68^{m,W}$ | $162.31 \pm 3.00^{k,M}$ |
| 4 | *Polyalthia suberosa* (PS) | $228.87 \pm 1.17^{f,P}$ | $76.48 \pm 0.36^{e,G}$ |
| 5 | *Uvaria rufa* (UR) | $275.63 \pm 2.38^{h,R}$ | $114.57 \pm 1.84^{i,K}$ |
| 6 | *Caesalpinia sappan* (CS) | $30.10 \pm 0.29^{a,A}$ | $28.61 \pm 0.04^{a,A}$ |
| 7 | *Celastrus paniculatus* (CP) | $162.38 \pm 4.28^{d,M}$ | $50.09 \pm 1.00^{b,C}$ |
| 8 | *Salacia chinensis* (SCh) | $171.39 \pm 1.75^{e,N}$ | $68.87 \pm 2.17^{d,F}$ |
| 9 | *Salacia verrucosa* (SV) | $53.58 \pm 0.53^{c,D}$ | $90.82 \pm 1.04^{g,I}$ |
| 10 | *Siphonodon celastrineus* (SCe) | $443.42 \pm 3.43^{n,X}$ | $80.29 \pm 0.44^{f,H}$ |
| 11 | *Clausena harmandiana* (CH) | $259.91 \pm 1.78^{g,Q}$ | $61.12 \pm 0.24^{c,E}$ |
| 12 | *Glycosmis pentaphylla* (GP) | $173.44 \pm 0.80^{e,N}$ | $50.00 \pm 0.55^{b,C}$ |
| 13 | *Micromelum minutum* (MM) | $338.03 \pm 2.34^{l,V}$ | $173.69 \pm 0.52^{l,N}$ |
| 14 | *Naringi crenulata* (NC) | $305.73 \pm 3.96^{i,S}$ | $103.78 \pm 1.60^{h,J}$ |
| 15 | *Diospyros ehretioides* (DE) | $318.53 \pm 2.20^{j,T}$ | $121.26 \pm 1.07^{j,L}$ |
| 16 | *Ochna integerrima* (OI) | $46.35 \pm 0.18^{b,B}$ | $173.69 \pm 0.52^{l,N}$ |

Different superscript lowercase and uppercase letters indicate statistically significant difference within the same and two column (ANOVA, post-hoc; $p < 0.05$). The $IC_{50}$ of ascorbic acid was 18.78 ± 0.06 µg/mL.

#### 4.1.2.3 FRAP

Fe (II) reducing activity power of the extracts is shown in **Table 7**. The high number of reduction expressed in μM of Fe (II) ($r^2$ = 0.9982) shows strong activity power of the reduction of Fe (III) to Fe (II). Similar results were observed for majority of the ethyl acetate extracts of each plant of having stronger reducing activity power than those of their methanol extracts except for PD, SV and OI. The strongest activity belonged to both of the methanol and ethyl acetate extracts of CS. However, this reducing activity was less active (~1.3 fold) when compared to ascorbic acid (3,567.16 ± 1.87 μM Fe (II)/g extract). Among the tested samples, the activity of the methanol extracts decreased in the following order of CS > OI > SV (2,695.66 ± 3.08, 1,387.79 ± 6.74 and 714.90 ± 1.87 μM Fe (II)/g extract) while the activity of the ethyl acetate extracts was CS > OI > CH = GP (2,812.70 ± 1.22, 950.21 ± 4.94, 627.63 ± 7.44 and 632.52 ± 3.24 μM Fe (II)/g extract) of decreasing sequence.

**Table 7.** Ferric reducing antioxidant power (FRAP assay) of the methanol and ethyl acetate extracts

| No. | Botanical name (Code) | FRAP assay ($\mu$M Fe (II)/g extract) | |
|---|---|---|---|
| | | **Methanol extract** | **Ethyl acetate extract** |
| 1 | *Hubera cerasoides* (HC) | $280.98 \pm 0.71^{f,P}$ | $566.46 \pm 6.47^{d,G}$ |
| 2 | *Polyalthia debilis* (PD) | $363.36 \pm 2.45^{e,M}$ | $279.76 \pm 7.77^{l,P}$ |
| 3 | *Polyalthia evacta* (PE) | $151.30 \pm 4.94^{i,j,T}$ | $283.84 \pm 3.24^{l,P}$ |
| 4 | *Polyalthia suberosa* (PS) | $150.08 \pm 2.55^{i,\ j,\ T}$ | $424.54 \pm 7.64^{g,J}$ |
| 5 | *Uvaria rufa* (UR) | $154.56 \pm 1.87^{i,T}$ | $342.97 \pm 2.55^{j,N}$ |
| 6 | *Caesalpinia sappan* (CS) | $2{,}695.66 \pm 3.08^{a,B}$ | $2{,}812.70 \pm 1.22^{a,A}$ |
| 7 | *Celastrus paniculatus* (CP) | $390.28 \pm 4.41^{d,L}$ | $539.13 \pm 4.30^{e,H}$ |
| 8 | *Salacia chinensis* (SCh) | $366.63 \pm 2.55^{e,M}$ | $456.34 \pm 4.41^{f,I}$ |
| 9 | *Salacia verrucosa* (SV) | $714.90 \pm 1.87^{c,E}$ | $303.41 \pm 2.12^{k,O}$ |
| 10 | *Siphonodon celastrineus* (SCe) | $70.55 \pm 1.87^{l,V}$ | $408.22 \pm 3.08^{h,K}$ |
| 11 | *Clausena harmandiana* (CH) | $97.47 \pm 1.87^{k,U}$ | $627.63 \pm 7.44^{c,F}$ |
| 12 | *Glycosmis pentaphylla* (GP) | $215.33 \pm 2.45^{g,R}$ | $632.52 \pm 3.24^{c,F}$ |
| 13 | *Micromelum minutum* (MM) | $195.75 \pm 2.45^{h,S}$ | $409.85 \pm 3.24^{h,K}$ |
| 14 | *Naringi crenulata* (NC) | $148.44 \pm 1.41^{j,T}$ | $258.96 \pm 5.52^{m,Q}$ |
| 15 | *Diospyros ehretioides* (DE) | $191.27 \pm 6.04^{h,S}$ | $369.89 \pm 7.77^{i,M}$ |
| 16 | *Ochna integerrima* (OI) | $1{,}387.79 \pm 6.74^{b,C}$ | $950.21 \pm 4.94^{b,D}$ |

Different superscript lowercase and uppercase letters indicate statistically significant difference within the same and two column (ANOVA, post-hoc; $p < 0.05$). The $Fe^{3+}$ reducing power capacity ($\mu$M Fe (II)/g extract) of ascorbic acid was 3,567.16 ± 1.87 µM Fe (II)/g extract.

#### 4.1.2.4 TPC and TFC

TPC and TFC are the two key factors to influence the overall antioxidant activity in the samples. Results of TPC ($r^2$ = 0.9989) and TFC ($r^2$ = 0.9985) of all the extracts are shown in **Table 8** and **9**. Most of cases, the TPC and TFC values of the ethyl acetate extracts of each plant were higher or slightly lower than their methanol extracts. The two highest TPC was detected in the CS and OI extracts and TPC values were found in the following order of CS > OI > SCh (840.96 ± 1.92, 463.61 ± 0.73 and 290.02 ± 1.45 mg GAE/g extract) for the methanol and CS > OI > CP (979.33 ± 6.93, 496.73 ± 4.54 and 270.73 ± 0.73 mg GAE/g extract) for the ethyl acetate extracts. For the TFC, the highest TFC were detected from the stem bark of methanol and ethyl acetate extracts of PD. The TFC of the methanol extracts decreased in the series of PD > CS > PE (207.17 ± 0.00, 193.33 ± 2.18 and 168.18 ± 2.18 mg QE/g extract) and PD > OI > PE (280.13 ± 4.36, 198.36 ± 2.18 and 192.08 ± 3.77 mg QE/g extract) for the ethyl acetate extracts.

**Table 8.** Total Phenolic Contents (TPC) of the methanol and ethyl acetate extracts

| No. | Botanical name (Code) | Total phenolic content (mg GAE/g extract) | |
|---|---|---|---|
| | | **Methanol extract** | **Ethyl acetate extract** |
| 1 | *Hubera cerasoides* (HC) | $106.79 \pm 0.00^{i,S}$ | $231.32 \pm 2.52^{e,H}$ |
| 2 | *Polyalthia debilis* (PD) | $95.47 \pm 2.18^{k,U}$ | $126.08 \pm 0.73^{m,P,Q}$ |
| 3 | *Polyalthia evacta* (PE) | $146.62 \pm 2.62^{e,N}$ | $145.37 \pm 3.17^{k,N}$ |
| 4 | *Polyalthia suberosa* (PS) | $51.45 \pm 2.18^{o,X}$ | $179.33 \pm 5.67^{h,K}$ |
| 5 | *Uvaria rufa* (UR) | $101.76 \pm 0.00^{j,T}$ | $175.97 \pm 1.26^{h,K}$ |
| 6 | *Caesalpinia sappan* (CS) | $840.96 \pm 1.92^{a,B}$ | $979.33 \pm 6.93^{a,A}$ |
| 7 | *Celastrus paniculatus* (CP) | $112.66 \pm 0.73^{h,R}$ | $270.73 \pm 0.73^{c,F}$ |
| 8 | *Salacia chinensis* (SCh) | $290.02 \pm 1.45^{c,E}$ | $204.91 \pm 2.18^{g,J}$ |
| 9 | *Salacia verrucosa* (SV) | $126.92 \pm 0.00^{g,P}$ | $122.73 \pm 0.73^{m,Q}$ |
| 10 | *Siphonodon celastrineus* (SCe) | $13.71 \pm 0.00^{p,M}$ | $165.49 \pm 0.73^{i,L}$ |
| 11 | *Clausena harmandiana* (CH) | $91.70 \pm 2.18^{l,U}$ | $214.97 \pm 1.26^{f,I}$ |
| 12 | *Glycosmis pentaphylla* (GP) | $139.50 \pm 3.33^{f,O}$ | $242.22 \pm 1.92^{d,G}$ |
| 13 | *Micromelum minutum* (MM) | $159.20 \pm 1.92^{d,M}$ | $136.56 \pm 0.73^{l,O}$ |
| 14 | *Naringi crenulata* (NC) | $71.15 \pm 0.73^{m,V}$ | $137.40 \pm 4.76^{l,O}$ |
| 15 | *Diospyros ehretioides* (DE) | $61.51 \pm 1.26^{n,W}$ | $160.04 \pm 0.73^{j,M}$ |
| 16 | *Ochna integerrima* (OI) | $463.61 \pm 0.73^{b,D}$ | $496.73 \pm 4.54^{b,C}$ |

Different superscript lowercase and uppercase letters indicate statistically significant difference within the same and two column (ANOVA, post-hoc; $p < 0.05$).

**Table 9.** Total Flavonoid Contents (TFC) of the methanol and ethyl acetate extracts

| No. | Botanical name (Code) | Total flavonoid content (mg QE/g extract) | |
|---|---|---|---|
| | | **Methanol extract** | **Ethyl acetate extract** |
| 1 | *Hubera cerasoides* (HC) | $102.77 \pm 2.18^{k,S}$ | $122.89 \pm 4.36^{j,N}$ |
| 2 | *Polyalthia debilis* (PD) | $207.17 \pm 0.00^{a,B}$ | $280.13 \pm 4.36^{a,A}$ |
| 3 | *Polyalthia evacta* (PE) | $168.18 \pm 2.18^{c,F}$ | $192.08 \pm 3.77^{c,D}$ |
| 4 | *Polyalthia suberosa* (PS) | $153.08 \pm 2.18^{d,H}$ | $134.21 \pm 2.18^{g,h,K,L}$ |
| 5 | *Uvaria rufa* (UR) | $116.60 \pm 3.77^{i,j,P,Q,R}$ | $168.18 \pm 2.18^{e,F}$ |
| 6 | *Caesalpinia sappan* (CS) | $193.33 \pm 2.18^{b,D}$ | $179.50 \pm 2.18^{d,E}$ |
| 7 | *Celastrus paniculatus* (CP) | $119.12 \pm 2.18^{i,N,O,P}$ | $121.64 \pm 2.18^{j,N,O}$ |
| 8 | *Salacia chinensis* (SCh) | $131.70 \pm 3.77^{g,h,L,M}$ | $161.89 \pm 0.00^{f,G}$ |
| 9 | *Salacia verrucosa* (SV) | $154.34 \pm 0.00^{d,H}$ | $129.18 \pm 2.18^{i,M}$ |
| 10 | *Siphonodon celastrineus* (SCe) | $114.09 \pm 2.18^{j,Q,R}$ | $131.70 \pm 3.77^{h,i,L,M}$ |
| 11 | *Clausena harmandiana* (CH) | $117.86 \pm 2.18^{i,O,P,Q}$ | $182.01 \pm 2.18^{d,E}$ |
| 12 | *Glycosmis pentaphylla* (GP) | $135.47 \pm 3.77^{f,g,J,K,L}$ | $165.66 \pm 0.00^{e,f,F,G}$ |
| 13 | *Micromelum minutum* (MM) | $148.05 \pm 2.18^{e,I}$ | $178.24 \pm 2.18^{d,E}$ |
| 14 | *Naringi crenulata* (NC) | $112.83 \pm 0.00^{j,R}$ | $132.96 \pm 2.18^{g,h,i,K,L,M}$ |
| 15 | *Diospyros ehretioides* (DE) | $129.18 \pm 2.18^{h,M}$ | $136.73 \pm 2.18^{g,J,K}$ |
| 16 | *Ochna integerrima* (OI) | $139.25 \pm 0.00^{f,J}$ | $198.36 \pm 2.18^{b,C}$ |

Different superscript lowercase and uppercase letters indicate statistically significant difference within the same and two column (ANOVA, post-hoc; $p < 0.05$).

## 4.2 Discussion

Result of chemical composition profile of Thai medicinal plant-derived galactogogue extracts by using Thin Layer Chromatography (TLC) technique were studied under 2 5 4 nm UV light together with chemical reagent, such as *p*-anisaldehyde-sulfuric reagent to detected poly(phenol). DPPH• reagent are used to check for antioxidants of extracts. All of the extracts revealed the antioxidant compounds by decolourizing of purple DPPH• reagent to colorless reduced form. Aluminium chloride reagent were detected of flavonoids. Bands of the flavonoids

were detected in the methanol extracts of PD, PE, UR, CS, CH, GP, MM and OI and the ethyl acetate extracts of PD, (PE) PS, (UR) CS, GP, MM, NC, DE and OI by observing orange fluorescence spots under the UV 365 nm of flavonoid-aluminium complexes after derivatization with aluminium chloride reagent. Bands of the alkaloids were detected in the methanol extracts of HC, PD, PE, PS, UR, SCe, CH, GP, MM and DE and the ethyl acetate extracts of HC, PD, PE, PS, UR, CP, SCh, SV, SCe, CS, CH, MM, DE and OI by observing orange brown spots of alkaloids with dragendorff reagents [54].

Results of the detection signal of aromatic protons using $^{1}$H NMR spectroscopy, for the methanol extracts, the aromatic proton signals in region $\delta_H$ 7 - 8 ppm were detected for HC, PS, CS, GP, MM and OI. All of the ethyl acetate extracts obviously revealed the aromatic proton signals of their phytochemicals except for SCh and NC. The HPLC chromatograms of the methanol and ethyl acetate extracts were analyzed and compared. HPLC chromatogram of the ethyl acetate extracts of CS and OI and the methanol extract of CS showed better separation of compound peaks more than other extracts by the system used.

Several *in vitro* antioxidant activity test procedures are carried out for evaluating antioxidant activity with the samples of interest, therefore, the antioxidant activity should not be concluded based on a single antioxidant test model [63]. Although the stable radicals of $DPPH^{\bullet}$ and $ABTS^{\bullet+}$ on the DPPH and ABTS assays are not found in biological system [63],[64] these two methods are two most rapid, simple and inexpensive methods among the radical scavenging activity tests. ABTS assay is also applicable for both hydrophilic and lipophilic antioxidants [63]. The FRAP technique is rapid, simple, reproducible and shows high correlation with both ascorbic acid and total phenolics. The DPPH, ABTS and FRAP assays gave comparable results for the antioxidant activity measured in methanolic extracts [64].

The results indicated strong and remarkable antioxidant property of CS stem bark methanol and ethyl acetate extracts. The antioxidant activity of CS extract, especially from heartwood, [65],[66] and brazillin, a major bioactive compound from CS, [67], [68], [69] are well researched. Previous studies point out that CS is an excellent natural antioxidant source which is in good agreement with our results from present study. The antioxidant activity reported for OI is limited [70]. The CS and OI extracts

which possessed strong antioxidant activity were also found to have high TPC and moderate TFC. These compounds are believed to be main active compounds responsible to their antioxidant capacities [34-35],[56-57],[71]. The highest TFC in both of the methanol and ethyl acetate extracts were found in PD. However, the TFC was much lower than the TPC in all of the plant extracts in comparison. In the case of PD, furans polyacetylenes and aporphine alkaloids have been isolated from the root of this plant [10], [72], [73].

# CHAPTER 5

# CONCLUSION

Galactogogue plants have been traditionally used worldwide to stimulate lactation. In the northeastern part of Thailand the galactogogue medicinal plant such as *Polyalthia debilis* (PD), *Micromelum minutum* (MM), *Caesalpinia sappan* (CS) and *Ochna integerrima* (OI) widely used in traditional galactogogue recipes. This work aims to screen for chemical composition (TLC, $^{1}$H-NMR and HPLC) and evaluate antioxidant activity (DPPH, ABTS and FRAP methods, total phenolic and total flavonoid contents) of the sixteen galactogogue medicinal plants from six families collected from northeastern Thailand. These sixteen plant samples are *Hubera cerasoides* (HC), *Polyalthia debilis* (PD), *Polyalthia evecta* (PE), *Polyalthia suberosa* (PS), *Uvaria rufa* (UR), *Celastrus paniculatus* (CP), *Salacia chinensis* (SCh), *Salacia verrucose* (SV), *Siphonodon celastrineus* (SCe), *Caesalpinia sappan* (CS), *Clausena harmandiana* (CH), *Glycosmis pentaphylla* (GP), *Naringi crenulate* (NC), *Micromelum minutum* (MM), *Diospyros ehretioides* (DE) and *Ochna integerrima* (OI). The plant samples were extracted and the methanol and ethyl acetate extracts were obtained from each plant.

The majority of chemical compositions in both methanol and ethyl acetate extracts were (poly)phenolic and antioxidant compounds. The results screening of phytochemical secondary metabolite of all extracts using TLC, $^{1}$H NMR and HPLC techniques, found that CS and OI from methanol and ethyl acetate extracts showed signals of interesting chemical compositions. Based on TLC analysis and reagent derivatization (*p*-anisaldehyde-sufuric acid, 0.4 mM DPPH$^{\bullet}$, 2% aluminium chloride and dragendorff reagents), phenolic and antioxidant compounds were detected in both of methanol and ethyl acetate of all plant extracts while flavonoids and alkaloids were detected in some of the plant extracts and found clear signals of aromatic protons of HC, PS, CS, GP, MM and OI from the methanol extracts and almost all ethyl acetate extracts except NC on the $^{1}$H NMR spectra, which is consistent with the results of the antioxidant activity. Moreover, The HPLC chromatograms of methanol and ethyl

acetate extracts showed poor separation system. The HPLC separation of active extracts need to be optimized further.

The results indicated that CS (Fabaceae) and OI (Ochnaceae) stem bark extracts showed the two most potent antioxidant property on the DPPH ($IC_{50}$ 6.70 ± 0.02 and 5.26 ± 0.04 μg/mL for the methanol and ethyl acetate extracts of CS and 16.86 ± 0.27 and 22.47 ± 0.46 μg/mL for the methanol and ethyl acetate extracts of OI), ABTS ($IC_{50}$ 30.10 ± 0.29 and 28.61 ± 0.04 μg/mL for the methanol and ethyl acetate extracts of CS and 46.35 ± 0.18 and 173.69 ± 0.52 μg/mL for the methanol and ethyl acetate extracts of OI) and FRAP (2,695.66 ± 3.08 and 2,812.70 ± 1.22 μM Fe (II)/g extract for the methanol and ethyl acetate extracts of CS and 1,387.79 ± 6.74 and 950.21 ± 4.94 μM Fe (II)/g extract for the methanol and ethyl acetate extracts of OI) methods. The CS and OI extracts which possessed strong antioxidant activity were also found to have high TPC (840.96 ± 1.92 and 979.33 ± 6.93 mg GAE/g extract for the methanol and ethyl acetate extracts of CS and 463.61 ± 0.73 and 496.73 ± 4.54 mg GAE/g extract for the methanol and ethyl acetate extracts of OI) and moderate TFC (193.33 ± 2.18 and 179.50 ± 2.18 mg QE/g extract for the methanol and ethyl acetate extracts of CS and 139.25 ± 0.00 and 198.36 ± 2.18 QE/g extract for the methanol and ethyl acetate extracts of OI). These antioxidant activities should be due to polyphenols, antioxidants and flavonoids in the extracts. These results support the use of CS extract as natural antioxidant source and suggest that OI can also be used as alternative sources for natural antioxidants.

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