สารต้านแบคทีเรียจากจันทน์แดง *Dracaena cochinchinensis* (Lour.) S.C.Chen และ
หอมแดง *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne

นางสาวริทเบย์ รูก้า

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บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)
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ANTIBACTERIAL AGENTS FROM *Dracaena cochinchinensis* (Lour.) S.C.Chen AND *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne

Miss Ritbey Ruga

จุฬาลงกรณ์มหาวิทยาลัย
Chulalongkorn University

A Dissertation Submitted in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy Program in Biotechnology
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ริทเบย์ รูก้า : สารต้านแบคทีเรียจากจันทน์แดง *Dracaena cochinchinensis* (Lour.) S.C.Chen และหอมแดง *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne (ANTIBACTERIAL AGENTS FROM *Dracaena cochinchinensis* (Lour.) S.C.Chen AND *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วรินทร ชวศิริ, 185 หน้า.

ได้แยกสารประกอบ 15 สาร ซึ่งประกอบด้วยแอนทราควิโนนใหม่ 2 สาร (118-119) สารประกอบใหม่ที่เกิดขึ้นในธรรมชาติ 2 สาร (116-117) และสารประกอบที่มีรายงานมาก่อนแล้วอีก 11 สาร จากสิ่งสกัดไดคลอโรมีเทนของ *Dracaena cochinchinensis* และ *Eleutherine americana* คือ dihydrochalcones (5 และ 23), pterostilbene (27) homoisoflavanones (15 และ 82-84) naphthalenes (85 และ 116) pyranonaphthoquinone (86) และ anthraquinones (99-100 และ 117-119) (+)-eleutherin (86) เป็นสารที่มีประสิทธิภาพในการต้านแบคทีเรีย *Propionibacterium acnes* KCCM 41747 *Staphylococcus aureus* ATCC 25923 *Streptococcus sobrinus* KCCM 11898 *Streptococcus mutans* ATCC 25175 และ *Salmonella typhi* ATCC 422 โดยมีบริเวณที่ยับยั้งการเจริญของเชื้อ 14.7-18.0 มม. และค่าความเข้มข้นต่ำสุดที่สามารถยับยั้งเชื้อแบคทีเรียที่ 0.24-7.8 ไมโครโมลาร์ สารประกอบ 86 มีฤทธิ์ยับยั้งการเจริญเติบโตของเชื้อ *P. acnes* ขณะที่พบว่าสารนี้เป็นสารฆ่าเชื้อแบคทีเรียอื่นๆ ที่ทดสอบ ในการใช้ chloramphenicol ร่วมกับสารประกอบ 86 มีปัจจัยเสริมผลกระทบมากในการยับยั้งเชื้อแบคทีเรียทุกชนิดที่ทดสอบ นอกจากนี้ได้ศึกษาฤทธิ์ต้านการแพ้ของสารประกอบ 9 สาร ในการปลดปล่อย β-hexosaminidase ในเซลล์หนูที่เป็น basophil leukemia (RBL-2H3) โดยการกระตุ้นเหนี่ยวนำโดยแคลเซียมไอออนอฟอร์ A23178 โดยพบว่า loureirin B (5) มีฤทธิ์ดีที่สุด ตามด้วย 23 และ 27 มีค่า IC_{50} 7.8 15.8 และ 17.5 ไมโครโมลาร์ ตามลำดับ สำหรับฤทธิ์ต้านมะเร็ง สารประกอบ 86 ยับยั้งการเติบโตของเซลล์มะเร็ง HeLa HCT116 และ HepG2 โดยมีค่า IC_{50} 0.3 4.6 และ 6.7 ไมโครโมลาร์ ตามลำดับ สารประกอบ 86 แสดงความเป็นพิษมากกว่าสารควบคุมแบบบวก (5-fluorourasil) ต่อเซลล์ HeLa สารประกอบ 23 และ 27 แสดงการยับยั้งเซลล์ โดยมีค่า IC_{50} 1.4 ไมโครโมลาร์

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สาขาวิชา เทคโนโลยีชีวภาพ ลายมือชื่อนิสิต .....

ปีการศึกษา 2560 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5572879023 : MAJOR BIOTECHNOLOGY
KEYWORDS: DRACAENA COCHINCHINENSIS (LOUR.) S.C.CHEN / ELEUTHERINE AMERICANA (AUBL.) MERR. EX K. HEYNE / ISOLATION / ANTIBACTERIAL ACTIVITY / ANTI-ALLERGIC ACTIVITY / ANTICANCER ACTIVITY

RITBEY RUGA: ANTIBACTERIAL AGENTS FROM *Dracaena cochinchinensis* (Lour.) S.C.Chen AND *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 185 pp.

Fifteen compounds including two new anthraquinones (118-119), two naturally occurring new compounds (116-117) and eleven known compounds were successfully isolated from the CH_2Cl_2 extracts of *Dracaena cochinchinensis* and *Eleutherine americana i.e.* dihydrochalcones (5 and 23), pterostilbene (27), homoisoflavanones (15 and 82-84), naphthalenes (85 and 116), pyranonaphthoquinone (86) and anthraquinones (99-100 and 117-119). Among isolated compounds, (+)-eleutherin (86) was the most potent antibacterial agent against *Propionibacterium acnes* KCCM 41747, *Staphylococcus aureus* ATCC 25923, *Streptococcus sobrinus* KCCM 11898, *Streptococcus mutans* ATCC 25175 and *Salmonella typhi* ATCC 422 with inhibition zones of 14.7 to 18.0 mm and MIC values of 0.24 to 7.8 μM. Compound 86 showed bacteriostatic effect against *P. acnes* while bactericidal agent was observed by this compound against other tested bacteria. The combination of chloramphenicol and 86 demonstrated synergistic effect against all tested bacteria. Moreover, anti-allergic activity of nine compounds on the release of β-hexosaminidase in rat basophil leukemia (RBL-2H3) cells by calcium ionophore A23178-induced stimulation were investigated. Loureirin B (5) showed the strongest activity followed by 23 and 27 with IC_{50} values of 7.8, 15.8 and 17.5 μM, respectively. For anticancer activity, 86 suppressed the growth of HeLa, HCT116 and HepG2 cancer cell lines with IC_{50} values of 0.3, 4.6 and 6.7 μM, respectively. Compound 86 displayed twice more toxic than positive control (5-fluorourasil) against HeLa cells. The suppression against the cell was also shown by 23 and 27 with IC_{50} value of 1.4 μM.

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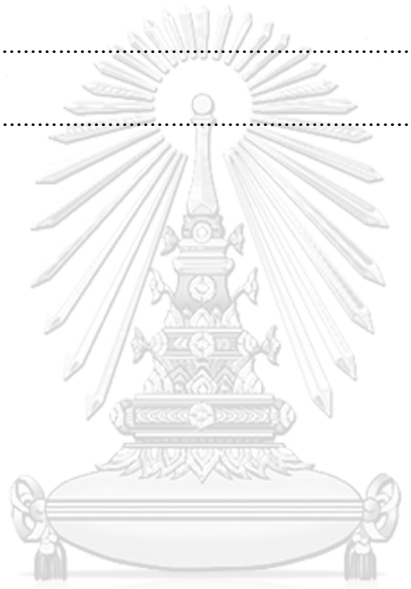

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LIST OF ABBREVIATIONS

acetone-d_6	deuterated acetone
ALP	alkaline phosphatase
ATCC	American type culture collection
brs	broad singlet (NMR)
^{13}C NMR	carbon-13 nuclear magnetic resonance
$CDCl_3$	deuterated chloroform
CD_3OD	deuterated methanol
CFU	colony-forming unit
$CHCl_3$	chloroform
CH_2Cl_2	dichloromethane
CHL	chloramphenicol
CLSI	clinical and laboratory standards institute
cm	centimeter
COX	cyclooxygenase
CO_2	carbondioxide
d	doublet (NMR)
dd	doublet of doublet (NMR)
dt	doublet of triplet (NMR)
dq	doublet of quartet (NMR)
DMEM	Dulbecco's modified eagle's medium
DMSO	dimethylsulfoxide
EMEM	Eagle's minimal essential medium

ESI	electron spray ionization
ETOH	ethanol
EtOAc	ethyl acetate
equiv	equivalent (s)
FICI	fractional inhibitory concentration index
FBS	fetal bovine serum
g	gram (s)
h	hour (s)
^{1}H NMR	proton nuclear magnetic resonance
HCl	hydrochloric acid
HeLa	Henrietta Lacks cervical cancer
HepG2	hepatoma G2 cells
HIV	human immunodeficiency virus
H_2O	dihydrogen monoxide
HPLC	high-performance liquid chromatography
HRMS	high resolution mass spectrum
hTopIIα	human topoisomerase II-alpha
Hz	Hertz
IC_{50}	concentration that required for 50% inhibition in vitro
IgE	immunoglubulin E
IL-2	interleukin 2
IL-6	interleukin 6
iNOS	inducible nitric oxide synthase
J	coupling constant

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KCCM	Korean culture center of microorganisms
kcal	kilocalorie
kg	kilogram (s)
LPS	lipopolisaccharide
m	multiplet (NMR)
MBC	minimum bactericidal concentration
MeOH	methanol
mg	milligram (s)
MHz	mega hertz
MIC	minimum inhibitory concentration
MICI	minimum inhibitory concentration idex
mix	mixture
Myc	myelocytomatosis
mL	milliliter (s)
mM	millimolar (s)
MS	mass spectrophotometry
MSCs	Mesenchymal stem cells
mRNA	messenger ribo nucleic acid
MRSA	multi-resistant *Staphylococcus aureus*
MSSA	methicillin-resistant *Staphylococcus aureus*
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5-diphenil tetrazodium bromide)
$NaHCO_3$	sodium bicarbonate
NA	nutrient agar

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NB	nutrient broth
NF-kB	nuclear factor-kappa B signaling
nM	nanomolar (s)
nm	nanometer
NMR	nuclear magnetic resonance
NO	nitric oxide
OCH_3	methoxyl
OH	hydroxyl
ORTEP	oak ridge thermal ellipsoid plot program
PBS	phosphate buffer saline
PHA	phytohemagglutanin
ppm	part per million
q	quartet (NMR)
QCC	quick column chromatography
RBL	rat basophyl leukemia
RMCP	rat mast cells protease
s	singlet (NMR)
SD	standard deviation
SCCmec	Staphylococcal chromosomal cassette mec
t	triplet (NMR)
TLC	thin layer chromatography
TNF-α	tumour necrosis factor alpha
TOF	time of flight
tRNA	transfer ribo nucleic acid

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UV	ultraviolet
w	weight
WHO	world health organization
δ	chemical shift
δ_C	chemical shift of carbon
δ_H	chemical shift of proton
°C	degree Celsius
µg	microgram (s)
µL	microliter (s)
µM	micromolar (s)
% yield	percentage yield
$[\alpha]_D$	specific optical rotation

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CHAPTER I

INTRODUCTION

1.1 The Scope of Research

Natural products have played very important role in treating and preventing human diseases. They have been a particularly rich source of therapeutic agents which can be obtained from organisms including plants, fungi, bacteria, lichens, algae, protozoans, insects and animals. They provide many benefits in the treatment of wide range of medical conditions, including infectious diseases caused by bacteria, fungi, and virus.

Plants and their metabolites have been recorded for a long history of use in modern and traditional medicine system [1]. The written evidence of the plant uses as source of medicines to overcome various diseases was documented by the great civilizations of ancient Chinese, Indians and North Africans [2]. Generally, secondary metabolites exhibit individuality and diversity in their molecular structures than primary metabolites. These substances are structurally distributed among very limited number of species within the plant kingdom. They can be obtained in only specific plants or specific group of plants and not necessarily in all condition such as for growth and reproduction of a plant [3, 4]. Production of these metabolites are assumed through biochemical "side track" in the cell of organism in response to external stimulus

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such as nutritional changes, infection and competition [5, 6]. In recent years, more than 50% of all the drugs in clinical use in the world derived from natural products and their derivatives, and approximately 25% of all medical prescriptions are based of substances contributed from higher plants and plants-derived synthetic analogue [7].

Medicinal plants have been one of the major sources of medicines that have biological activities as therapeutic agents such as antimicrobial, anticancer, anti-oxidant and anti-inflammatory properties. Approximately, 25% of the drugs prescribed in industrialized countries contain active compounds extracted from medicinal plants and some naturally occurring compounds serve as models for a large percentage clinically proven drugs and being re-assessed as antimicrobial agents [8]. In addition, among 109 new antibacterial drugs approved in the period of 1981–2006, it was about 69% originated from natural products and 21% of antifungal drugs were natural derivatives or compounds mimicking natural products [9].

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The primary reason for finding the new drugs from nature especially from plants is the fact that infectious diseases, cancer and allergic remain significant causes of ill health. The resistance of pathogenic bacteria caused by the abuse of the antibacterial drugs extensively in both humans and food-producing animals. Consequently, antibacterial drugs have become less effective or even ineffective, resulting the multidrug resistance among the microbial pathogen has been one of the great concerns over the world [10, 11]. Furthermore, the rates of detection of different types of cancers have increased and became leading of the death. In recent years, cancer

develops and involves at least 100 different diseases which are abnormal cells in which processes regulating normal cell division are disrupted [12]. The other chronic disease health problems that also cause high mortality in most countries are allergies such as allergic rhinitis, atopic dermatitis, food allergy and asthma. Approximately, 50% of people worldwide suffer from the diseases and up to now the disease rapidly increasing chronic health problem [13, 14].

Certain studies were addressed on chemical constituents and biological activities of *Dracaena cochinchinensis* (Lour.) S.C.Chen and *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne which are medicinal plants in Thailand and Indonesia, respectively. Some compounds including dihydrochalcone, homoisoflavonoid and stilbenoid derivatives were isolated from *D. loureiri* and showed anti-inflammatory, cytotoxicity, antibacterial and hyperthropic scar formation inhibitory effects [15-17]. Certain naphthoquinone, anthraquinone, naphthalene and their glycoside derivatives were isolated from the bulbs of *E. americana* [18] with their inhibitory effect against the growing of tumour cells [19] and pathogenic bacteria [20]. α-Glucosidase inhibitory effect was also exhibited from glycoside derivatives from this plant [21]. Hence, based on the broad spectrum of their biological activity, it was needed to explore more on the chemical constituents and biological activities of those plants.

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1.2 The Objective of the Research

This study was focused on searching for bioactive compounds from *D. cochinchinensis* and *E. americana* with potent antibacterial, anti-allergic and anticancer effects. This study composes of three main steps including isolation and purification, structure elucidation and bioassay evaluation of extracts and isolated compounds. For the isolation step, chemical and biological approaches will be used. The extracts are screened for antibacterial activity as complement to the bioassay-guided approach: TLC and HPLC are used to guide the isolation and purification of the compounds responsible for the activity of extracts or compounds that are most interesting.

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CHAPTER II

CHEMICAL CONSTITUENTS AND ANTIBACTERIAL ACTIVITY OF *Dracaena cochinchinensis* (Lour.) S.C.Chen

2.1 Introduction

The family Dracaenaceae or Agavaceae with perhaps more than 480 species has distributed in tropical and sub-tropical dry climate regions throughout the world [22]. Many species of *Dracaena* have been used as traditional medicine such as *Dracaena manii* Bak. [23], *Dracaena draco* L. Spp. draco [24, 25], *Dracaena reflexa* Lam. [26], *Dracaena thalioides* Makoy ex Regel [27], and *Dracaena deisteliana* Engl.. *Dracaena cochinchinensis* (Lour.) S.C.Chen [28] is another species widely used in China and Thailand as folk medicine.

2.1.1 General Characteristics of *D. cochinchinensis*

Figure 2.1 The stems wood of *Dracaena cochinchinensis*

D. cochinchinensis or dragon's blood tree (synonym: *Dracaena loureiri* Gagnep, *Aletris cochichinensis* (Lour.) and *Pleomele cochichinensis* (Lour.) Merr. Ex Gagnep) (**Figure 2.1**) [29] belongs to Agavaceae family is an evergreen tree and native to the tropical regions of south western China, Myanmar, Laos [30], and can also be found in every part of Thailand, especially on high mountain. This plant was well known as Chan-daeng or Jun-par. *D. cochinchinensis* is a part of the large, very robust and ornamental plants, which in its numerous shapes and sizes play an important role in tropical gardens all over Thailand. It is a shrub or slender much branched tree with a red core in the stems when become old.

2.1.2 Literature Review of *D. cochinchinensis* and Its Biological Activities

The red resin produced by artificial wounding of the stem wood of *D. cochinchinensis* [31] mainly contained flavonoids such as homoisoflavanones, flavanones, chalcones and dihydrochalcones. Stilbenoids and several steroid-saponins were also addressed [32]. Traditionally, the stem wood of *D. cochinchinensis* has been used as antipyretic, analgesic and for preventing infectious diseases [16, 17]. In China, *D. cochinchinensis* is commonly used as traditional medicine to treat traumatic injury, fractures, diarrhea and dysmenorrhea [33].

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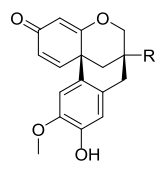
Significant numbers of reports have been revealed from this folk medicine including the chemical constituents and its pharmacological evaluation with a broad spectrum of healthy benefits such as antimicrobial, anti-platelet aggregation,

antithrombotic, anti-inflammatory, radio protective, wound healing, anti-diabetic and cerebral protective activities [28].

In 1988, Meksiruyen and Cordell [29] reported that the dried stems of *D. loureiri* were successfully extracted with petroleum ether, $CHCl_3$ and EtOH. The antibacterial activity of three extracts against *Staphylococcus aureus* ATCC 25923, *Bacillus subtilis* ATCC 6633 and *Escherichia coli* ATC 25922 was investigated by disc diffusion technique. Only the $CHCl_3$ extract displayed antibacterial activity against *S. aureus* and *B. subtilis*. From this active extract, fifteen flavonoids including two new cyclized homoisoflavans (**1–2**), a chromane derivative (**3**), four retrohydrochalcones (**4-7**) and eight flavonoids (**8–15**) were isolated.



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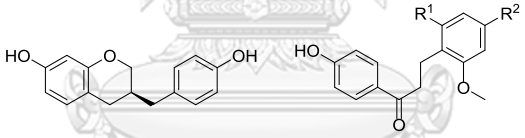
2: R= OH



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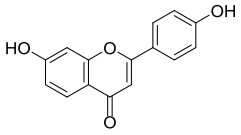
4: R^1 = H, R^2= OCH_3
5: R^1 = OCH_3, R^2 = OCH_3
6: R^1 = H, R^2 = OH
7: R^1 = OH, R^2 = OH



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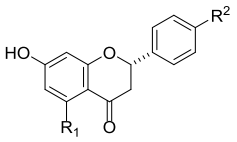
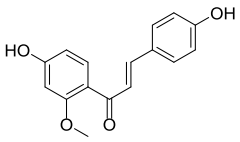


9: R^1 = H, R^2 = H
10: R^1 = OH, R_2 = H
11: R^1 = OCH_3, R^2 = OH



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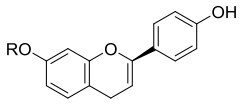
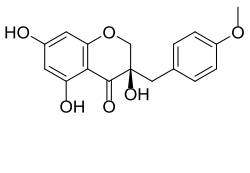


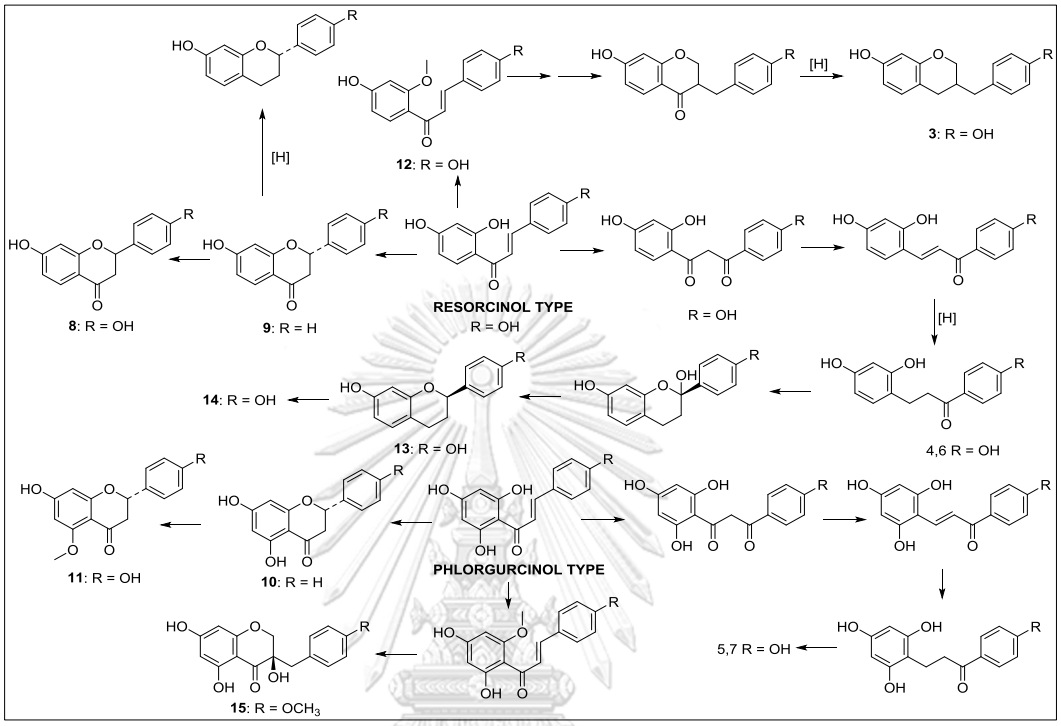
13: R= H
14: R= CH_3



15

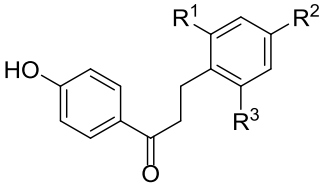
Based on the structures of the isolated compounds from *D. loureiri*, this genus displayed the ability to accumulate a series of structurally and biosynthetically inter-

related flavonoids at different levels of oxidation and substitution. Hence, they proposed biogenesis of the flavonoids of *D. loureiri* as shown in **Scheme 2.1**.

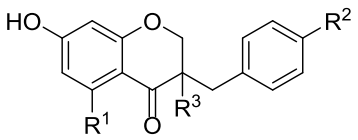


Scheme 2.1 Proposed biogenesis of the flavonoids of *D. loureiri*

Estrogen agonist activity of retrohydrochalcones (**5**, **7**, **16-17**) and homoisoflavones (**18-20**) from *D. loureiri* was investigated by Ichikawa and co-workers in 1997 [16]. 4´-Dihydroxy-2,6-dimethoxydihydrochalcone (**5**) and 5,7-dihydroxy-3-(4-hydroxybenzyl)-4-chromanone (**19**) appreciably inhibited [^{3}H]-estradiol binding to the estrogen receptor comparing to genistein and daidzein. The apparent IC_{50} for **19** was 375 nM, and 865 nM was observed for **5**, while estradiol itself, genistein and daidzein exhibited IC_{50} of 0.9, 225 and 2600 nM, respectively. Compound **5** and **17** stimulated the cell proliferation in a concentration-dependent manner between 10^{-8} and 10^{-5} M.

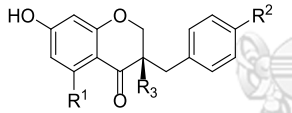


5: $R^1 = OCH_3$, $R^2 = OCH_3$, $R^3 = OCH_3$
7: $R^1 = OH$, $R^2 = OH$, $R^3 = OCH_3$
16: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = OCH_3$
17: $R^1 = OCH_3$, $R^2 = OCH_3$, $R^3 = OH$

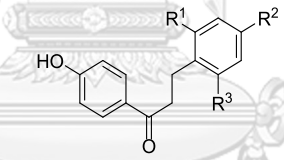


18: $R^1 = OH$, $R^2 = OCH_3$, $R^3 = OH$
19: $R^1 = OH$, $R^2 = OH$, $R^3 = OH$
20: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = H$

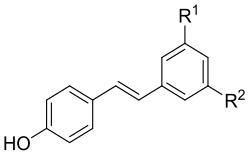
In 2002, Likhitwitayawuid and co-workers [34] reported the isolation of ten compounds including three homoisoflavanones (**15**, **21-22**), four dihydrochalcones (**5**, **16**, **23-24**) and three stilbenes (**25-27**) from *D. loureiri*. Among the isolated compounds, stilbenes inhibited the activity of COX-1 and COX-2 enzymes with IC_{50} of 1.29 – 4.92 μM and more active than a standard aspirin (IC_{50} = 11.41 – 19.80 μM).



15: $R^1 = OH$, $R^2 = OCH_3$, $R^3 = OH$
21: $R^1 = OH$, $R^2 = OH$, $R^3 = OH$
22: $R^1 = OH$, $R^2 = OH$, $R^3 = H$



5: $R^1 = OCH_3$, $R^2 = OCH_3$, $R^3 = OCH_3$
16: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = OCH_3$
23: $R^1 = OH$, $R^2 = OCH_3$, $R^3 = OCH_3$
24: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = OH$



25: $R^1 = OH$, $R^2 = OH$
26: $R^1 = OH$, $R^2 = OCH_3$
27: $R^1 = OCH_3$, $R^2 = OCH_3$

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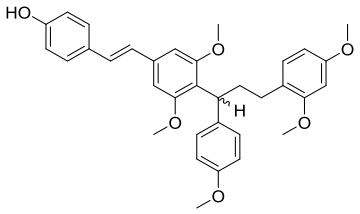
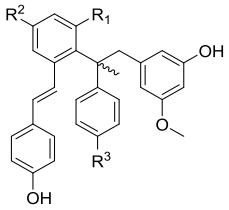
Zhu and co-workers [32] in 2007 discovered eight new flavonoid derivatives (**28-35**) and fourteen known compounds. The effect of all compounds on antibacterial activity against *Helicobacter pylori* ATCC 43504, a spiral Gram negative bacteria strongly associated with human gastritis, peptic ulcer and gastric cancer was investigated. The thrombin inhibitory effect of those compounds was also evaluated. New flavonoids (**6-7**) and **11** showed the most efficacious against *H. pylori* with MIC of 29.5, 29.5 and 31.3

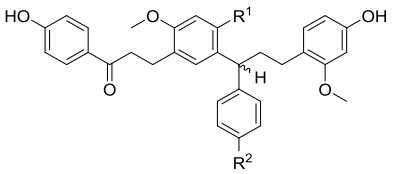
μM, respectively. Ten compounds including seven new flavonoids (**28-34**) and a known biflavonoid (**9**) exhibited moderate thrombin inhibitory activity.



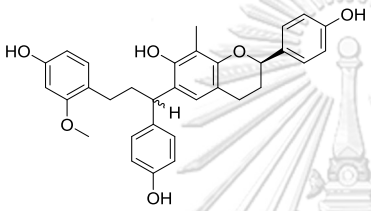
28



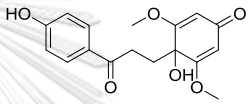
29: R^1 = H, R^2 = OCH_3, R^3 = OCH_3
30: R^1 = OCH_3, R^2 = OH, R^3 = OCH_3
31: R^1 = OH, R^2 = OH, R^3 = OH



29: R^1 = OCH_3, R^2 = H
30: R^1 = H, R^2 = H



34



35

In 2014, Su and co-workers [28] isolated two new dihydrochalcones (**36-37**), a new homoisoflavane (**41**) and twelve known compounds from the red resin of *D. cochinchinensis*. All compounds were evaluated for their inhibitory effects on NO production in lipopolysaccharide-induced RAW 264.7 macrophages. Compound **7** exhibited mild inhibition of NO production with IC_{50} of 50.3 μM.

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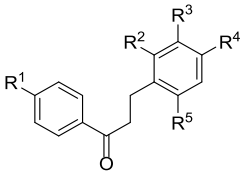
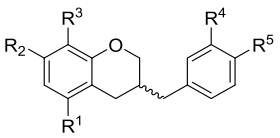
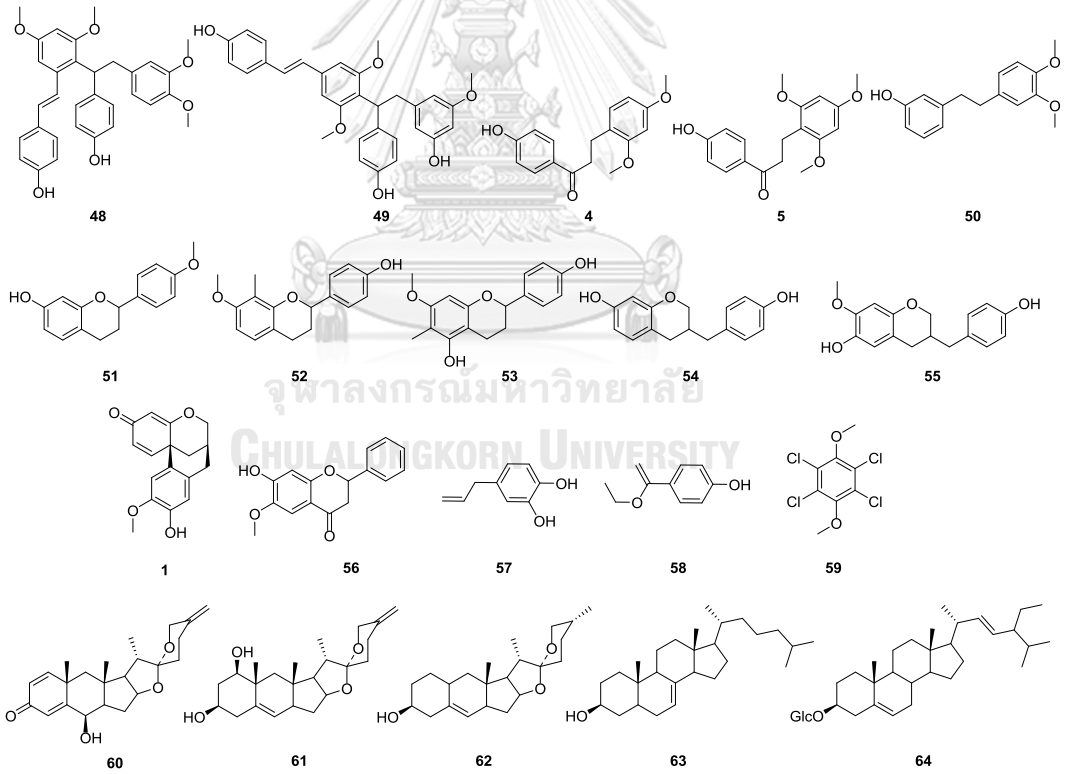


5: R^1 = OH, R^2 = OCH_3, R^3 = H, R^4 = OCH_3, R^5 = OCH_3
7: R^1 = OH, R^2 = OH, R^3 = H, R^4 = OH, R^5 = OCH_3
23: R^1 = OH, R^2 = OH, R^3 = H, R^4 = OCH_3, R^5 = OCH_3
36: R^1 = OCH_3, R^2 = OCH_3, R^3 = H, R^4 = OH, R^5 = OCH_3
37: R^1 = OH, R^2 = OCH_3, R^3 = OH, R^4 = OCH_3, R^5 = H
38: R^1 = OH, R^2 = OCH_3, R^3 = H, R^4 = OCH_3, R^5 = H
39: R^1 = OH, R^2 = OCH_3, R^3 = H, R^4 = OH, R^5 = H
40: R^1 = OH, R^2 = OH, R^3 = H, R^4 = OH, R^5 = OH



41: R^1 = H, R^2 = OH, R^3 =OCH_3, R^4 = OH, R^5 = OCH_3
42: R^1 = H, R^2 = OCH_3, R^3 = OCH_3, R^4 = H, R^5 = OH
43: R^1 = H, R^2 = -OCH_2-O, R^3 = -OCH_2-O, R^4 = H, R^5 = OH
44: R^1 = H, R^2 = OH, R^3 = OCH_3, R^4 = H, R^5 = OH
45: R^1 = OCH_3, R^2 = OCH_3, R^3 = H, R^4 = H, R^5 = OH
46: R^1 = H, R^2 = OH, R^3 = H, R^4 = H, R^5 = OH
47: R^1 = OCH_3, R^2 = OH, R^3 = H, R^4 = H, R^5 = OH

Li and co-workers [35] in 2014 addressed the isolation of natural potential therapeutic agents of neurodegenesis diseases from *D. cochinchinensis*. Twenty effective constituents, including two new polymers, cochinchinenene E (**48**) and cochinchinensis F (**49**), and a new steroid dracenol C (**60**). Compounds **4-5**, **50**, **53-55** were responsible for anti-inflammatory activities while **1**, **5**, **48**, **50** and showed good NQO1 inducing activities. Compounds **4** and **50** were potential candidate therapeutic agents with low toxicity and leading compounds for medicine design for neurodegenerative diseases.



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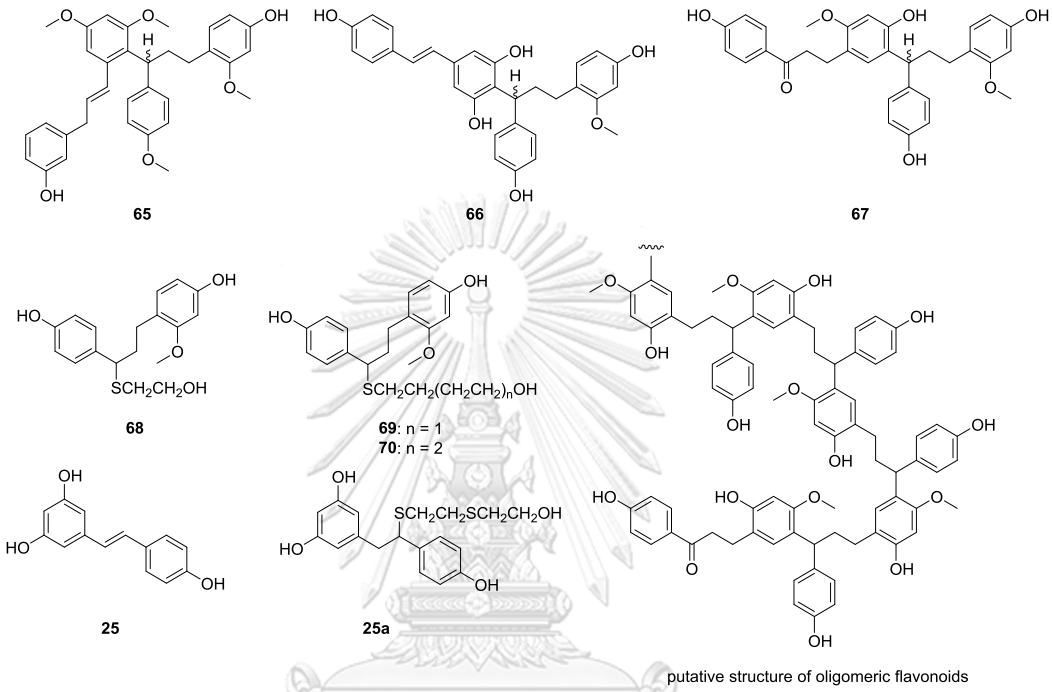
In 2015, Hao and co-workers [30] isolated two chalcone-stilbene conjugates namely chochinchinenenes G (**65**) and H (**66**) together with twenty-five known compounds from the resins of *D. chochinchinensis*.



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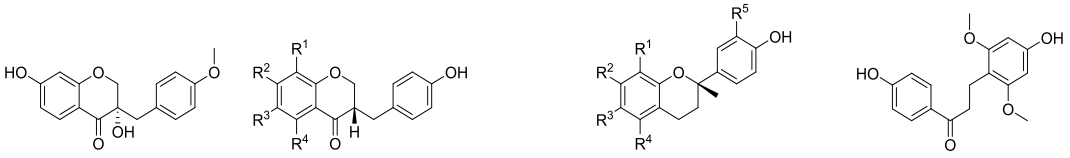
Two new homoisoflavonoids (**71-72**) and ten known compounds were isolated from the red resin of *D. cochinchinensis* and evaluated their effects on mouse bone marrow-derived mesenchymal stem cells (MSCs) proliferation using CCK8 assay by Xu and co-workers in 2016 [33]. Their abilities in promoting MSCs differentiating into osteoblast through the assay of alkaline phosphatase (ALP) activity *in vitro* were conducted. With 10 μM, **72–74**, **77**, **79** and **81** without cytotoxicity significantly promoted MSC estrogenic differentiation by increasing the levels of ALP activity to percent of **151**, **160**, **168** and **170-171** in relative to control, respectively.



71

72: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = H$, $R^4 = OCH_3$
73: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = H$, $R^4 = H$
74: $R^1 = H$, $R^2 = OH$, $R^3 = H$, $R^4 = H$
75: $R^1 = H$, $R^2 = OH$, $R^3 = H$, $R^4 = OCH_3$
76: $R^1 = OCH_3$, $R^2 = H$, $R^3 = OH$, $R^4 = H$

77: $R^1 = CH_3$, $R^2 = OH$, $R^3 = H$, $R^4 = H$, $R^5 = H$
78: $R^1 = CH_3$, $R^2 = OH$, $R^3 = H$, $R^4 = OCH_3$, $R^5 = H$
79: $R^1 = CH_3$, $R^2 = OCH_3$, $R^3 = H$, $R^4 = OH$, $R^5 = H$
80: $R^1 = H$, $R^2 = OH$, $R^3 = H$, $R^4 = H$, $R^5 = OCH_3$
81: $R^1 = H$, $R^2 = OH$, $R^3 = H$, $R^4 = H$, $R^5 = H$

16

2.2 Experimental

2.2.1 Plant Materials

The dried stems of *D. cochinchinensis* were purchased from a medicinal herb shop in Bangkok, Thailand in February 2013.

2.2.2 Instruments and Equipment

Analytical thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel Kieselgel 60 F_{254} (Merck, Germany) for qualitative analysis purpose, spots on the plate were observed under UV light and visualized by dipping in vanillin staining as detecting agent followed by heating and dried to detect spots of some compounds with no UV absorption. Column chromatography was carried out on silica gel no. 7729, 7734 and 9385 (Merck, Germany), sephadex LH-20 (Merck, Germany) and reverse phase-18 (Merck, Germany). Preparative TLC was used for purification using silica gel no. 7730 (Merck, Germany). The ^1H and ^{13}C NMR spectra were recorded in deuterated chloroform ($CDCl_3$), dimethylsulfoxide-d_6 (DMSO-d_6), methanol-d_4 (CD_3OD)

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or acetone-d_6 on a Bruker Ultrashield 400 plus NMR spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The chemical shifts are assigned by comparison with residue solvent protons. The optical rotation was measured on Perkin-Elmer 341 polarimeter using a cell with 300 µL - 0.5 mL capacity and a 1.5 cm path length.

2.2.3 Media and Chemicals

Nutrient broth and commercial agar media were used for antibacterial activity assay. Chloramphenicol (CHL) ≥ 98% (Aldrich Sigma) was used as positive control and resazurin sodium salt (Aldrich Sigma) was used as oxidation-reduction reaction and bacterial growth indicator. All organic solvents were commercial grade purified prior to use by standard methodology except for those which were reagent grades.

2.2.4 Extraction of Dried Plant Materials

Ten kilograms of the dried-powder of *D. cochinchinensis* stems were extracted by soxhlet with CH_2Cl_2 to obtain the crude CH_2Cl_2 extract as dark-red (471 g, 4.71% yield). The residue was continued extracting with MeOH to yield dark-red of MeOH extract (352 g, 3.52% w/w).

2.2.5 Isolation and Purification of Compounds from *D. cochinchinensis*

Two hundred grams of the CH_2Cl_2 extract was further separated by quick column using silica gel no. 7729 to yield six fractions (**CD1-CD6**). Fractions **CD5** and **CD6** showed very good antibacterial activity. Recrystallization of an active fraction **CD5**

(12 g) from hexane-$CHCl_3$ gave pure **82** (160 mg) and a mother liquor which was further separated on silica gel column eluting with CH_2Cl_2-EtOAc (4.5:0.5) to yield 7 sub-fractions. **CD5-3** (2 g) was purified by sephadex LH-20 using MeOH to obtain **27** (100 mg). Fraction **CD5-4** was purified by preparative TLC using CH_2Cl_2-EtOAc to yield **83** (1.2 mg). **CD6** (16 g) was subjected to silica gel column and eluted with hexane (100%), hexane-EtOAc (9:1 to 1:9), EtOAc (100%), EtOAc -MeOH (9:1) to obtain 7 sub-fractions. **CD6-5** (23 g) was subjected to silica gel column eluting with hexane-EtOAc (3:2) to give 8 sub-fractions. **CD6-5.4** (12 g) was applied on sephadex LH-20 column to yield **15** (765 mg). The separation of **CD6-5.5.5** by sephadex LH-20 column furnishing 6 sub-fractions. Compound **84** (50 mg) was derived from **CD6-5.5.5.3** by sephadex LH-20 column (MeOH). **CD6-5.5.5.4** was purified by RP-18 column with MeOH-H_2O (2:3) to give **23** (17.7 mg). Purification of **5** (200 mg) from **CD6-5.5.5.5** on silica gel column with hexane-CH_2Cl_2 (2:8) and 5 drops of acetic acid. The structural identification of the isolated compounds were conducted by comparing spectroscopic data and optical rotation with previous studies.

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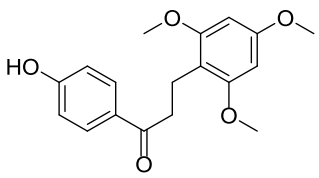
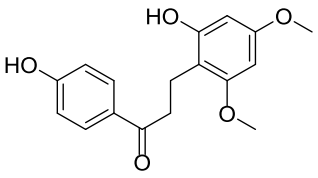


5

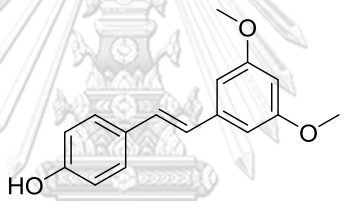


23

Loureirin B (**5**): 200 mg, $C_{18}H_{20}O_5$, colourless crystal from hexane and $CHCl_3$. ^1H NMR (400 MHz, $CDCl_3$): **δ** (ppm) 2.98 (m, 2H-**β**), 3.05 (m, 2H-**α**), 3.76 (s, 6H), 3.80 (s, 3H), 6.12

(s, 2H), 6.38 (br s, 1H), 6.88 (d, *J* = 8.8 Hz, 2H), 7.93 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): **δ** (ppm) 18.8, 38.7, 55.5, 55.8, 90.8, 110.3, 115.4, 130.3, 131.0, 158.9, 159.8, 160.2, 200.2.

4´,2-Dihydroxy-4,6-dimethoxydihydrochalcone (**23**): 17.7 mg. white amorphous powder. ^{1}H NMR (400 MHz, DMSO-d_6): **δ** 2.62 (dd, *J* = 5.6, 2.8 Hz, 2H-**β**), 2.79 (dd, *J* = 10.0, 6.8 Hz, 2H-**α**), 3.55 (s, 3H), 3.58 (s, 3H), 5.93 (dd, *J* = 6.0, 2.4 Hz, 2H), 6.71 (d, *J* = 8.4 Hz, 2H), 7.72 (d, *J* = 8.8 Hz, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): **δ** (ppm) 18.5, 37.9, 54.9, 55.5, 89.8, 93.7, 107.5, 115.2, 128.2, 130.5, 156.3, 158.8, 158.9, 161.9, 198.0.



27

Pterostilbene (**27**): 100 mg, $C_{16}H_{16}O_3$, brown semi-solid. ^{1}H NMR (400 MHz, $CDCl_3$): **δ** (ppm) 3.83 (s, 6H), 6.38 (t, *J* = 2.00 Hz, 1H), 6.65 (d, *J* = 2.0 Hz, 2H), 6.83 (d, *J* = 8.4 Hz, 2H), 6.89 (d, *J* = 16.4 Hz, 1H-**α**), 7.02 (d, *J* = 16.0 Hz, 1H-**β**), 7.39 (d, *J* = 8.4 HZ, 2H). ^{13}C NMR (400 MHz, $CDCl_3$): **δ** 55.2, 99.8, 104.6, 115.8, 126.7, 128.1, 128.9, 139.9, 155.7, 161.1.

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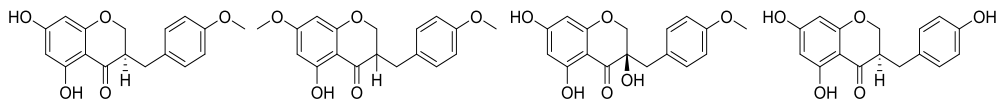

82 **83** **15** **84**

(3*R*)-5,7-Dihydroxy-3-(4-methoxybenzyl) chroman-4-one (**82**): 160 mg, $C_{17}H_{16}O_5$, pale yellow needles, $[\alpha]_D^{21}$-2.16 (c 1, MeOH). ^{1}H NMR (acetone-d_6): **δ** (ppm) 2.70 (dd, *J* =

14.0, 10.0 Hz, 1H), 2.94 (m, 1H), 3.16 (dd, *J* = 14.0, 4.8 Hz, 1H), 3.77 (s, 3H), 4.13 (dd, *J* = 11.6, 8.4 Hz, 1H), 4.31 (dd, *J* = 11.6, 4.8 Hz, 1H), 5.94 (dd, *J* = 14.0, 2.0 Hz, 2H), 6.88 (d, *J* = 8.4, 2H), 7.19 (d, *J* = 8.4, 2H), 9.62 (br s, 1H), 12.22 (s, 1H). ^{13}C NMR (400 MHz, acetone-d_6): δ (ppm) 32.2, 47.2, 55.5, 70.0, 95.6, 96.9, 102.7, 114.8, 130.9, 131.0, 159.4, 164.2, 165.6, 167.3, 198.8.

7-*O*-Methyl-eucomol (**83**): 1.2 mg, $C_{18}H_{18}O_6$, red solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.99 (dd, *J* = 10, 2 Hz, 1H), 3.05 (dd, *J* = 8, 1.2 Hz, 1H), 3.75 (s, 3H), 3.79 (s, 3H), 4.04 (d, *J* = 10.8, 1H), 4.18 (d, *J* = 10.8 Hz, 1H), 6.02 (d, *J* = 2.0 Hz, 1H), 6.07 (d, *J* = 2.0 Hz, 1H), 6.12 (br s, 1H), 6.84 (dd, *J* = 6.4, 2.0 Hz, 2H), 7.12 (dd, *J* = 6.8, 2.0 Hz, 2H), 11.32 (s, 1H). ^{13}C NMR (400 MHz, $CDCl_3$): δ (ppm) 29.8, 55.4, 55.7, 71.8, 72.4, 96.0, 97.3, 100.4, 113.9, 131.2, 131.7, 158.9, 163.1, 164.2, 166.5, 198.1.

(3*R*)-Eucomol (**15**): 765 mg, $C_{17}H_{16}O_6$, red needles, $[\alpha]_D^{21}$-52.62 (c 1.0, MeOH). ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 2.86 (s, 2H), 3.72 (s, 3H), 3.95 (d, *J* = 24.0 Hz, 2H), 5.91 (dd, *J* = 8.8, 2.4 Hz, 2H), 5.97 (br s, 1H), 6.84 (d, *J* = 8.4 Hz, 2H), 7.15 (d, *J* = 8.4 Hz, 2H), 10.86 (br s, 1H), 11.95 (s, 1H). ^{13}C NMR (400 MHz, DMSO-d_6): δ (ppm) 38.5, 54.9, 71.5, 71.7, 94.9, 96.1, 99.9, 113.3, 126.9, 131.5, 158.1, 162.5, 163.9, 166.8, 198.2.

(3*R*)-5,7-Dihydroxy-3-(4-hydroxybenzyl) chroman-4-one (**84**): 50 mg, $C_{16}H_{14}O_5$, pale yellow amorphous powder. $[\alpha]_D^{21}$-1.417 (c 1.02, MeOH). ^{1}H NMR (acetone-d_6): δ (ppm) 2.68 (dd, *J* = 14.0, 10.0 Hz, 1H), 2.88 (s, 1H), 3.13 (dd, *J* = 14.0, 4.8 Hz, 1H), 4.14 (dd, *J* = 11.6, 8.4 Hz, 1H), 4.32 (dd, *J* = 11.6, 4.4 Hz, 1H), 5.93 (dd, *J* = 12.4, 2.0 Hz, 2H), 6.80 (d, *J* = 8.4 Hz, 2H), 7.11 (d, *J* = 8.4 Hz, 2H), 12.22 (s, 1H). ^{13}C NMR (400 MHz, acetone-

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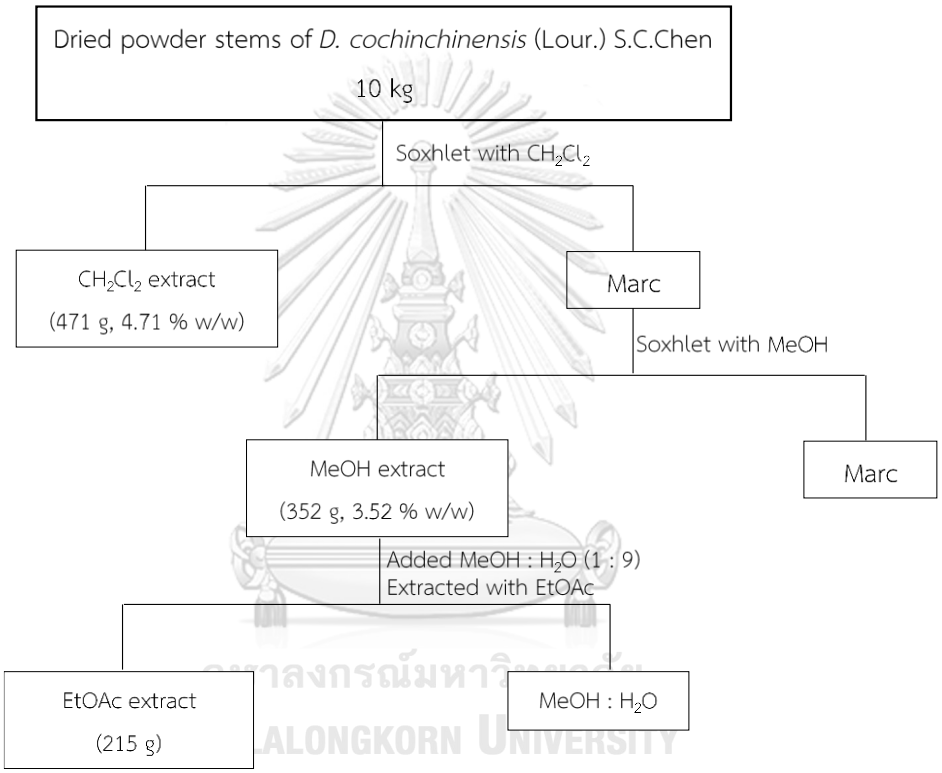
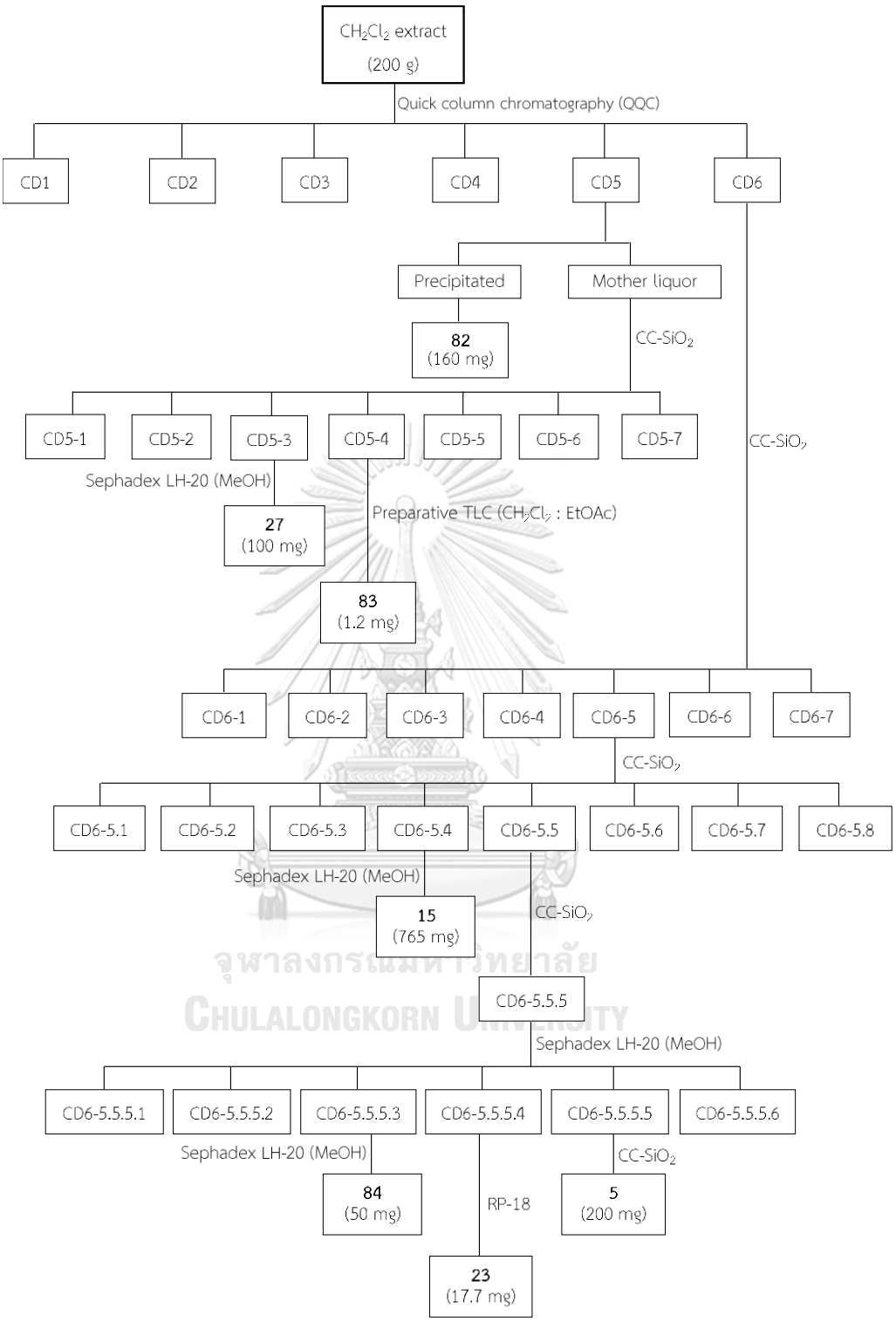
d_6): δ (ppm) 32.4, 47.5, 70.1, 95.7, 97.0, 116.3, 129.9, 131.1, 157.1, 164.4, 165.7, 167.4, 199.0.

The extraction and purification of isolated compounds from the CH_2Cl_2 extract of *D. cochinchinensis* stems are briefly summarized in **Schemes 2.2–2.3**.



Scheme 2.2 Extraction procedure of *D. cochinchinensis* stems



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Scheme 2.3 Isolation procedure of the CH_2Cl_2 extract of *D. cochinchinensis*

2.2.6 The Investigation of Antibacterial Activity

2.2.6.1 Bacterial Strains

The pathogenic bacteria used were four Gram-positive bacteria including *Propionibacterium acnes* KCCM 41747 (Faculty of Forestry, Mulawarman University, East Kalimantan, Indonesia), *Staphylococcus aureus* ATCC 25923 (Laboratory of Microbiology, Faculty of Science, Chulalongkorn University), *Streptococcus sobrinus* KCCM 11898 (Faculty of Forestry, Mulawarman University), *Streptococcus mutans* ATCC 25175 (Department of Microbiology, Faculty of Dentistry, Chulalongkorn University) and *Salmonella typhi* ATCC 422 (Sawanpracharuk Hospital) as representative for Gram-negative bacteria. The tested bacteria were periodically sub-cultured and maintained in nutrient agar under suitable conditions depending upon the tested bacteria.

2.2.6.2 Agar Well Diffusion Method

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The preliminary screening antibacterial activity of crude extracts and compounds were investigated against five bacteria pathogens using modified agar well diffusion method [36]. Nutrient broth was inoculated with the tested microorganisms and incubated at 37^{o}C for 24 h. The inoculum bacteria were adjusted with the sterile NB solution to obtain turbidity by comparison with 0.5 McFarland turbidity standard (1.5X10^{6} CFU/mL). The nutrient agar (NA) was poured into the plate and let it harden. The required number of wells was cut using sterile cork borer ensuring proper

distribution of wells. The suspension of tested bacteria was uniformly spread using sterile cotton swab on a sterile petri dish NA. Crude extracts and compounds were prepared at the concentration of 1 mg/mL and 1 mM, respectively, then put it into the well. The suitable solvent to dissolve samples was used as a negative control and chloramphenicol (CHL) was performed as a positive control. These plates were incubated at 37°C for 24 h. The results were conducted by the presence of inhibition zones compared to the control and expressed as average value. All experiments were performed in triplicate.

2.2.6.3 The Broth Micro-dilution Method for Determination of MIC, MBC and MIC Index

Determination of MIC was conducted by the broth micro-dilution method, according to the Clinical and Laboratory Standards Institute (CLSI, 2012) protocol [37], and modified by using resazurin colorimetric assay on 96-well micro plate [38, 39]. The MIC value recorded is defined as the lowest concentration of the assayed antimicrobial agent that inhibits the visible growth of tested microorganisms. Firstly, the bacterial culture was inoculated in the nutrient broth and incubated at 37°C for 18-24 h. The bacterial suspensions were adjusted to the 0.5 McFarland standard ($1.5X10^6$ CFU/mL). The final two-fold dilutions of compound and CHL were prepared volumetrically in the broth. Six various concentrations were used for each compound. To all wells were added 50 µL of NB and the serial dilution was performed using a

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multichannel pipette. Tips were discarded after using such that each well had 50 µL of the compounds in serially descending concentrations. After that, each well was added 30 µL NB and bacteria suspension of 10 µL. The plates were prepared in triplicate and incubated at 37°C for 18-24 h. For colorimetric assay, 30 µL of 0.01% resazurin were added into each well of the 96-well micro plates and the plates were incubated for 10 min. Colour change of the dye from blue to pink indicated cell viability. The lowest concentrations which do not change the blue colour of resazurin was considered as MIC. MBC was evaluated by re-inoculating on agar plate with 10 µL each culture medium from the micro plates. The test was performed and concurrently with commercial antibiotic (CHL) as a positive control. The MIC index (MBC/MIC) was calculated to indicate whether those compounds possessed bactericidal or bacteriostatic agents by following this criteria: MBC/MIC ≤ 4 (bactericidal), MBC/MIC **>** 4 (bacteriostatic) [40].

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2.3 Results and Discussion

2.3.1 Extraction of Dried Stems of *D. cochinchinensis*

The dried-powder of *D. cochinchinensis* stems were extracted with CH_2Cl_2 and MeOH, respectively by soxhlet yielding dark-red of CH_2Cl_2 extract (471 g, 4.71% w/w) and dark-red MeOH extract (352 g, 3.52% w/w). Those extracts were preliminary investigated on antibacterial activity.

2.3.2 Isolation and Purification of the Compounds from *D. cochinchinensis*

About 200 g of the CH_2Cl_2 extract was further subjected to silica gel quick column by stepwise elution with hexane (100%) and followed by increasing the polarity with EtOAc and finally with 10% MeOH in EtOAc. Based on TLC profile, the fractions were combined to yield six fractions (**CD1-CD6**). Among the fractions, **CD5** and **CD**6 displayed very good antibacterial activity which were further separated. The precipitate from an active fraction **CD5** was recrystallized with hexane and $CHCl_3$ to give pure **82** (160 mg) while **27** (100 mg) and **83** (1.2 mg) were afforded from a mother liquor of **CD5**. Four compounds including **5** (200 mg), **15** (765 mg), **23** (17.7 mg) and **84** (50 mg) were yielded from **CD6**.

2.3.3 Properties and Structural Elucidation of Isolated Compounds

By comparing spectroscopic data and optical rotation with previous studies, seven compounds isolated from *D. cochinchinensis* were identified as loureirin B (**5**), 4′,2-dihydroxy-4,6-dimethoxydihydrochalcone (**23**), pterostilbene (**27**) [16, 35, 41], (3*R*)-5,7-dihydroxy-3-(4-methoxybenzyl) chroman-4-one (**82**), 7-*O*-methyl-eucomol (**83**), (3*R*)-eucomol (**15**) and (3*R*)-5,7-dihydroxy-3-(4-hydroxybenzyl) chroman-4-one (**84**) [42-44].

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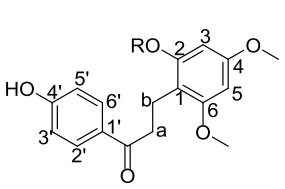
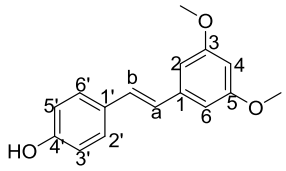
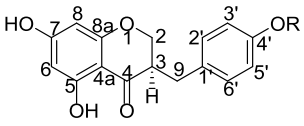


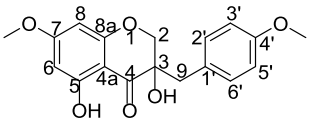
5: R = CH_3
23: R = H



27



82: R = CH_3
84: R = H



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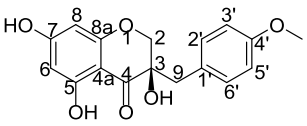

15

2.3.3.1 Dihydrochalcones (5 and 23)

The ^{1}H NMR spectrum of compound **5** (**Figure 2.2**) revealed two doublets of aromatic proton signals in the downfield region at δ_H 7.93 (J = 8.8 Hz) and 6.88 (J = 8.8 Hz) ppm, two singlets at δ_H 6.12 ppm for aromatic proton and the signal for hydroxyl proton at δ_H 6.38 ppm. The presence of two methoxyl singlet signals could be detected at δ_H 3.80 and 3.76 ppm. In addition, four aliphatic signals at δ_H 3.05 and 2.98 ppm were visualized. The ^{13}C NMR spectrum of **5** (**Figure 2.3**) presented 18 carbon resonances comprising a carbonyl group, twelve aromatic carbons, two methylene and three methoxyl carbons. On the basis of above evidence, the dihydrochalcone skeleton was proposed for **5**. The spectroscopic evidence lucidly confirmed that **5** should be 4′-hydroxy-2,4,6-trimethoxydihydroxychalcone or loureirin B. The ^{13}C NMR data of **23** displayed seventeen carbon signals comprising a carbonyl group, two methylene, twelve aromatic and two methoxyl carbons. These indicated that the main skeleton of **23** should belong to dihydrochalcone. The ^{1}H and ^{13}C NMR spectroscopic

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data (**Figures 2.5** and **2.6**) of **23** was quite similar to those of **5** except for the presence of a substituted hydroxyl group at C-2 in **23**. The structure of **23** was identified as 2′,2-dihydroxy-4,6-dimethoxydihydroxychalcone. The comparative ^{1}H and ^{13}C NMR spectral data of **5**, **23** lourerin B and 4′,2-dihydroxyl-4,6-dimethoxydihydrochalcone are presented in **Table 2.1**.

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Table 2.1 The ^{1}H and ^{13}C NMR spectral assignment of **5**, **23**, loureirin B and 4′,2-dihydroxyl-4,6-dimethoxydihydrochalcone [16, 35]

Position	Chemical shift (ppm)							
	5[a]		**loureirin B** [16]		**23**[b]		**4′,2-dihydroxyl-4,6-dimethoxydihydrochalcone** [35]	
	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C	δ_H	δ_C
1	-	110.1	-	108.7	-	107.5	-	109.2
2	-	159.8	-	158.3	-	158.9	-	160.6
3	6.12 (s, 1H)	90.8	6.21 (s, 1H)	90.8	5.93 (dd, *J* = 6.0, 2.4 Hz, 1H)	93.7	6.10 (s, 1H)	95.1
4	-	158.9	-	159.3	-	156.3	-	157.3
5	6.12 (s, 1H)	90.8	6.21 (s, 1H)	90.8	5.93 (dd, *J* = 6.0, 2.4 Hz, 1H)	89.8	6.10 (s, 1H)	91.2
6	-	159.8	-	158.3	-	158.8	-	160.1
1′	-	130.3	-	128.1	-	128.2	-	129.9
2′	7.93 (d, *J* = 8.8 Hz, 1H)	131.0	7.83 (d, *J* = 8.4 Hz, 1H)	130.4	7.72 (d, *J* = 8.8 Hz, 1H)	130.5	7.95 (d, *J* = 9.0 Hz, 1H)	131.6
3′	6.88 (d, *J* = 8.8 Hz, 1H)	115.4	6.83 (d, *J* = 8.4 Hz, 1H)	115.1	6.71 (d, *J* = 8.4 Hz, 1H)	115.2	6.92 (d, *J* = 9.0 Hz, 1H)	116.1
4′	-	160.2	-	161.8	-	161.9	-	161.9
5′	6.88 (d, *J* = 8.8 Hz, 1H)	115.4	6.83 (d, *J* = 8.4 Hz, 1H)	115.1	6.71 (d, *J* = 8.8 Hz, 1H)	115.2	6.92 (d, *J* = 9.0 Hz, 1H)	116.1
6′	7.93 (d, *J* = 8.8 Hz, 1H)	131.0	7.83 (d, *J* = 8.4 Hz, 1H)	130.4	7.72 (d, *J* = 8.8 Hz, 1H)	130.5	7.95 (d, *J* = 9.0 Hz, 1H)	131.6
4′-OH	6.38 (br s, 1H)	-	-	-	10.26 (br s, 1H)	-	8.75 (br s, 1H)	-
H-α	3.05 (m, 2H)	38.7	2.88 (m, 2H)	37.8	2.79 (dd, *J* = 10.0, 6.8 Hz, 2H)	37.9	3.15 (t, *J* = 7.0 Hz, 2H)	39.0
H-β	2.98 (m, 2H)	18.8	2.77 (m, 2H)	18.3	2.62 (dd, *J* = 5.6, 2.8 Hz, 2H)	18.5	2.90 (t, *J* = 7.0 Hz, 2H)	18.6
2-OH	-	-	-	-	9.29 (br s, 1H)	-	8.75 (br s, 1H)	-
2-OCH_3	3.76 (s, 3H)	55.8	3.73 (s, 3H)	55.6	-	-	-	-
4-OCH_3	3.80 (s, 3H)	55.5	3.75 (s, 3H)	55.1	3.58 (s, 3H)	54.9	3.79 (s, 3H)	55.4
6-OCH_3	3.76 (s, 3H)	55.8	3.73 (s, 3H)	55.6	3.55 (s, 3H)	55.5	3.72 (s, 3H)	55.9
C=O	-	200.2	-	198.0	-	198.4	-	200.4

^{1}H and ^{13}C NMR: [a]$CDCl_3$ (400 MHz); [b]DMSO-d_6 (400 MHz)

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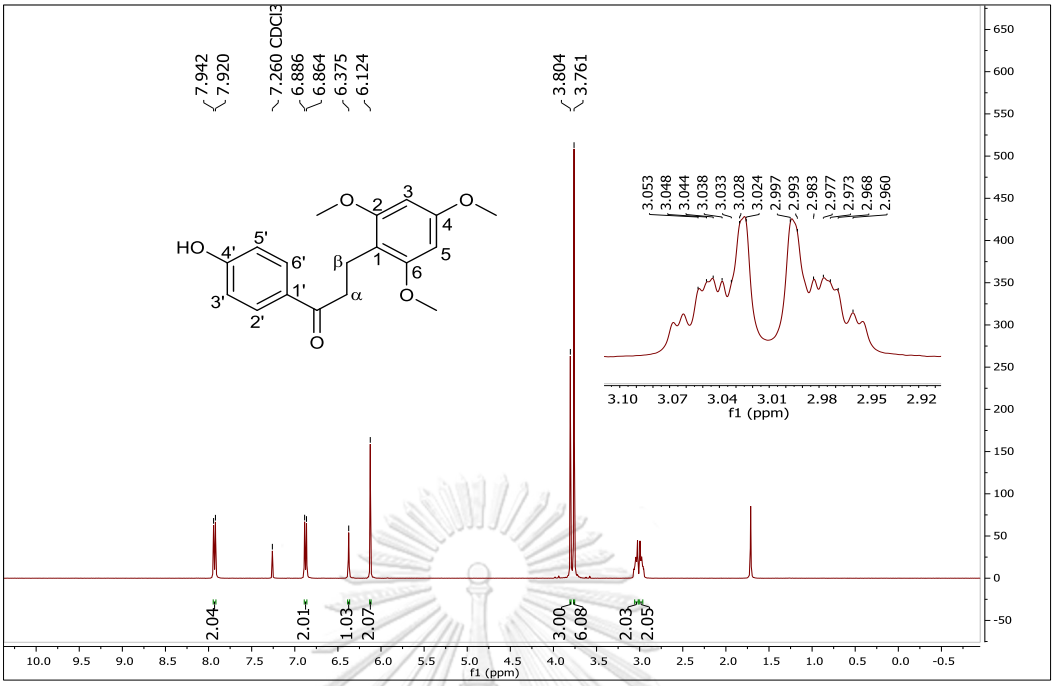

Figure 2.2 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **5**

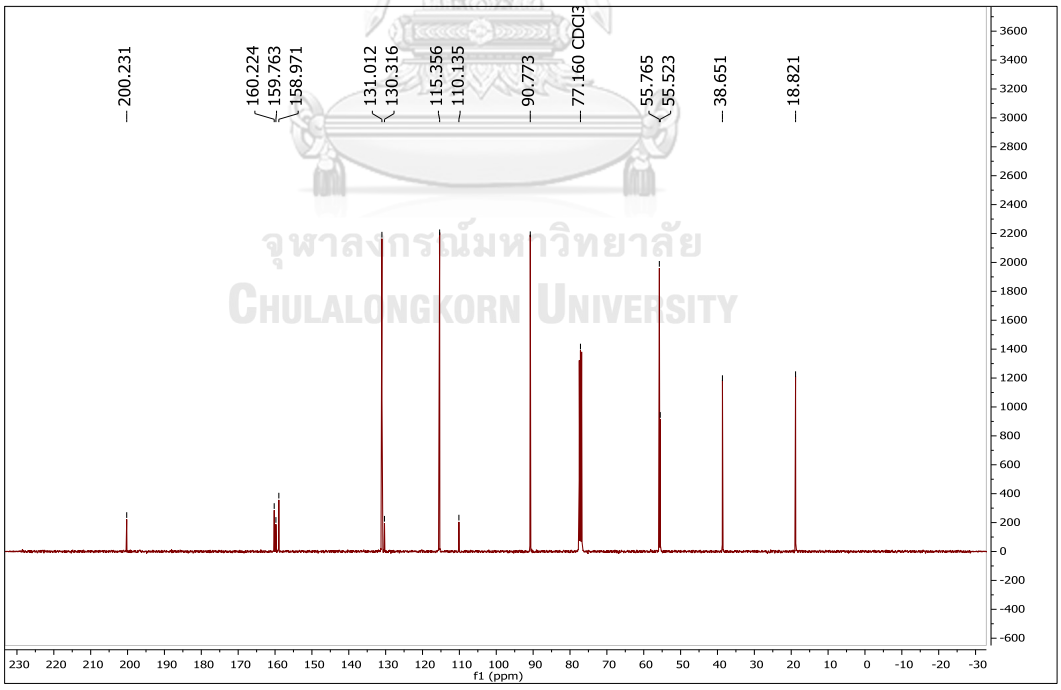

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Figure 2.3 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **5**

Compound **5** was also confirmed its structure by single crystal X-ray diffraction and proposed ORTEP is presented in **Figure 2.4**.

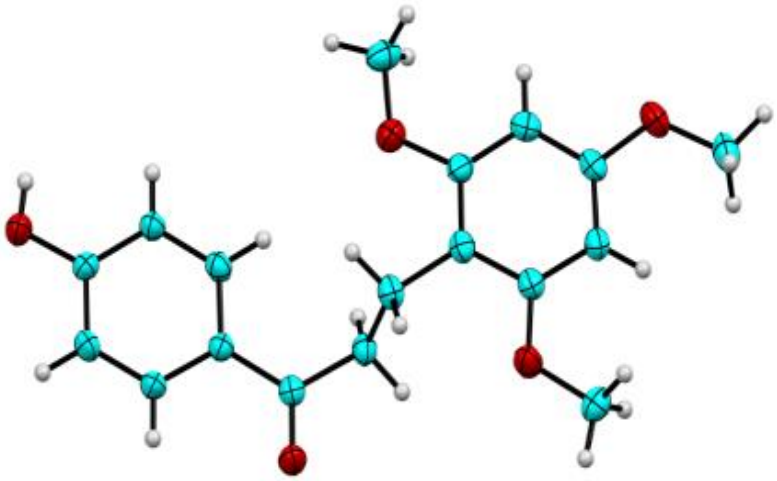

Figure 2.4 The proposed ORTEP of loureirin B (**5**)

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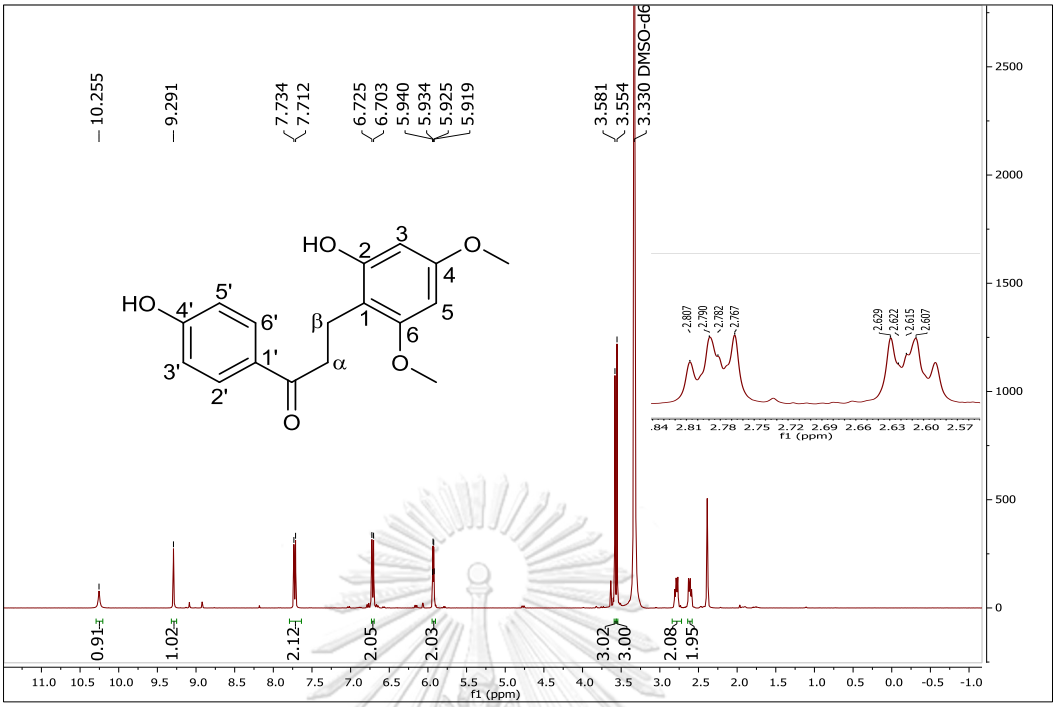

Figure 2.5 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **23**

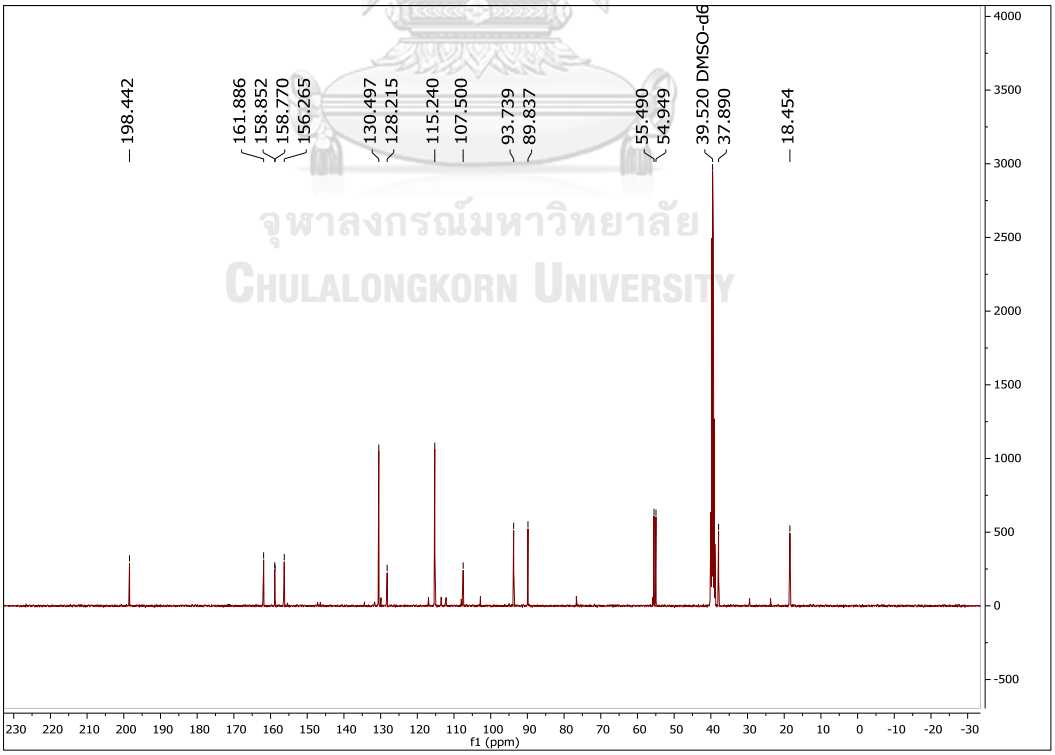

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Figure 2.6 The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of **23**

2.3.3.2 Pterostilbene (27)

Pterostilbene (**27**) was isolated as brown solid. It has a C_6-C_2-C_6 basic skeleton based on the information derived from the ^{1}H NMR spectrum (**Figure 2.7**). Besides two methoxyl signals appeared at **δ**H 3.83 ppm, other two signals of methene were observed at **δ**H 6.89 (H-**α**) and 7.02 ppm (H-**β**) with the coupling constant of 16.4 and 16.0 Hz, respectively. The aromatic signals in the downfield region were visualized: one triplet at **δ**H 6.38 ppm (*J* = 2.0 Hz) and one doublet at **δ**H 6.65 ppm (*J* = 2.0 Hz). Two doublet signals at **δ**H 6.83 (*J* = 8.4 Hz) and 7.39 ppm (*J* = 8.4 Hz) were also detected. The ^{13}C NMR spectrum (**Figure 2.8**) presented two methoxyl carbons at **δ**C 55.4 ppm. The aromatic carbons were inferred from the presence of signals at **δ**C 99.7, 104.4, 115.7, 128.0, 128.8, 139.8, 155.6 and 161.0 ppm. Two carbon signals (C-**β** and C-**α**) were visualized at **δ**C 126.6 ppm. According to the spectral data and information from previous study, it can be identified that **27** was pterostilbene. The ^{1}H and ^{13}C NMR spectral data of **27** are summarized in **Table 2.2**.

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Table 2.2 The ^{1}H and ^{13}C NMR spectral assignment of **27** and pterostilbene [41]

Position	Chemical shift (ppm)			
	27[a]		**pterostilbene** [41]	
	δ_H	δ_C	δ_H	δ_C
1	-	139.9	-	128.7
2	6.65 (d, *J* = 2.0 Hz, 1H)	104.6	6.65 (s, 1H)	104.5
3	-	161.1	-	155.3
4	6.38 (t, *J* = 2.0 Hz, 1H)	99.8	6.38 (s, 1H)	99.6
5	-	161.1	-	155.3
6	6.65 (d, *J* = 2.0 Hz, 1H)	104.6	6.65 (s, 1H)	104.5
1′	-	128.1	-	126.5
2′	7.39 (d, *J* = 8.4 HZ, 1H)	128.9	7.4 (d, *J* = 16.0 Hz, 1H)	128.0
3′	6.83 (d, *J* = 8.4 Hz, 1H)	115.8	6.83 (d *J* = 9.5 Hz, 1H)	115.6
4′	-	155.7	-	160.8
5′	6.83 (d, *J* = 8.4 Hz, 1H)	115.8	6.83 (d *J* = 9.5 Hz, 1H)	115.6
6′	7.39 (d, *J* = 8.4 HZ, 1H)	128.9	7.4 (d, *J* = 16.0 Hz, 1H)	128.0
H-α	6.89 (d, *J* = 16.4 Hz, 1H)	126.7	6.89 (d, *J* = 16.0 Hz, 1H)	130.0
H-β	7.02 (d, *J* = 16.0 Hz, 1H)	126.7	7.02 (d, *J* = 16.0 Hz, 1H)	139.7
4′-OH	-	-	5.04 (s, 1H)	-
3-OCH_3	3.83 (s, 3H)	55.2	3.85 (s, 3H)	55.4
5-OCH_3	3.83 (s, 3H)	55.2	3.85 (s, 3H)	55.4

^{13}C NMR: [a]$CDCl_3$ (400 and 100 MHz)

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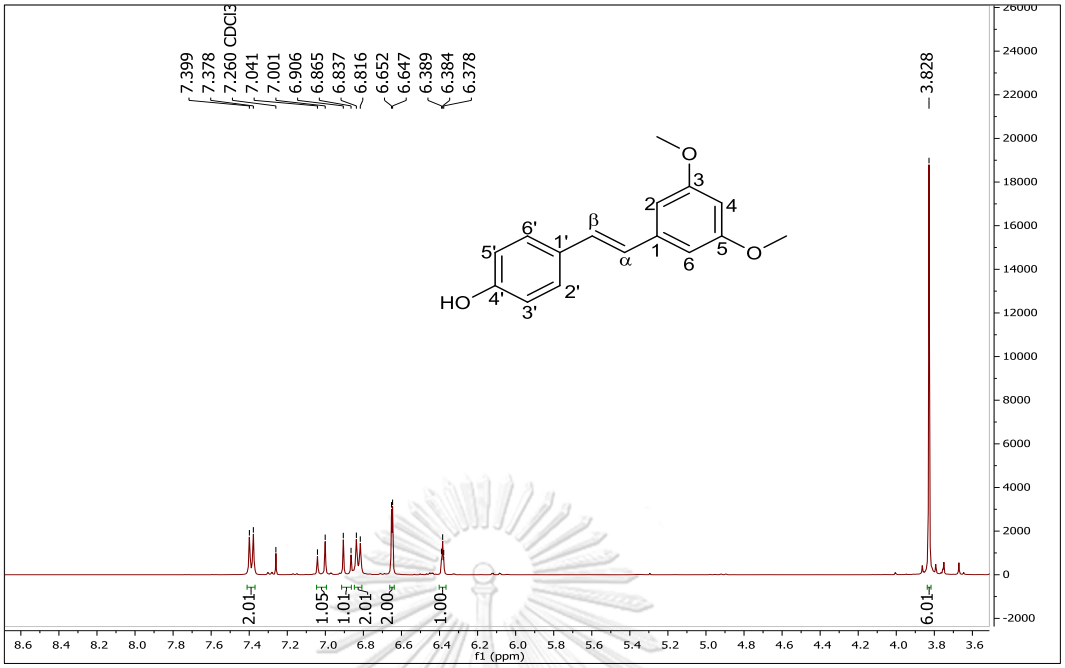

Figure 2.7 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **27**

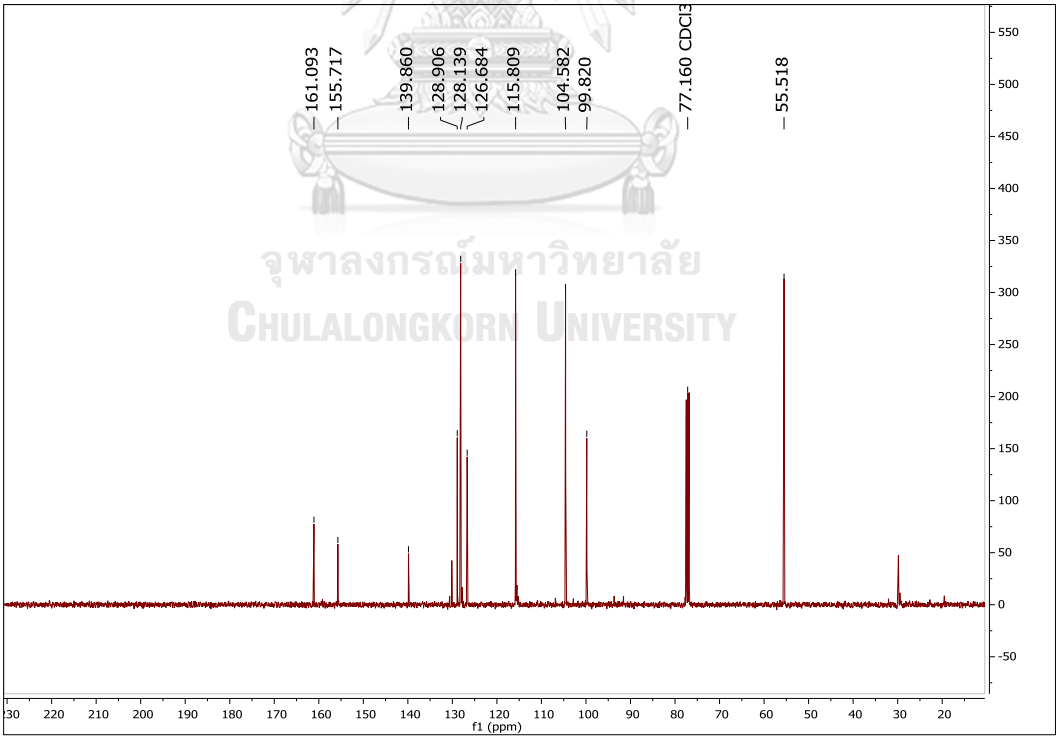

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Figure 2.8 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **27**

2.3.3.4 Homoisoflavanones (15 and 82-84)

The spectroscopic data of **15** and **82–84** (**Tables 2.3** and **2.4**) indicated the presence of sixteen carbon atoms in the basic skeleton with their B and C rings connected with an additional ethylene group. The typical signals of homoisoflavanone of 3-benzylchroman-4-one structural type are two sets of two geminal protons at C-2 and C-9. The ^{1}H NMR spectrum of **82** (**Figure 2.9**) shows the double doublets at δ_H 4.31 (*J* = 11.6 and 4.8 Hz) and 4.13 ppm (*J* = 11.6 and 8.4 Hz) at C-2. The other geminal protons at C-9 were assigned as double doublets at δ_H 3.16 and 2.70 ppm. Two doublet of doublets of aromatic protons were detected at δ_H 5.94 ppm (*J* = 14.0, 2.0 Hz). The signal at δ_H 3.77 ppm with three protons indicated the presence of methoxyl group at C-4′. The carbonyl chemical shift of all four compounds were observed at δ_C 198 – 199 ppm. A quaternary carbon signal (C-3) in the spectra of **82** (**Figure 2.10**) and **84** (**Figure 2.16**) were observed at δ_C 47.2 and 47.5 ppm while those of **83** (**Figure 2.12**) and **15** (**Figure 2.14**) appeared at δ_C 72.4 and 71.2 ppm, respectively. The comparison of the ^{1}H and ^{13}C NMR spectral data of **15** and **82–84** are summarized in **Tables 2.3** and **2.4**. The optical rotations of **15** and **82** were determined and compared with previous studies [29, 42]. The configurations at C-3 position of both **15** and **82** possessed (*R*)-configuration. Moreover, (*R*)-configuration was also shown for **84** after comparing with **82**. However, the configuration of **83** was not determined due to the limited amount of the compound.

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Table 2.3 The ^{1}H NMR spectral assignment of **15**, **82–84**, 7-O-methyl-eucomol and (3*R*)-eucomol [29, 43]

Position	Chemical shift (ppm)					
	82 [a]	**83** [b]	**7-*O*-methyl-eucomol** [43]	**15** [c]	**(3*R*)-eucomol** [29]	**84** [d]
2a	4.31 (dd, *J* = 11.6, 4.8 Hz, 1H)	4.18 (d, *J* = 10.8 Hz, 1H)	4.21 (d, *J* = 11.2 Hz, 1H)	3.95 (d, *J* = 2.4, 2H)	3.99 (d, *J* = 11.3, 2H)	4.32 (dd, *J* = 11.6, 4.4 Hz, 1H)
2b	4.13 (dd, *J* = 11.6, 8.4 Hz, 1H)	4.04 (d, *J* = 10.8 Hz, 1H)	4.06 (d, *J* = 11.2 Hz, 1H)			4.14 (dd, *J* = 11.6, 8.4 Hz, 1H)
3	2.94 (m, 1H)	-	-	-	-	2.88 (s, 1H)
6	5.94 (dd, *J* = 14.0, 2.0 Hz, 2H)	6.07 (d, *J* = 2.0 Hz, 1H)	6.08 (d, *J* = 2.3 Hz, 1H)	5.91 (dd, *J* = 8.8, 2.4 Hz, 2H)	5.90 (d, J = 2.2 Hz, 1H)	5.93 (dd, *J* = 12.4, 2.0 Hz, 2H)
8		6.02 (d, J = 2.0 Hz, 1H)	5.96 (d, J = 2.3 Hz, 1H)		5.93 (d, *J* = 2.2 Hz, 1H)	
9a	3.16 (dd, *J* = 14.0, 4.8 Hz, 1H)	3.05 (dd, *J* = 8.0, 1.2 Hz, 1H)	2.95 (s, 2H)	2.86 (s, 2H)	2.91 (d, *J* = 14.1 Hz, 2H)	3.13 (dd, *J* = 14.0, 4.8 Hz, 1H)
9b	2.70 (dd, *J* = 14.0, 10.0 Hz, 1H)	2.99 (dd, *J* = 10.0, 2.0 Hz, 1H)				2.68 (dd, *J* = 14.0, 10.0 Hz, 1H)
2′, 6′	7.19 (d, *J* = 8.4, 2H)	7.12 (dd, *J* = 6.8, 2.0 Hz, 2H)	7.12 (d, *J* = 8.8, Hz, 2H)	7.15 (d, *J* = 8.4, 2H)	7.14 (d, *J* = 8.6, 2H)	7.11 (d, *J* = 8.4 Hz, 2H)
3′, 5′	6.88 (d, *J* = 8.4, 2H)	6.84 (dd, *J* = 6.4, 2.0 Hz, 2H)	6.85 (d, *J* = 8.8, Hz, 2H)	6.84 (d, *J* = 8.4 Hz, 2H)	6.82 (d, *J* = 8.6 Hz, 2H)	6.80 (d, *J* = 8.4 Hz, 2H)
3-OH	-	6.12 (br s, 1H)	3.34 (s, 1H)	5.97 (br s, 1H)	-	-
5-OH	12.22 (s, 1H)	11.32 (s, 1H)	11.24 (s, 1H)	11.95 (s, 1H)	-	12.22 (s, 1H)
7-OH	9.62 (br s, 1H)	-	-	10.86 (br s, 1H)	-	-
4′-OCH_3	3.77 (s, 3H)	3.79 (s, 3H)	3.80 (s, 3H)	3.72 (s, 3H)	3.75 (s, 3H)	-
7-OCH_3	-	3.75 (s, 3H)	3.89 (s, 3H)	-	-	-

^{1}H NMR: [a]$CDCl_3$ (400 MHz); [b]DMSO-d_6 (400 MHz; [c]DMSO-d_6 (400 MHz); [d]Acetone-d_6 (400 MHz)

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Table 2.4 The ^{13}C NMR spectral assignment of **15**, **82–84** and (3*R*)-eucomol [29]

Position	Chemical shift (ppm)				
	82 [a]	**83** [b]	**15** [c]	**(3*R*)-eucomol** [29]	**84** [d]
2	70.0	71.8	71.5	72.6	70.1
3	47.2	72.4	71.7	73.4	47.5
4	198.8	198.1	198.2	199.6	199.0
4a	102.7	100.4	99.9	101.3	97.0
5	165.6	163.1	163.9	165.5	165.7
6	95.6	96.0	94.9	96.1	95.7
7	167.3	166.5	166.8	168.5	167.4
8	96.9	97.3	96.1	97.4	95.7
8a	164.2	164.2	162.5	164.2	164.4
9	32.2	29.8	38.5	40.6	32.4
1′	131.0	131.2	126.9	127.9	129.9
2′, 6′	130.9	131.7	131.5	132.7	131.1
3′, 5′	114.8	113.9	113.3	114.4	116.3
4′	159.4	158.9	158.1	160	157.1
4′-OCH_3	55.5	55.4	54.9	55.5	-
7-OCH_3	-	55.7	-	-	-

^{13}C NMR: [a]$CDCl_3$ (100 MHz); [b]DMSO-d_6 (100 MHz); [c]DMSO-d_6 (100 MHz); [d]Acetone-d_6 (100 MHz)

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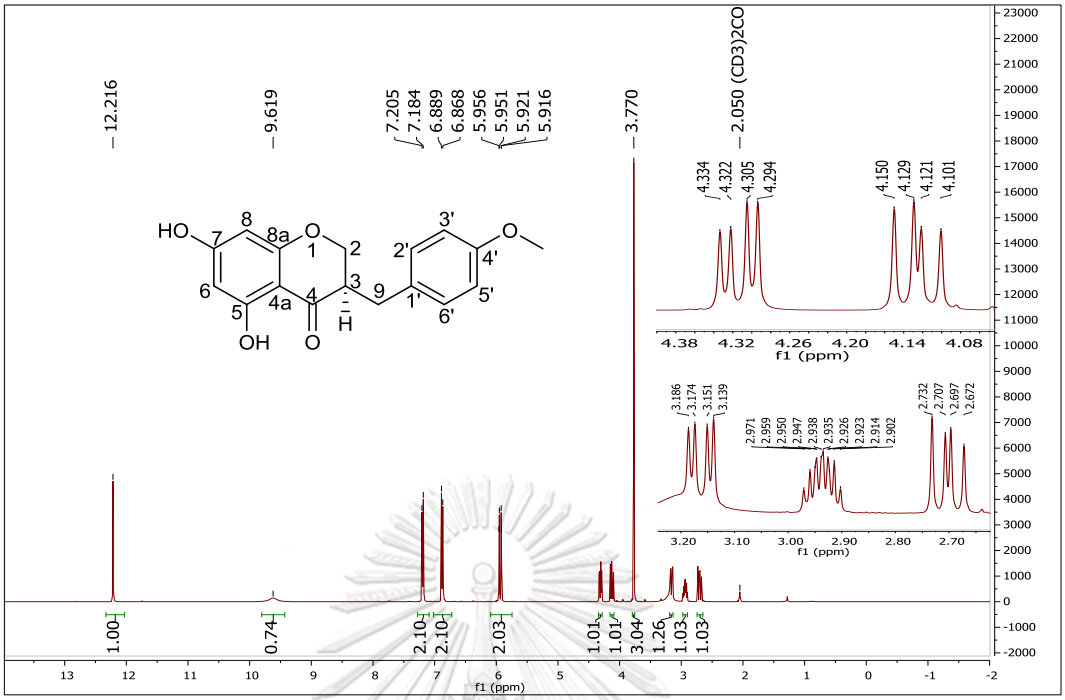

Figure 2.9 The ^{1}H NMR spectrum (acetone-d_6, 400 MHz) of **82**

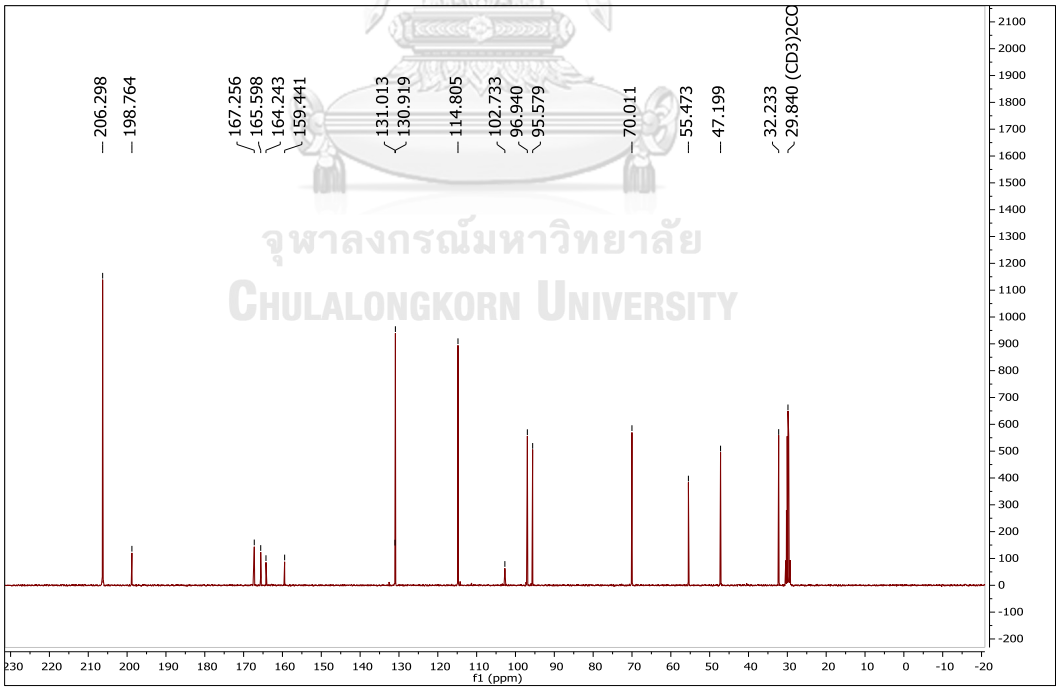

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Figure 2.10 The ^{13}C NMR spectrum (acetone-*d6*, 100 MHz) of **82**

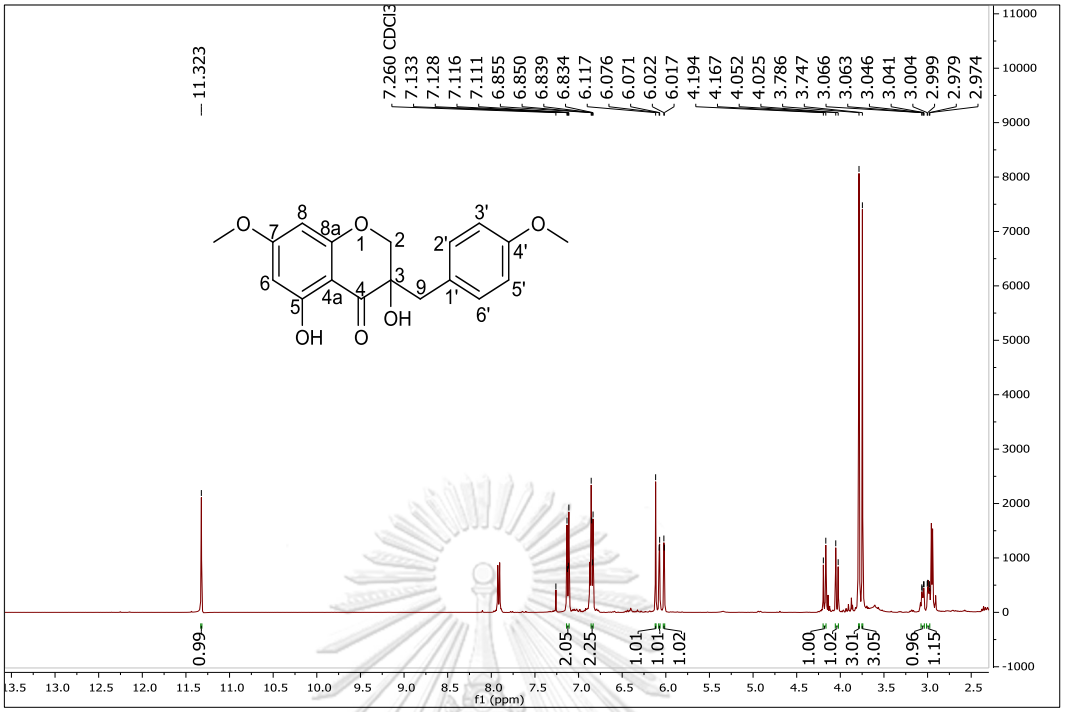

Figure 2.11 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **83**

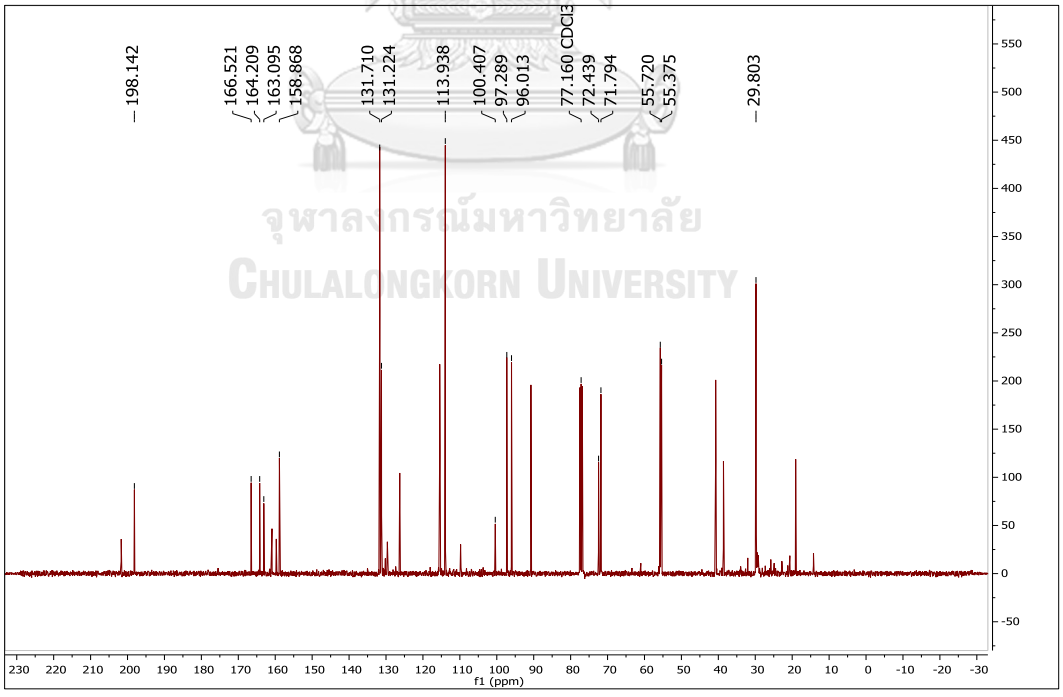

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Figure 2.12 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **83**

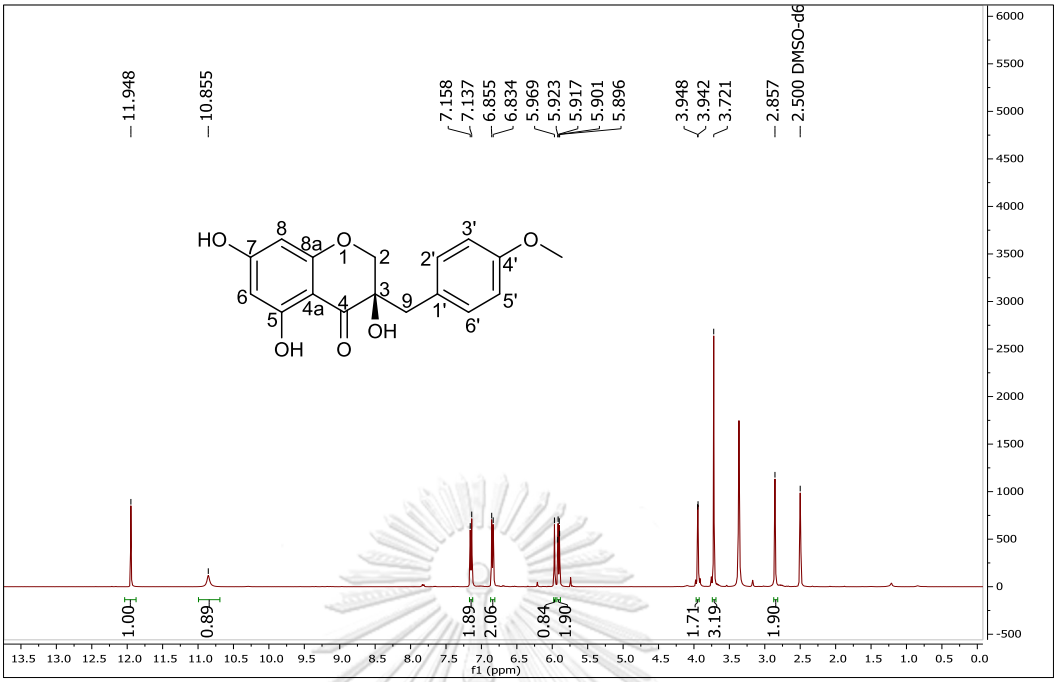

Figure 2.13 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **15**

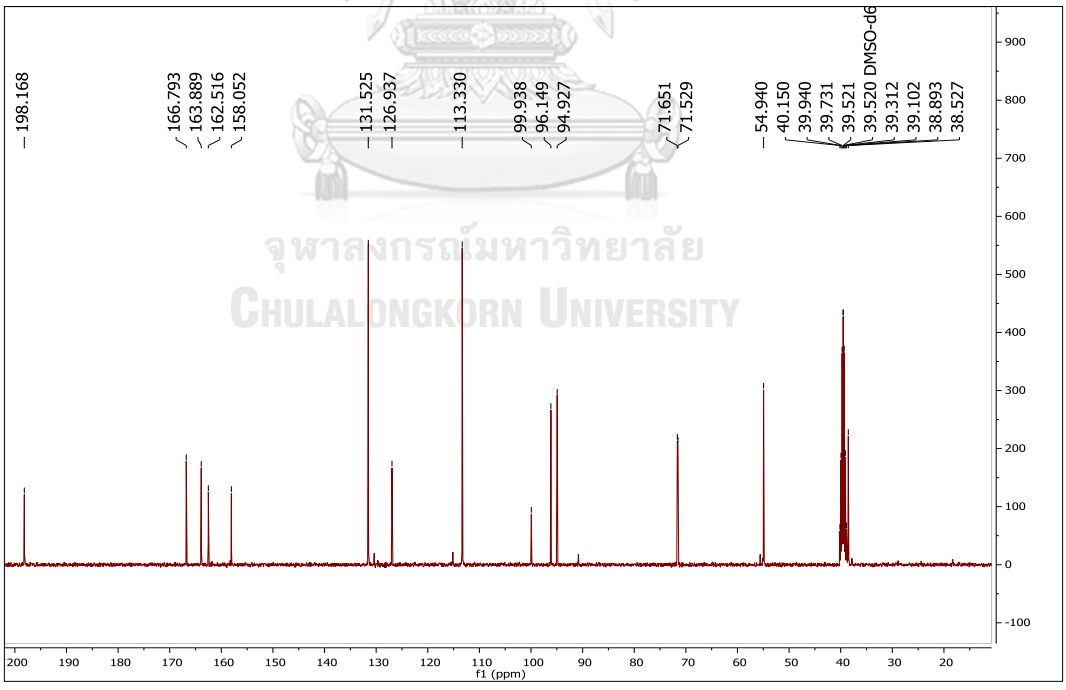

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Figure 2.14 The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of **15**

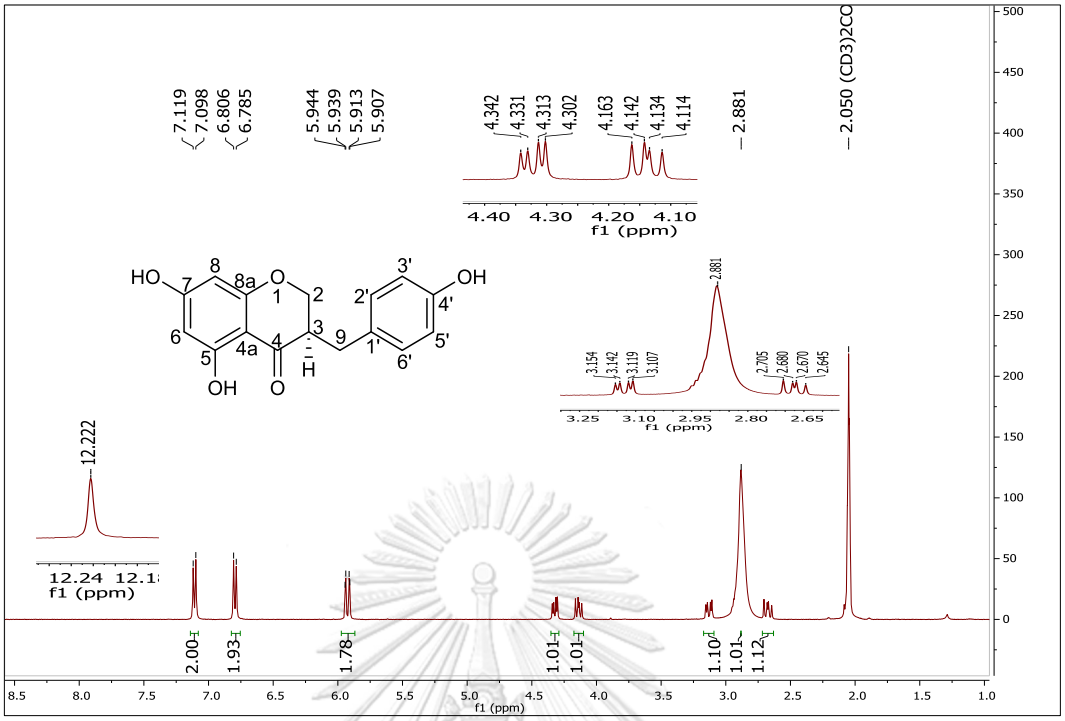

Figure 2.15 The ^{1}H NMR spectrum (acetone-d_6, 400 MHz) of **84**

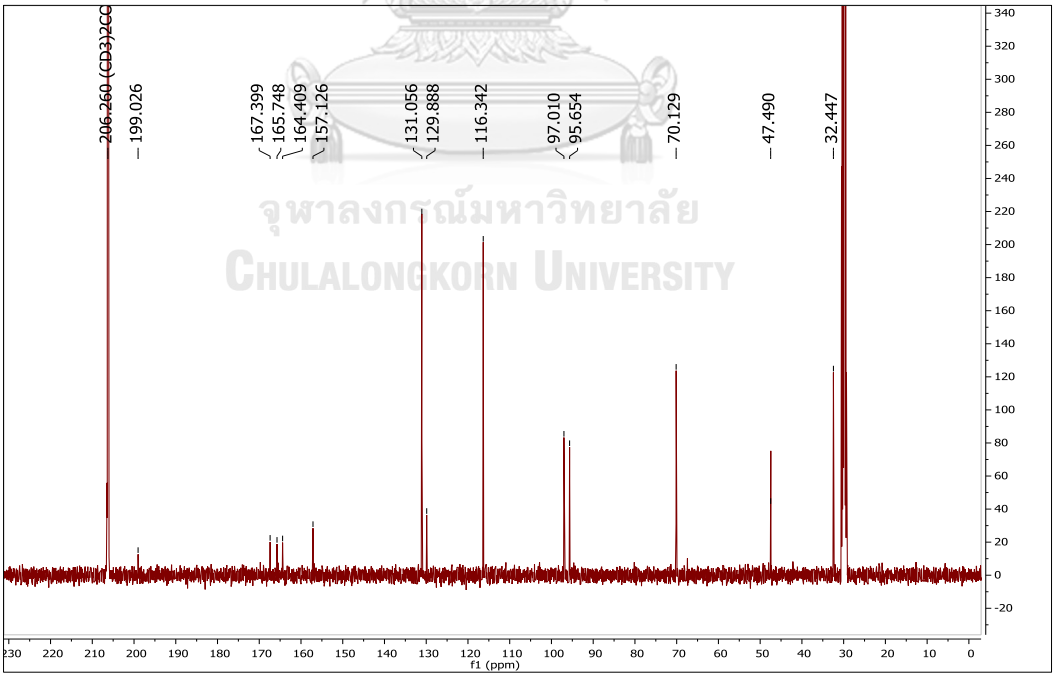

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Figure 2.16 The ^{13}C NMR spectrum (acetone-d_6, 100 MHz) of **84**

2.3.4 Antibacterial Activity

2.3.4.1 Preliminary Screening of the Antibacterial Activity

The crude extracts were screened for antibacterial activity as complement to the bioassay-guided approach. TLC and HPLC were used to guide the isolation and purification of the compounds responsible for the activity. The antibacterial activity test was conducted against bacteria pathogenic: *Propionibacterium acnes* KCCM 41747, *Staphylococcus aureus* ATCC 25923, *Streptococcus sobrinus* KCCM 11898, *Streptococcus mutans* ATCC 25175 (Gram-positive bacteria) and *Salmonella typhi* ATCC 422 (Gram-negative bacteria) by agar well diffusion method. The results are presented in **Table 2.5**.

Table 2.5 Antibacterial activity of crude extracts of *D. cochinchinensis*

Entry	Extracts (1mg/mL)	Inhibition zone average (mm) ± SD				
		P. acnes KCCM 41747	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175	*S. typhi* ATCC 442
1	CH_2Cl_2	19.0±0.4	24.7±0.5	15.7±0.5	18.3±0.9	23.0±1.4
2	MeOH	17.0±1.4	22.3±1.9	14.3±0.5	17.7±0.5	23.0±0.5
3	CHL	25.0±0.0	26.0±0.8	26.0±0.0	29.3±0.5	20.0±0.0

The experiment was repeated for three times and the inhibition zone is shown as average values of three independent determinations; Cholramphenicol (CHL) as positive control at 500 μM; Key to the inhibition zone activity (mm) >15 = Excellent, 13.1 – 15.0 = Very good, 10.1 – 13.0 = Good, 8.1 – 10.0 = Moderate, 6.1 – 8.0 = Weak, 6.0 = No activity

As shown in **Table 2.5**, the CH_2Cl_2 extract displayed excellent activity against all bacteria with diameter of inhibition zone ranging from 15.7–24.7 mm. Excellent antibacterial activity was also observed from the MeOH extract against *P. acnes*, *S. aureus*, *S. mutans* and S*. typhi* (17.0–23.0 mm), while the extract revealed very good activity against *S. sobrinus* with diameter inhibition zone 14.3 mm. CHL as a positive control exhibited excellent activity against all bacteria with the range of inhibition zone at 20.0–29.3 mm. The CH_2Cl_2 extract was selected for further separation.

The CH_2Cl_2 extract was fractionated to silica gel quick column chromatography (QCC) with 100% hexane and followed by increasing polarity solvent system with EtOAc and finally eluted with a mixture of 10% MeOH and EtOAc to obtain 6 subfractions (**A–F**). Fractions E and F showed high antibacterial activity against all tested bacteria with inhibition zone ranging from 12.0 to 15.0 mm (**Table 2.6**). Therefore, these two fractions were further separated and evaluated for their antibacterial activity, MIC, MBC and MIC index.

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Table 2.6 Antibacterial activity of sub-fractions from the CH_2Cl_2 extract of *D. cochinchinensis*

Entry	Fractions (1 mg/mL)	Inhibition zone average (mm) ± SD				
		P. acnes KCCM 41747	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175	*S. typhi* ATCC 442
1	**A**	10.0±0.0	8.0±0.0	9.0±0.0	9.0±0.8	10.0±0.0
2	**B**	9.0±0.0	8.0±0.0	13.0±0.0	12.0±0.0	13.0±0.0
3	**C**	8.0±0.0	7.0±0.0	9.0±0.0	9.0±0.0	9.3±0.5
4	**D**	8.0±0.0	7.0±0.0	8.0±0.0	9.3±0.5	10.3±0.5
5	**E**	13.0±0.0	12.7±0.5	12.0±0.0	12.7±0.5	15.0±0.0
6	**F**	13.7±0.5	13.0±0.0	12.0±0.0	12.0±0.0	14.7±0.5
7	CHL	25.0±0.0	26.0±0.0	26.0±0.0	29.3±0.5	20.0±0.0

The experiment was repeated three times and the inhibition zone is shown as average values of three independent determinations; Chloramphenicol (CHL) as positive control at 500 μM; Key to the inhibition zone activity (mm) >15 = Excellent, 13.1 – 15.0 = Very good, 10.1 – 13.0 = Good, 8.1 – 10.0 = Moderate, 6.1 – 8.0 = Weak, 6.0 = No activity

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Seven isolated compounds (**5**, **15**, **23**, **27** and **82–84** were further tested for their antibacterial activity against five pathogenic bacteria by agar well diffusion method. The results are presented in **Table 2.7**.

Table 2.7 Antibacterial activity of isolated compounds from *D. cochinchinensis*

Entry	Cpds	Inhibition zone average (mm) ± SD				
		P. acnes KCCM 41747	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175	*S. typhi* ATCC 442
1	**5**	12.7±1.1	12.3±0.5	12.0±0.0	10.3±0.5	12.0±0.0
2	**15**	10.7±0.5	11.3±0.5	11.0±0.8	13.3±0.5	10.3±0.5
3	**23**	11.0±0.0	10.0±0.0	12.3±0.5	12.7±0.5	12.7±0.5
4	**27**	12.7±0.5	12.3±0.5	12.7±0.5	9.7±0.5	12.7±0.5
5	**82**	17.3±0.5	16.7±0.5	13.0±0.0	12.0±0.0	13.0±0.0
6	**83**	8.0±0.0	7.7±0.5	9.0±0.0	9.0±0.0	11.3±1.1
7	**84**	11.7±1.1	10.3±0.5	9.0±0.0	8.3±0.5	9.0±0.0

The concentration of compounds used for testing was 1 mM.
The experiment was repeated for three times and the inhibition zone is shown as average values of three independent determinations.
Criteria of inhibition zone activity (mm): inhibition zone >15.0: excellent, 13.1-15.0: very good, 10.1-13.0: good, 8.1-10.0: moderate, 6.1-8.0: weak, ≤6.0: no activity

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As seen in **Table 2.7**, **5** displayed good activity against *P. acnes* and *S. aureus* while 23 showed slightly lower inhibitory effect than 5 against both bacteria. It could be concluded that **5** was more potent than **23** toward both *P. acnes* and *S. aureus* which are one of the factors involving in the pathogenesis of acnes and superficial skin infections [45-47]. However, the antibacterial activity of **5** against *S. sobrinus* and *S. mutans* was less than **23**. Both bacteria are *Streptococcus* bacterial types having a major role in oral diseases possessing cariogenic properties inhabiting the oral cavity

[48, 49]. Furthermore, **5** and **23** revealed good activity against *S. typhi* with inhibition zone of 12.0 and 12.7 mm, respectively. The replacement of hydroxyl group in **5** by methoxyl group at the same position (C-2) in **23** was slightly different in antibacterial activity against *S. typhi* which is known to contribute a typhoid fever. Overall, the results indicated that the antibacterial effect of both **5** and **23** was broad spectrum due to their ability to inhibit the growth of both Gram positive and negative bacteria.

On the other hand, the antibacterial effect of **27** was good to inhibit the growth of all tested Gram-positive and negative bacteria except *S. mutans* with the inhibition zone ranging from 12.3 to 12.7 mm. This indicated that **27** also had a broad spectrum for antibacterial activity. Compound **27** is a structural analogue of resveratrol (**25**) as shown in **Figure 2.17**. These compounds belonging to stilbenoids are known as potent antibacterial agents against Gram positive and negative bacteria [50]. According to previous studies, the beneficial effects of **27** were successfully recorded having several therapeutic agents such as anti-inflammatory [51], anticancer, antioxidant [52] and antimicrobial activity against more than a hundred strains of bacteria [53]. Specifically, it is capable to inhibit the growth of dermatophytes, *P. acnes* and other bacteria pathogens of skin [54, 55]. Based on the structural activity relationship, **27** has well documented its pharmacological effects that more advantage than **25** due to the presence of methoxyl groups [56]. To date, there have been no report on the antibacterial activity of **27** against *S. sobrinus* and *S. mutans*.

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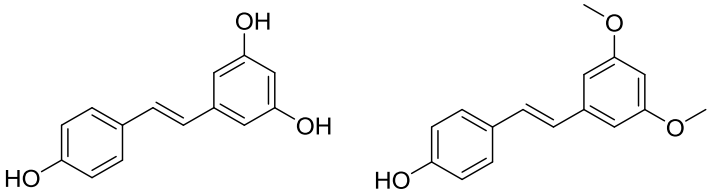

25 **27**

Figure 2.17 The structures of resveratrol (**25**) and pterostilbene (**27**)

In addition, the antibacterial activity of **15** and **82–84** which contained hydroxyl and methoxyl groups was screened. Among the homoisoflavanone series, **82** presented a significant excellent inhibitory effect against *P. acnes* and *S. aureus* with the inhibition zone of 17.3 and 16.7 mm, respectively. Good antibacterial activity against *S. sobrinus*, *S. mutans* and *S. typhi* was also observed for **82** with the diameter of inhibition zones of 13.0, 12.0 and 13.0 mm, respectively. Due to its capability to inhibit the growth of both Gram-positive and negative bacteria, it could be concluded that **82** displayed broad spectrum in antibacterial activity and was a promising candidate for the treatment of staphylococcal skin infection such as wound infection, scabies, dermatitis and cellulitis. Thus lending support for its use would be a promising way to discover anti-methicillin resistant *S. aureus* (anti-MRSA). On the other hand, **82** also had an ability to cure typhoid fever caused by *S. typhi*. Furthermore, the inhibitory effect of **83** showed moderate activity with the diameter inhibition zone of 9.0 mm against *S. sobrinus* and *S. mutans* while weak effect was displayed against *P. acnes* and *S. aureus* with the inhibition zone of 8.0 and 7.0 mm, respectively. The diameter

of inhibition zone of **83** displayed good activity at 11.3 mm against *S. typhi*. Compound **15** revealed very good inhibitory effect (13.3 mm) against *S. mutans* while its ability to inhibit the growth of *P. acnes*, *S. aureus*, *S. sobrinus* and *S. typhi* showed good activity with inhibition zone ranging from 10.3 to 11.3 mm. The antibacterial activity of **84** revealed good effect (10.3–11.7 mm) against *P. acnes* and *S. aureus* while moderate effect was shown by **84** against *S. sobrinus*, *S. mutans* and *S. typhi* with the diameter inhibition zone of 9.0, 8.3 and 9.0, respectively.

Based on the results of antibacterial activity, the presence of hydroxyl group at C-7 together with methoxyl group at C-4′ in aromatic ring increased the potency of inhibition of the compound. Compound **82** had both hydroxyl and methoxyl groups at the positions showed enhancing of the activity. In contrast with **84**, the changing of methoxyl to hydroxyl at C-4′ caused the decreasing of the activity. However the inhibitory effect of **82** against *S. mutans* was slightly lower than **15** which contained hydroxyl at C-3 position. Moreover, with changing hydroxyl to methoxyl at C-7 as shown by **83** caused the decreasing of the activity against Gram-positive bacteria but its activity against *S. typhi* was slightly higher than **15** and **84**. The result could be concluded that with hydroxyl (C-7) and methoxyl (C-4′) and with the absence of hydroxyl at C-3 influenced the inhibitory of Gram-positive and negative bacteria as shown by **82**. This result indicated the ability of **82** to easily infiltrate and diffuse or penetrate into membrane cell wall of the following bacterial strains. The antibacterial

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activity of the isolated homoislofvanones against *P. acnes*, *S. sobrinus* and *S. mutans* would be the new report.

2.3.4.2 MIC, MBC values and MIC index of the Isolated Compounds

The MIC values of isolated compounds were performed by broth micro-dilution method as previously reported which could be used as a standard to measure the susceptibility of bacteria to antibacterial agents. The assay was performed in 96-well micro plate by using colorimetric assay with some modifications that be more simple, sensitive, rapid and reliable. It could successfully be used as a standard to assess the susceptibility of bacteria to antibacterial properties of natural products. In addition, resazurin as a blue non-fluorescent and non-toxic dye was used as an indicator in the oxidation-reduction reaction. It could change the blue colour become pink and fluorescent when oxidoreductases reduced it to resorufin within viable cells which were subsequently reduced to uncoloured and non-fluorescent namely hydroresufurin. The MBC value was further evaluated to determine the lowest concentration of compounds that can kill bacteria. From the results, MIC index could be conducted by calculation of MBC/MIC value of each compound to determine whether the compounds possessed bactericidal or bacteriostatic properties. The results are tabulated in **Table 2.8**.

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Table 2.8 MIC, MBC values and MIC index of isolated compounds from *D. cochinchinensis*

Entry	Cpds	*P. acnes* KCCM41747			*S. aureus* ATCC25923			*S. sobrinus* KCCM11898			*S. mutans* ATCC25175			*S. typhi* ATCC 422		
		MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index	MIC	MBC	MIC index
1	**5**	62.5	125	2	62.5	250	4	62.5	125	2	62.5	125	2	62.5	250	4
2	**23**	62.5	62.5	1	62.5	250	4	62.5	125	2	62.5	250	4	125	250	2
3	**27**	62.5	62.5	1	62.5	125	2	62.5	125	2	62.5	250	4	62.5	250	4
4	**82**	62.5	62.5	1	62.5	125	2	62.5	125	2	62.5	125	2	62.5	250	4
5	**15**	125	125	1	62.5	125	2	125	250	4	62.5	62.5	1	125	250	4
6	**84**	62.5	125	2	62.5	250	4	125	>250	>4	125	>250	>4	250	>250	>4
7	**CHL**	15.6	250	16	62.5	500	8	15.6	250	16	15.6	250	16	15.6	250	16

CHL (Chloramphenicol). Serial concentration of compounds (1,000–0.488 μM) by two fold serial dilution. The experiment was repeated three times and the concentrations are shown as average values of three independent determinations

MIC index ≤ 4 (bactericidal), MIC index **>** 4 (bacteriostatic)

Determination of MIC value of isolated compounds was performed except for **83** due to the limited amount of the compound. MIC value of **5** was 62.25 μM towards all those Gram positive and negative bacteria. Compounds **27** and **82** also suppressed the growth of all tested bacteria with MIC value of 62.25 μM. At the same MIC value (62.25 μM), **23** was recorded to inhibit only Gram-positive bacteria while *S. typhi* was inhibited by **23** with MIC value of 125 μM. Its ability displayed twice lower than **5** (62.25

μM). Compound **15** revealed MIC value of 62.25 μM against *S. aureus* and *S. mutans* while it showed its activity against *P. acnes*, *S. sobrinus* and *S. typhi* at 125 μM. Comparing to **82**, only **84** presented MIC value of 62.25 μM against *P. acnes* and *S. aureus* while this compound revealed MIC value of 125 μM against *S. sobrinus* and *S. mutans*, and was inactive against *S. typhi* with MIC value of 250 μM. CHL as a standard exhibited MIC value of four times more effective (15.6 μM) than **5**, **27** and **82** against *P. acnes*, *S. sobrinus*, *S. mutans* and *S. typhi* while the same MIC value (62.25 μM) as those compounds was performed by CHL against *S. aureus*.

Based on the results shown in Table 2.9, CHL was a bacteriostatic agent against all tested bacteria with MIC index of 8–16 together with **84** against *S. sobrinus*, *S. mutans* and *S. typhi*. According to Mayaud (2008) and Bernatov (2011), the mechanisms of bacteriostatic agents were to inhibit the growth of bacteria in the lower concentration interfering bacterial protein synthesis, DNA replication or bacterial cellular metabolism but it prevented or kept the growth of bacteria in stationary phase [57, 58]. CHL, one of the bacteriostatic antibiotics was produced by *Streptomyces venezuelae*. It was active to many Gram-positive and negative bacteria by binding to 50S ribosomal subunit and blocking aminoacyl-tRNA attachment in inhibitory protein synthesis pathway [59, 60]. Bactericidal effect was exerted by **5**, **15**, **23**, **27** and **82** against all tested bacteria with the MIC index of 1 to 4, whereas **84** only displayed bactericidal effect against *P. acnes* and *S. aureus* at 62.25 μM. The results indicated that those compounds could kill all tested bacteria at each concentration used. The

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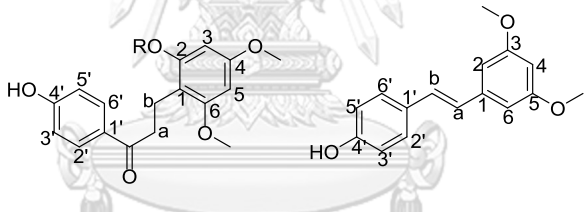
mode of action of the bactericidal agents would be interference with addition of new cell wall subunit by inhibiting recycling of membrane lipid carrier [61].

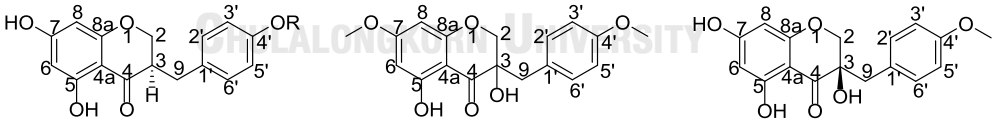
2.4 Conclusions

Seven compounds have successfully been isolated by chromatographic techniques from the CH_2Cl_2 extract of *Dracaena cochinchinensis* (Lour.) S.C.Chen. There are two dihydrochalcones (**5** and **23**), one pterostilbene (**27**) and four homoisoflavonones (**15** and **82–84**). The structures of all isolated compounds were characterized by spectroscopic techniques (^{1}H and ^{13}C NMR) and x-ray analysis as well as comparison with previous literature data.



5: R = CH_3
23: R = H

27



82: R = CH_3
84: R = H

83

15

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The antibacterial activity of isolated compounds was conducted by agar well diffusion method against five pathogenic bacteria including *Propionibacterium acnes* KCCM 41747, *Staphylococcus aureus* ATCC 25923, *Streptococcus sobrinus* KCCM

11898, *Streptococcus mutans* ATCC 25175 (Gram-positive bacteria) and *Salmonella typhi* ATCC 442 (Gram-negative bacteria).

Compound **82** exhibited excellent activity against *P. acnes* and *S. aureus* followed by **5**, **15**, **23**, **27** and **84** with good activity while **83** showed weak effect against those bacteria. Good inhibitory effect was performed by **5**, **15**, **23**, **27** and **82** against *S. sobrinus* while **83** and **84** revealed moderate inhibitory effect. The highest effect was noticed by **15** against *S. mutans* while **5**, **23** and **82** showed good activity. However, moderate effect was displayed by **27**, **83** and **84**. All isolated compounds presented good inhibitory effect against *S. typhi* except for **84** with moderate activity. The antibacterial activity of **27** against *S. sobrinus* and *S. mutans* has not previously been reported. Also there is no report about the antibacterial activity of isolated dihydrochalcones and homoisoflavonones against *P. acnes*, *S. sobrinus* and *S. mutans*.

By the broth micro-dilution method, the MIC values of isolated compounds (except **83**) were evaluated. The susceptibility of **5**, **27** and **82** was performed against all tested bacteria with MIC value of 62.25 μM while **23** exhibited the same MIC value against Gram-positive bacteria only. However, **15** inhibited the growth of *S. aureus* and *S. mutans* at 62.25 μM and with MIC value of 125 μM against other tested bacteria. On the other hand, **84** could inhibit the growth of *P. acnes* and *S. aureus* with MIC value of 62.25 μM and displayed MIC value of 125 μM against other Gram-positive bacteria whereas the MIC value of the compound was 250 μM against *S. typhi*. Bactericidal

effect was performed by all isolated compounds except **84** which only showed the same effect against *P. acnes* and *S. aureus*.

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CHAPTER III

CHEMICAL CONSTITUENTS AND ANTIBACTERIAL ACTIVITY OF *Eleutherine americana* (Aubl.) Merr. Ex K. Heyne

3.1 Introduction

Eleutherine belongs to Iridaceae family which comprises of more than 1200 species [62]. *E. americana* has widely been cultivated in China, Indonesia and Thailand as ornamental and medicinal purposes [18, 63, 64]. The local tribe (Dayak tribe) in Kalimantan was used as folk medicine for treatment numerous diseases such as diabetes, breast cancer, stroke, hypertension and sexual disorders [21]. Traditionally, it could be used as carminative in its pure form or in combination with galangal to prevent nasal congestion in children [65].

3.1.1 General Characteristics of *E. americana*

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Figure 3.1 The rhizomes of *Eleutherine americana*

Eleutherina americana (**Figure 3.1**) is a red rhizome originated from tropical America. It can be found in plantation around the world particularly in South Africa and South East Asia. This plant was also known as *E. bulbosa* [Mill.] Urban and *E. palmifolia* (L.) Merr [21, 66]. For local name, *E. americana* is known as Bawang Tiwai or Bawang Dayak by Dayak tribe in Kalimantan, Indonesia. In China, the plant is known as Hong-cong while Thai people calls Hom-daeng. It is a predominant red or wine colour herbal with scales similar to onion. It presents simple leaves and the colour of flowers is white or roses [67].

3.1.2 Literature Review of *E. americana* and Its Biological Activities

Many reports have been documented on *E. americana* concerning chemical constituents and their biological activities including anticancer, antibacterial, antiviral, anti-proliferation, anti-inflammatory, antioxidant and *etc*.

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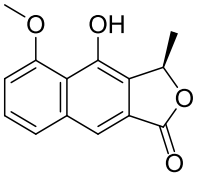
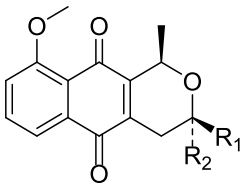
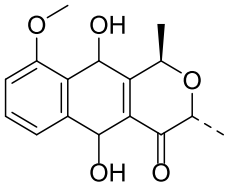
Zhengxiong and co-workers [64] reported in 1986 that three naphthalene derivatives namely eleutherol (**85**), eleutherin (**86**), isoeleutherin (**87**) as well as a novel new naphthalene (hongconin, **88**) were successfully derived from the rhizomes of *E. americana*. Those four isolated compounds could increase coronary blood flow in an isolated guinea pig heart and be effective as anti-anginal.



85

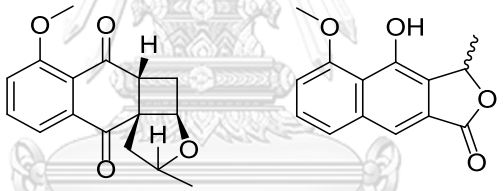


86: $R_1 = CH_3$, $R_2 = H$
87: $R_1 = H$, $R_2 = CH_3$



88

Two novel naphthoquinones (elecanacin, **89**) and naphthalene (isoeleutherol, **90**) together with two known naphthoquinones (**86**, **87**) were isolated from the bulbs of *E. americana*. Among those compounds, **86** exhibited inhibitory effect against human topoisomerase II while **87** and **90** were active against human immunodeficiency virus (HIV) inhibitory [68].



89 **90**

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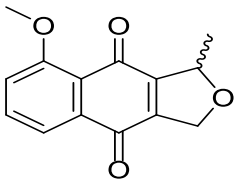
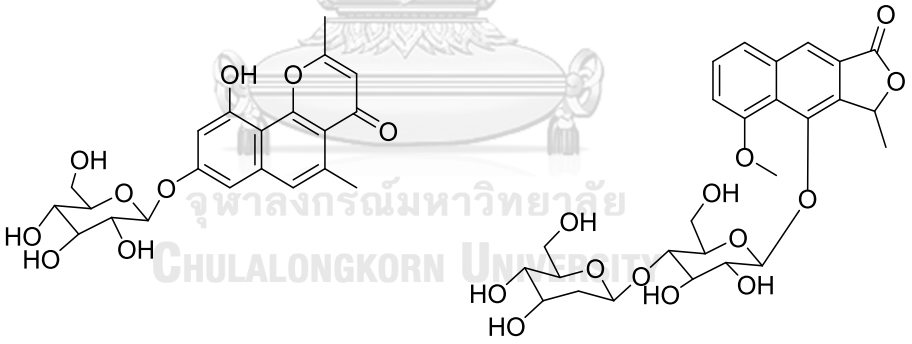
In 2003, Alves and co-workers [69] isolated four compounds including eleutherol (**85**) eleutherin (**86**), isoeuleutherin (**87**) and eleutherinone (**91**), and investigated their antifungal activity against *Cladosporium sphaerospermum* in bioautography assay. Compounds **86**, **87** and **91** revealed strong antifungal activity at 100 µg/spot.



91

Paramapojn and co-workers [63] in 2008 separated and purified five compounds from *E. americana* by column chromatography using silica gel, sephadex LH-20 and RP-18. The compounds were gained by TLC and HPLC, and elucidated their structures by comparison of ^{1}H and ^{13}C NMR, and MS data with previous literatures. Those compounds were confirmed as eleutherol (**85**), eleutherin (**86**), isoeleutherin (**87**), 2,5-dimethyl-10-hydroxynaphthopyrone-8-*O*-D-glucopyranoside or eleutherinoside A (**92**) and eleuthoside B (**93**).



92 **93**

To find the potent anti-inflammatory agents, Song and co-workers [70] in 2009 investigated the potential of isoeleutherin (**89**) to inhibit inducible nitric oxide synthase (iNOS) through the regulation of NF-kB activity. The results revealed that **89** inhibited lipopolysaccharide (LPS)-stimulated induction of nitric oxide (NO) with IC_{50} of 7.4 μM

dose-dependent manner. By western blots analysis and reverse transcription-polymerase chain reaction (RT-PCR), it suppressed the expression of iNOS and mRNA. The compound also blocked the activation of transcriptional activity of NF-kB by LPS.

The anti-methicillin resistant *S. aureus* (anti-MRSA) activity and mechanism of action determination of the crude extract from *E. americana* were conducted. This extract inhibited the growth of *S. aureus* and MRSA. Under electron microscope, the crude extract also caused damage of membrane morphology in *S. aureus* [71].

Mahabusarakam and co-workers [18] in 2009 isolated four compounds including eleuthinone A (**94**), eleuthraquinone A (**95**), eleuthraquinone B (**96**), eleucanarol (**97**) from *E. americana* and investigated their antibacterial activity together with eleven compounds including hongconin (**88**), 3,4-dihydro-9-hydroxy-1,3-dimethyl-1*H*-naphtho[2,3-c]pyran-5,10-dione (**98**), 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**99**), 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**100**), eleutherol (**85**), eleutherin (**86**), elecanacin (**89**), 3-[2-(acetyloxy)propyl]-2-hydroxy-8-methoxy-1,4-naphthoquinone (**101**), isoeleutherin (**87**), erithrolaccin (**102**), 1,2-dihydroxy-8-methoxy-3-methylanthraquinone (**103**), 6,8-dihydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**104**) against *Staphylococcus aureus* ATCC 25923 and ATCC 27664. Previously, the result showed antibacterial activity of **85**- **89**, **94** and **99**-**100** against *S. aureus* ATCC 25293 and ATCC 27664. The

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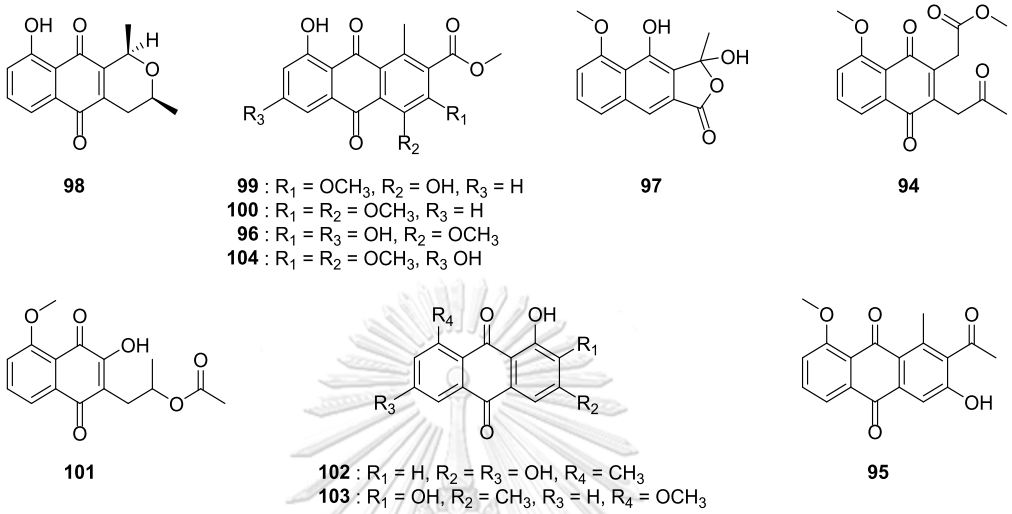
further investigation of antibacterial activity resulted the MIC values of **95-96**, **98**, **113**, **102** and **103** were ranging from 62.5 to ≥ 1,000 µg/mL.



Ifesan and co-workers [72] in 2010 evaluated antimicrobial effect of the EtOH extract from *E. americana* against six Gram positive, seven Gram negative bacteria, six fungal species and two yeasts by disc agar diffusion method and found that the crude extract suppressed the growth of all tested Gram positive bacteria with inhibition zone ranging from 13.0–20.0 mm. However, its sensitivity was effective against one Gram negative bacteria only. The extract also displayed antifungal activity against *A. niger*, *Rhizopus* spp. and *Penicillium* spp. while all dermatophytic fungi and yeast resistant to the extract treatment. By time-killing study, the extract revealed the bacteriostatic and fungistatic effects by 5- and 3-log reduction, respectively.

The preliminary screening antibacterial activity of the EtOH extract of *E. americana* against sixty-five *Campylobacter* spp. was investigated by Sirisak and co-

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workers [73] in 2010. The result displayed good inhibitory effect against all tested bacteria with the inhibition zone ranging from 10.0 to 37.0 mm. The MIC value of the extract was further determined against five *Campylobacter* spp. by counting viable cells after time interval. The EtOH extract at 4 MIC suppressed the growth of all tested bacteria by decreasing the level of tested bacteria in 2 and 5-log within 8 h.

Ieyama and co-workers [21] in 2011 isolated **85**, **92** and **93** from the bulbs of this plant. Compound **92** demonstrated high inhibitory effect against α-glucosidase with IC_{50} of 0.5 mM.

In 2013, Ha and co-workers [74] reported the inhibitory effect of sixteen compounds from *E. bulbosa* in the inflammatory response by bone marrow-derived dendritic cells. The chemical constituents were eleutherin (**86**), isoeleutherin (**87**), (-)-hongconin (**89**), eleutherinoside A (**92**), eleuthoside B (**93**), (2*S*)-dihydroeleutherinol-8-*O*-β-D-glucopyranoside (**105**), dihydroeleutherinol (**106**), eleutherinol (**107**), eleuthoside C (**108**), eleutherinoside C (**109**), eleutherinoside B (**110**), (*R*)-7-acetyl-3,6-dihydroxy-8-methyltetralone (**111**), eleuthoside A (**112**), eleutherinoside D (**113**), 3,6,8-trihydroxy-1-methyl-anthraquinone (**114**), and 2-acetyl-3,6,8-trihydroxy-1-methyl-anthraquinone (**115**). Compounds **89**, **92**, **105** and **107** could inhibit the production of TNF-α, IL-6 and IL-12 in LPS-stimulated dendritic cells.

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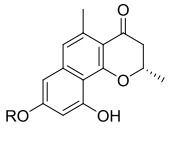
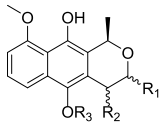
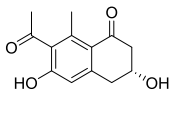


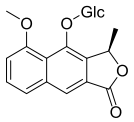
105 : R = Glc
106 : R = H
107 : R = H, D^{2-3}
92 : R = Glc, D^{2-3}



108 : R_1 = CH_3 (b), R_2 = H, R_3 = Dsac
109 : R_1 = CH_3 (a), R_2 = H, R_3 = Dsac
110 : R_1 = CH_3 (a), R_2 = OH (b), R_3 = Dsac



111



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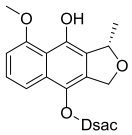

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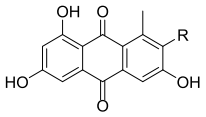
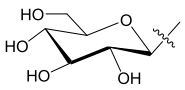
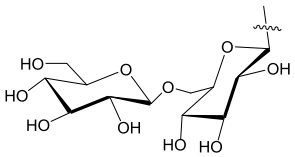


114 : R = H
115 : R = $COCH_3$



Glc



Dsac

Amelia and co-workers [75] in 2014 investigated eight compounds from *E. americana* including **86**, **87**, **91**, **96**, **95**, **89** and **104** as ligands on human estrogen receptor alpha as potential anti-breast cancer by docking method. Compound **96** displayed the highest affinity with binding free energy of -6.43 kcal/mol compared to the other compounds.

3.2 Experimental

3.2.1 Plant Materials

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The dried rhizomes of *E. americana* were collected from East Kalimantan, Indonesia in April 2014.

3.2.2 Instruments and Equipment

Analytical thin layer chromatography (TLC) was performed on aluminium sheets precoated with silica gel Kieselgel 60 F_{254} (Merck, Germany) for qualitative analysis purpose, spots on the plate were observed under UV light and visualized by dipping

in vanillin staining as detecting agent followed by heating and dried to detect spots of some compounds with no UV absorption. Column chromatography was carried out on silica gel no. 7729, 7734 and 9385 (Merck, Germany) and sephadex LH-20 (Merck, Germany). The ^{1}H and ^{13}C NMR spectra were recorded in deuterated chloroform ($CDCl_3$) or dimethylsulfoxide-d_6 (DMSO-d_6), acetone-d_6 on a Bruker Ultrashield 400 plus NMR spectrometer at 400 MHz for ^{1}H NMR and 100 MHz for ^{13}C NMR. The chemical shifts are assigned by comparison with residue solvent protons. High resolution mass spectra (HRMS) were recorded on a Bruker Daltonics micro-time of flight (TOF) using electron spray ionization (ESI). The optical rotation was measured on Perkin-Elmer 341 polarimeter using a cell with 300 µL-0.5 mL capacity and a 1.0 cm path length.

3.2.3 Media and Chemicals

Nutrient broth (NB) and commercial agar media were used for antibacterial activity assay. Chloramphenicol (CHL) ≥ 98% (Aldrich Sigma) was used as positive control and resazurin sodium salt (Aldrich Sigma) was used as oxidation-reduction and bacterial growth indicator. All organic solvents were commercial grade purified prior to use by standard methodology except for those which were reagent grades.

3.2.4 Extraction of Dried Plant Materials

The dried and powdered *E. americana* rhizomes (8.5 kg) were extracted by soxhlet with CH_2Cl_2 for 5 days. After that, the extract was filtered and concentrated by rotary evaporator under vacuum to obtain dark red CH_2Cl_2 extract (139 g, 1.64% yield

of the dried rhizome). The residue was further successively extracted with MeOH to yield MeOH extract (350 g, 4.12% yield).

3.2.5 Isolation and Purification of Compounds from *E. americana*

The CH_2Cl_2 extract (130 g) was subjected to silica gel quick column with hexane, and followed by increasing the polarity with EtOAc and then 10% MeOH in EtOAc. According to TLC pattern, ten fractions (**HD1**-**HD10**) were obtained. Based on antibacterial activity results, active fractions **HD5** and **HD8**-**10** were further separated on silica gel column. **HD5** (5.94 g) was subjected to silica gel column (No. 9385) using 100% CH_2Cl_2 and obtain six fractions (**HD5-1** to **HD5-6**). Fraction **HD5-3** was chromatographed on silica gel column using 100% CH_2Cl_2 to afford six fractions (**HD5-31** to **HD5-36**). Eleutherin (**86**, 194 mg) was yielded from both sub-fractions **HD5-34** and **HD5-4** by recrystallization using *n*-hexane and CH_2Cl_2. Further separation of **HD5-33** obtained two compounds, *i.e.* **99** (0.6 mg) and **100** (15 mg). The fraction **HD8** was subjected to column chromatography over silica gel with the eluent of 100% CH_2Cl_2 to afford fourteen subfractions (**HD8-1** to **HD8-14**). Subfraction **HD8-7** was washed with CH_2Cl_2 to yield **118** (6 mg) while **85** (2 g) was obtained from both subfractions **HD8-3** and **HD9-2** by recrystallization using hexane and CH_2Cl_2. Separation of subfraction **HD8-10** by silica gel column using CH_2Cl_2 to yield seven subfractions (**HD8-101** to **HD8-107**). The subfraction **HD8-105** was chromatographed on silica gel column (No. 9385) using hexane, CH_2Cl_2 and EtOAc (5: 1: 0.5) to yield **116** (2.4 mg). The subfraction **HD8-107**

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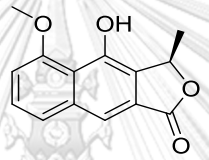
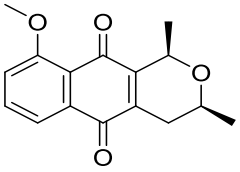
was purified by sephadex LH-20 column using 100% MeOH and washed by acetone to afford **117** (4.7 mg). Compound **119** (2.2 mg) was obtained from subfraction **HD10-2** by chromatography column on silica gel (No. 9385) using hexane, CH_2Cl_2 and EtOAc (10:1:0.5). The structural elucidation of isolated compounds was supported by ^1H and ^{13}C NMR (Bruker, 400 and 100 MHz), HR-MS and comparison with literature data. Optical rotation was measured on a Perkin-Elmer 341 polarimeter using a cell with 300 µL - 0.5 mL capacity and a 1.5 cm path length.



85

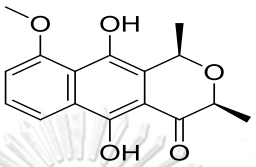
(+)-Eleutherol (**85**): 2 g. $C_{14}H_{12}O_4$. Yellow crystal. $[\alpha]_D^{22}$ +44.93 (c 1.0, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.65 (d, *J* = 6.4 Hz, 1H), 4.11 (s, 3H), 5.72 (q, *J* = 6.8 Hz, 1H), 6.93 (d, *J* = 8.0 Hz, 1H), 7.41 (t, *J* = 8.0 Hz, 1H), 7.58 (d, *J* = 8.4 Hz, 1H), 7.88 (s, 1H), 9.64 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 19.3, 56.5, 77.5, 106.4, 116.7, 117.7, 126.1, 126.7, 123.8, 128.1, 137.4, 149.3, 156.7, 170.6.



86

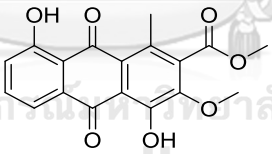
(+)-Eleutherin (**86**): 194.5 mg. $C_{16}H_{16}O_4$. Crystal orange. $[\alpha]_D^{21}$+203.73 (c 1.0, $CHCl_3$). ^1H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.38 (d, *J* = 6.3 Hz, 3H), 1.55 (d, *J* = 6.4 Hz, 3H), 2.22

(dq, *J* = 18.0, 3.6 Hz, 1H), 2.77 (dt, *J* = 15.6, 2.8 Hz, 1H), 3.60 (m), 4.01 (m), 4.01 (s, 3H), 7.29 (dd, *J* = 7,6, 1.2 Hz, 1H), 7.66 (d, *J* = 8.0 Hz, 1H), 7.75 (dd, *J* = 7.6, 1.2 Hz, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 20.9, 21.4, 30.1, 56.6, 68.9, 70.4, 117.9, 119.2, 120.6, 134.2, 134.7, 140.1, 148.9, 159.6, 183.9, 184.2.



116

(+)-3-*epi*-Hongconin (**116**): 2.4 mg. $C_{16}H_{16}O_5$. Orange solid. $[\alpha]_D^{20}$+13.56 (c 0.77, $CHCl_3$). ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 1.53 (d, *J* = 6.4 Hz, 3H), 1.64 (d, *J* = 6.8 Hz, 3H), 4.07 (s, 3H), 4.69 (q, *J* = 6.4 Hz, 1H), 5.48 (q, *J* = 6.8 Hz, 1H), 7.01 (d, *J* = 7.6 Hz, 1H), 7.05 (d, *J* = 8.4 Hz, 1H), 7.38 (t, *J* = 8.0 Hz, 1H), 8.97 (s, 1H), 12.81 (s, 1H).



99

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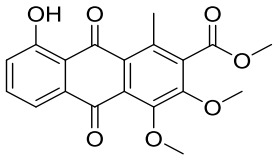
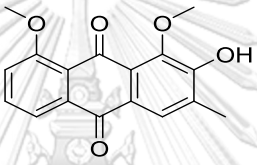
4,8-Dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester **(99)**: 0.6 mg. $C_{18}H_{14}O_7$. Orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.64 (s, 3H), 3.98 (s, 3H), 4.09 (s, 3H), 7.33 (dd, *J* = 8.4, 0.8 Hz, 1H), 7.66 (t, *J* = 8.0 Hz, 1H), 7.83 (dd, *J* = 7.6, 0.8 Hz, 1H), 12.91 (br s, 1H), 13.70 (br s, 1H).



100

8-Hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**100**): 15 mg. $C_{19}H_{16}O_7$. Orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.67 (s, 3H), 3.96 (s, 3H), 3.98 (s, 3H), 4.02 (s, 3H), 7.23 (dd, *J* = 8.4, 1.2 Hz, 1H), 7.60 (t, *J* = 8.0 Hz, 1H), 7.67 (dd, *J* = 7.6, 1.2 Hz, 1H), 12.55 (br s, 1H).



117

1,8-Dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone (**117**): 4.7 mg, $C_{17}H_{14}O_5$, orange solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ (ppm) 2.38 (s, 3H), 4.02 (s, 3H), 4.03 (s, 3H), 6.73 (br s, 1H), 7.30 (d, *J* = 8.4 Hz, 1H), 7.66 (t, *J* = 8.0 Hz, 1H), 7.88 (d, *J* = 6.8 Hz, 1H), 7.89 (s, 1H). ^{13}C NMR (100 MHz, $CDCl_3$): δ (ppm) 16.3, 56.8, 62.5, 117.9, 119.4, 122.7, 125.8, 126.0, 130.4, 134.5, 135.5, 138.7, 145.8, 154.0, 160.0, 182.8, 182.9.

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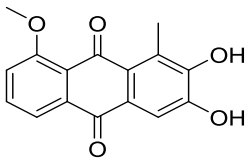
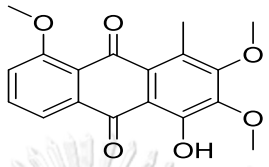


118

2,3-Dihydroxy-8-methoxy-1-methyl-9,10-anthraquinone (**118**): 6.0 mg. $C_6H_{12}O_5$, orange solid. ^{1}H NMR (400 MHz, DMSO-d_6): δ (ppm) 2.26 (s, 3H), 3.97 (s, 3H), 7.51 (s, 1H), 7.57

(d, *J* = 8.0 Hz, 1H), 7.79 (d, *J* = 7.6 Hz, 1H), 7.85 (dd, *J* = 7.6 Hz, 1H). ^{13}C NMR (100 MHz, DMSO-d_6): δ (ppm) 16.3, 56.5, 115.2, 118.7, 119.1, 119.9, 121.6, 122.3, 130.9, 135.5, 136.2, 149.5, 150.5, 160.5, 180.7, 188.3. HRMS (ESI): calcd for $C_6H_{12}O_5$ $[M+Na]^+$: 307.0582, found 307.0580.



119

4-Dihydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthraquinone (119): 2.2 mg. C18H16O6, orange solid. 1H NMR (400 MHz, CDCl3): δ (ppm) 2.69 (s, 3H), 3.97 (s, 3H), 3.99 (s, 3H), 4.03 (s, 3H), 7.26 (d, J = 7.2 Hz, 1H), 7.63 (d, J = 8.0 Hz, 1H), 7.70 (dd, J = 6.8, 1.2 Hz, 1H), 12.57 (s, 1H).

The extraction and purification of isolated compounds of the CH_2Cl_2 extracts of the rhizomes of *E. americana* were briefly summarized in **Schemes 3.1 – 3.2.**

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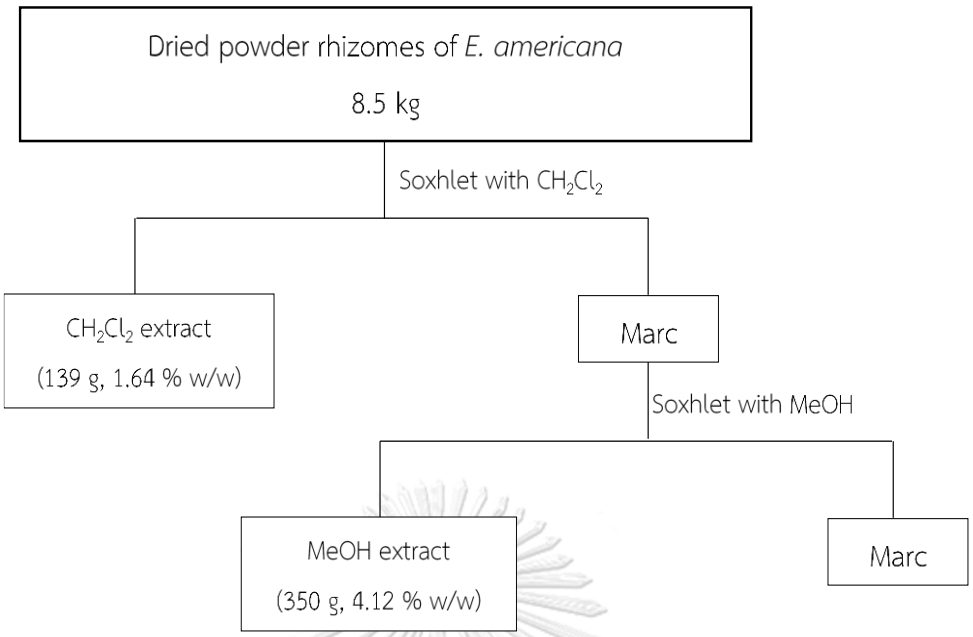

Scheme 3.1 Extraction procedure of *E. americana* rhizomes

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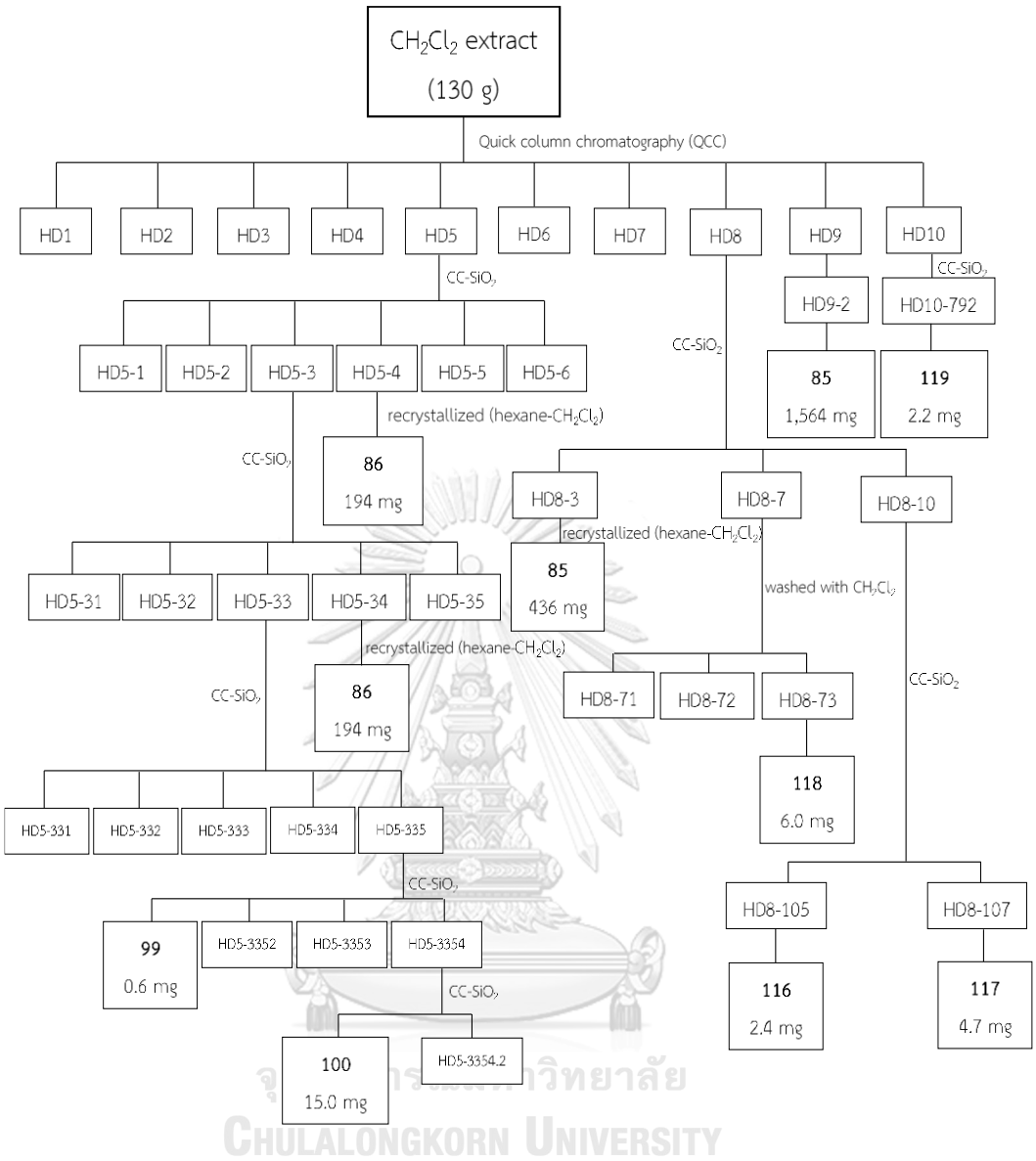

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Scheme 3.2 Isolation procedure of the CH_2Cl_2 extract of *E. americana*

3.2.5 The Investigation of Antibacterial Activity

3.2.5.1 Bacterial Strains

The pathogenic bacteria used in this bioassay were four Gram-positive bacteria including *Propionibacterium acnes* KCCM 41747, *Staphylococcus aureus* ATCC 25923, *Streptococcus sobrinus* KCCM 11898, *Streptococcus mutans* ATCC 25175 and

Salmonella typhi ATCC 422 as representative for Gram-negative bacteria. The test bacteria were periodically sub-cultured and maintained in nutrient agar under suitable conditions depending upon the test bacteria.

3.2.5.2 Agar Well Diffusion Method

The preliminary screening antibacterial activity of crude extracts and compounds were investigated against five bacteria pathogens by using a modified agar well diffusion method as described in **2.2.6.2**.

3.2.5.3 The Broth Micro-dilution Method for Determination of MIC, MBC and MIC index

Determination of the MIC value was conducted by the broth micro-dilution method as reported in **2.2.6.3**.

3.3 Results and Discussion

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3.3.1 Extraction of Dried Rhizomes of *E. americana*

The dried-powder of *E. americana* rhizomes were extracted with CH_2Cl_2 by soxhlet yielding dark-red of CH_2Cl_2 extract (139 g, 1.64% w/w). Then, the residue was extracted with MeOH to obtain MeOH extract as dark-red (350 g, 4.12% yield of the dried-powdered). Those extracts were preliminary investigated on antibacterial activity.

3.3.2 Isolation and Purification of Compounds from *E. americana*

About 130 g of the CH_2Cl_2 extract was further subjected to silica gel quick column by stepwise elution with 100% hexane and followed by increasing the polarity with EtOAc and final with a mixture of 10% MeOH in EtOAc. Each fraction was collected and concentrated under reduced pressure using vacuum rotary evaporator. Based on TLC profile, ten fractions (**HD1** to **HD10**) were obtained. Using bioassay guided approach, fractions **HD5**, **HD8**-**10** showed potential antibacterial activity against tested pathogenic bacteria. The separation of subfractions **HD5-34** and **HD5-4** by recrystallization furnishing **86** (194 mg) while **99** (0.6 mg) and **100** (15 mg) were derived from the separation by silica gel column from **HD5-33**. Subfractions **HD8-3** and **HD9-2** were recrystallized using hexane and CH_2Cl_2 affording **85** (2 g) whereas **118** (6 mg) was obtained from **HD8-7** by washing with CH_2Cl_2. Compound **116** (2.4 mg) and **119** (2.2 mg) were attained by silica gel column from subfractions **HD8-105** and **HD10-2**, respectively. The purification of subfractions **HD8-107** by sephadex LH-20 column using 100% MeOH provided **117** (4.7 mg).

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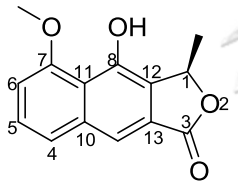
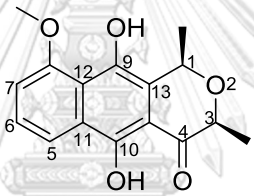
3.3.3 Properties and Structural Elucidation of Isolated Compounds

The structural elucidation of the isolated compounds were determined by analyses of ^{1}H, ^{13}C NMR spectroscopies, optical rotation and HRMS-ESI analysis as well as comparison of their data with those reported in previous literatures [40, 64, 76, 77]. Eight compounds were isolated from the CH_2Cl_2 extract of *E. americana* rhizomes and

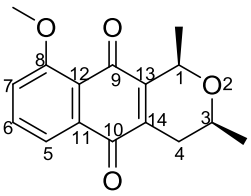
confirmed as (+)-eleutherol (**85**), (+)-eleutherin (**86**), 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**99**), 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**100**), (+)-3-*epi*-hongconin (**116**), 1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone (**117**), 2,3-dihydroxy-8-methoxy-1-methyl-9,10-anthraquinone (**118**) and 4-hydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthraquinone (**119**). Among those compounds, **118** and **119** were identified as new compounds while **116** and **117** were reported for the first time as natural occurring compounds.



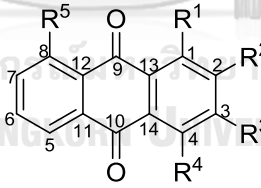
85



116



86



99: $R^1 = CH_3$, $R^2 = CO_2CH_3$, $R^3 = OCH_3$, $R^4 = OH$, $R^5 = OH$
100: $R^1 = CH_3$, $R^2 = CO_2CH_3$, $R^3 = OCH_3$, $R^4 = OCH_3$, $R^5 = OH$
117: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = CH_3$, $R^4 = H$, $R^5 = OCH_3$
118: $R^1 = CH_3$, $R^2 = R^3 = OH$, $R^4 = H$, $R^5 = OCH_3$
119: $R^1 = CH_3$, $R^2 = R^3 = OCH_3$, $R^4 = OH$, $R^5 = OCH_3$

3.3.3.1 Naphthalenes (85 and 116)

Compound **85** was isolated as an orange powder. After recrystallization with hexane and CH_2Cl_2, the compound becomes yellow crystal. The ^1H NMR spectrum

(**Figure 3.2**) displayed aromatic proton signals in the downfield region for one triplet (*J* = 8.0 Hz) at δ_H 7.41 ppm, two doublets at δ_H 7.58 and 6.93 ppm with the coupling constant of 8.4 and 8.0 Hz, respectively and one singlet at δ_H 7.88 ppm. A proton signal at C-1 was detected as quartet (*J* = 6.8 Hz) at δ_H 5.72 ppm. The signal of hydroxyl proton was assigned at δ_H 9.64 ppm. The ^{13}C NMR spectrum (**Figure 3.3**) presented aromatic carbons at δ_C 106.7 to 156.7 ppm. Optical rotation of this compound was also determined, then compared to the previous literature [68]. The result showed that the specific rotation of **85** is +44.93°. Consequently, **85** was identified as (+)-eleutherol. The ^{1}H and ^{13}C NMR data of **85** are tabulated in **Table 3.1**.

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Table 3.1 The ^{1}H and ^{13}C NMR spectral assignment of **85** and eleutherol [21]

Position	Chemical shift (ppm)		
	85[a]		**eleutherol** [21]
	δH	δC	δH
1	5.72 (q, *J* = 6.8 Hz, 1H)	77.5	5.76 (q, *J* = 6.5 Hz, 1H)
3	-	170.6	-
4	7.58 (d, *J* = 8.4 Hz, 1H)	128.1	7.63 (d, *J* = 8.3 Hz, 1H)
5	7.41 (t, *J* = 8.0 Hz, 1H)	123.8	7.47 (t, *J* = 8.1 Hz, 1H)
6	6.93 (d, *J* = 8.0 Hz, 1H)	106.4	7.12 (d, *J* = 7.8 Hz Hz, 1H)
7	-	156.7	-
8	-	149.3	-
9	7.88 (s, 1H)	126.7	7.87 (s, 1H)
10	-	137.4	-
11	-	116.7	-
12	-	117.7	-
13	-	126.1	-
1-CH_3	1.65 (d, *J* = 6.4 Hz, 1H)	19.3	1.72 (d, *J* = 6.5 Hz, 1H)
8-OH	9.64 (s, 1H)	-	-
7-OCH_3	4.11 (s, 3H)	56.5	4.11 (s, 3H)

^{1}H and ^{13}C NMR: [a]$CDCl_3$ (400 and 100 MHz)

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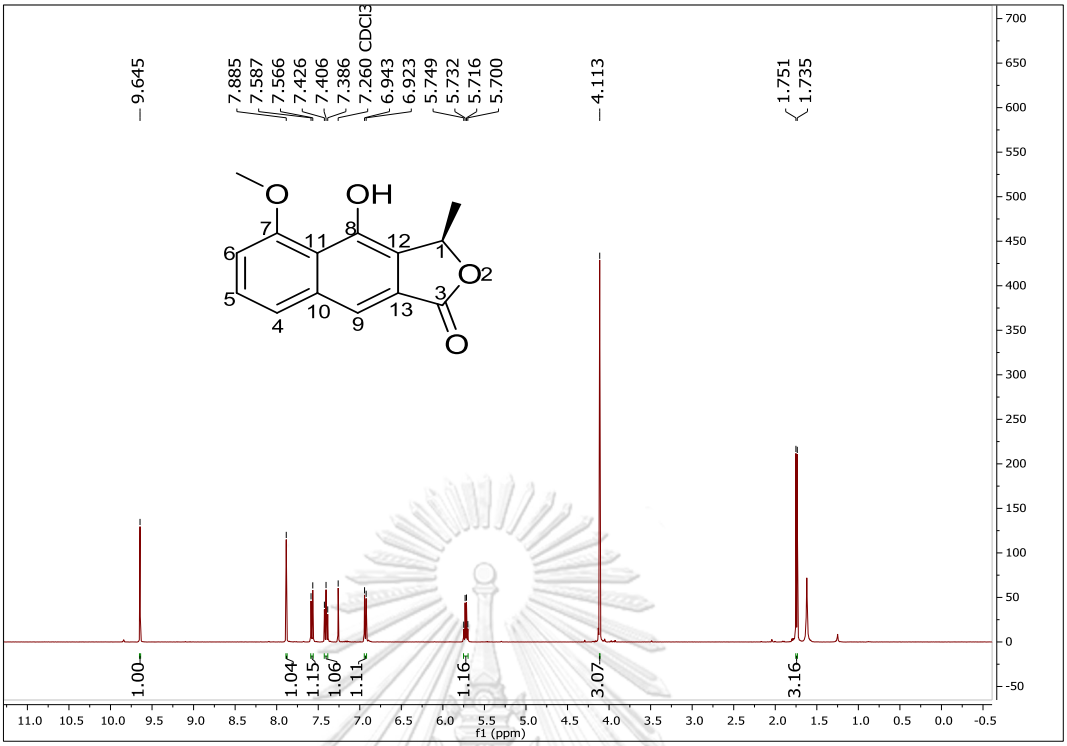

Figure 3.2 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **85**

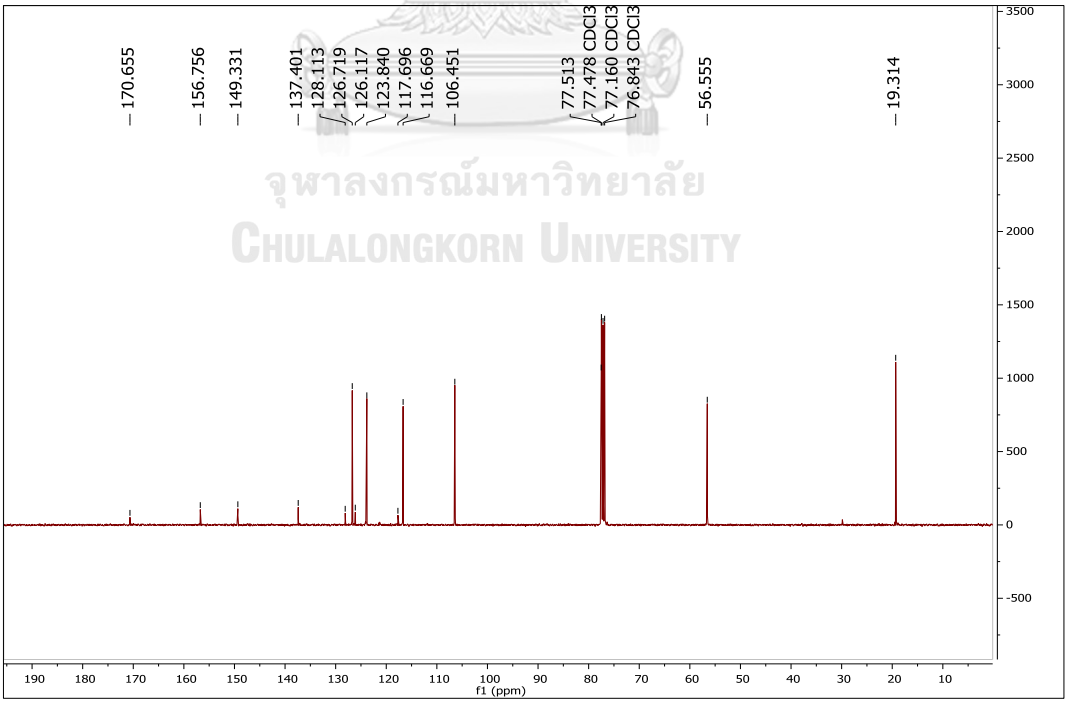

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Figure 3.3 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **85**

Compound **85** was also confirmed its structure by single crystal X-ray diffraction and proposed ORTEP crystal is presented in **Figure 3.4**.

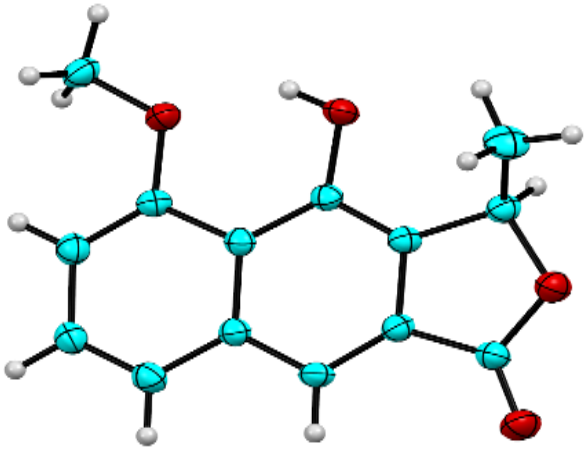

Figure 3.4 The proposed ORTEP of (+)-eleutherol (**85**)

Another naphthalene derivative (**116**) was isolated as orange powder. The ^{1}H NMR spectrum (**Figure 3.5**) showed the aromatic signals in downfield regions for one triplet at δ_H 7.38 ppm (1H, t, *J* = 8.0 Hz, H-5), two doublets at δ_H 7.05 ppm (1H, d, *J* = 8.4 Hz, H-6) and δ_H 7.01 ppm (1H, d, *J* = 7.6 Hz). Two quaternary protons were assigned to C-1 and C-3 at δ_H 4.69 and 5.48 ppm with coupling constants of 6.4 and 6.8 Hz, respectively. The presence of hydroxyl group was assigned at δ_H 8.97 ppm (H-9) and the other hydroxyl group was placed at C-10 to the C-4 carbonyl group at δ_H 12.81 ppm. The appearance of one methoxyl group displayed one signal at δ_H 4.07 ppm which placed at C-8. The doublet signal of two methyl groups was assigned to C-1 at δ_H 1.64 ppm (*J* = 6.8 Hz) and C-3 at δ_H 1.53 ppm (*J* = 6.4 Hz). Based on optical rotation analysis, the specific rotation of **116** was $[\alpha]_D^{20}$+ 13.56 (C 0.77, $CHCl_3$). This was compared with that of (-)-hongconin (**88**) as $[\alpha]_D^{20}$ – 26.0 (C 1.947, $CHCl_3$). Based on the

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comparison with previous study, the stereostructure of **116** should be (+)-3-*epi*-hongconin. There are four possibilities of stereoisomers of this compound with two chiral centers as shown in **Figure 3.6**. Consequently, the stereostucture of (-)-hongconin (**88**) was indicated to be the same as the enantiomer of (+)-*ent*-hongconin (**121**) and the diastereomer with (-)-1-*epi*-hongconin (**120**). (+)-3-*epi*-Hongconin (**116**) isolated from *E. americana* which was an enantiomer of **120** and diastereomer of **121.** The latter two compounds were synthesized and reported [40, 76]. (+)-3-*epi*-Hongconin (**116**) was isolated from nature for the first time. The ^{1}H NMR data of **116** is tabulated in **Table 3.2**.

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Table 3.2 The ^{1}H NMR spectral assignment of **116** and (+)-3-epi-hongconin [76]

Position	Chemical shift (ppm)	
	116[a]	**(+)-3-*epi*-hongconin** [76]
1	4.69 (q, *J* = 6.4 Hz, 1H)	4.26 (q, *J* = 6.6 Hz, 1H)
3	5.48 (q, *J* = 6.8 Hz, 1H)	5.15 (q, *J* = 6.3 Hz, 1H)
5	7.38 (t, *J* = 8.0 Hz, 1H)	8.02 (d, *J* = 8.4 Hz, 1H)
6	7.05 (d, *J* = 8.4 Hz, 1H)	7.37 (t, *J* = 8.1 Hz, 1H)
7	7.01 (d, *J* = 7.6 Hz, 1H)	6.99 (d, *J* = 7.8 Hz, 1H)
1-CH_3	1.64 (d, *J* = 6.8 Hz, 3H)	1.77 (d, *J* = 6.3 Hz, 3H)
3-CH_3	1.53 (d, *J* =6.4 Hz, 3H)	1.53 (d, *J* = 6.5 Hz, 3H)
8-OCH_3	4.07 (s, 3H)	4.06 (s, 3H)
9-OH	8.97 (s, 1H)	9.19 (s, 1H)
10-OH	12.81 (s, 1H)	12.69 (s, 1H)

^{1}H NMR: [a]$CDCl_3$ (400 MHz)

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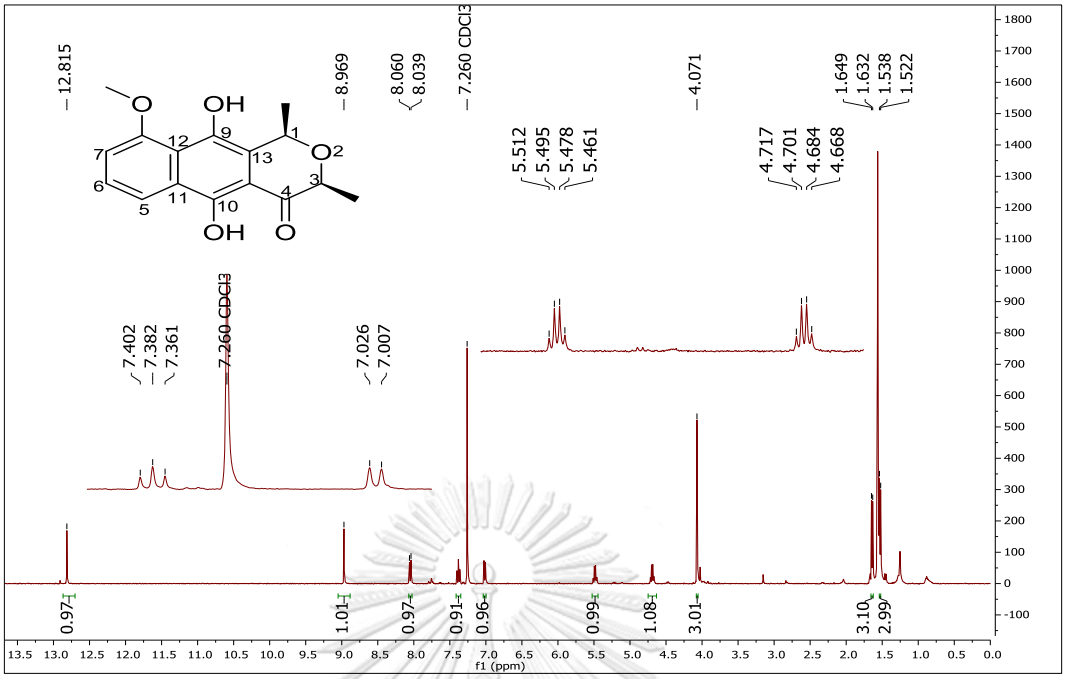
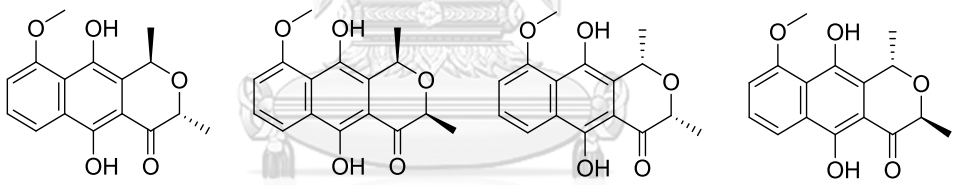


Figure 3.5 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **116**



88 **116** **120** **121**

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Figure 3.6 The possible structures of hongconin

3.3.3.2 Naphthoquinone (86)

(+)-Eleutherin (**86**) was isolated as orange crystal from hexane and CH_2Cl_2. The ^{1}H NMR spectrum of **86** (**Figure 3.7**) displayed three aromatic protons at **δ**H 7.75 ppm (dd, *J* = 7.6, 1.2 Hz), 7.29 ppm (dd, *J* = 7.6, 1.2 Hz) and 7.66 ppm (d, *J* = 8.0 Hz) which could be assigned for H-6, H-5 and H-7, respectively. The singlet signal of an aromatic

methoxyl appeared at **δ**H 4.03 ppm (C-8) while two doublets of methyl group exhibited at **δ**H 1.55 ppm (*J* = 6.4 Hz, C-1) and 1.38 ppm (*J* = 6.3 Hz, H-3). The presence of methylene signals could be assigned at **δ**H 4.01 and 3.60 ppm (C-4). Moreover, according to ^{13}C NMR spectrum (**Figure 3.8**), two carbonyl groups at **δ**C 184.2 (C-9) and 183.9 ppm (C-10), two methyl carbons at **δ**C 20.9 and 21.4 ppm and the methoxyl carbon at **δ**C 56.6 ppm were detected. The ^{1}H and ^{13}C NMR spectral assignment of **86** is summarized in **Table 3.3**.

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Table 3.3 The ^{1}H and ^{13}C NMR spectral assignment of **86**, eleutherin and isoeleutherin [68]

Position	Chemical shift (ppm)			
	86[a]		**eleutherin** [68]	**isoeleutherin** [68]
	δ_H	δ_C	δ_H	δ_H
1	4.01 (m, 1H)	70.4	5.01 (q, *J* = 6.7 Hz, 1H)	5.01 (q, *J* = 6.7 Hz, 1H)
3	3.60 (m, 1H)	68.9	3.96 (m, 1H)	3.96 (m, 1H)
4a	2.22 (dq, *J* = 18.0, 3.6 Hz, 1H)	30.1	2.20 (dd, *J* = 18.1, 10.3 Hz, 1H)	2.23 (dd, *J* = 19.0, 11.0 Hz, 1H)
4b	2.77 (dt, *J* = 15.6, 2.8 Hz, 1H)	30.1	2.75 (dd, *J* = 18.1, 2.4 Hz, 1H)	2.69 (dd, *J* = 19.0, 3.5 Hz, 1H)
5	7.29 (dd, *J* = 7.6, 1.2 Hz, 1H)	119.2	7.28 (d, *J* = 6.7 Hz, 1H)	7.27 (d, *J* = 6.7 Hz, 1H)
6	7.75 (dd, *J* = 7.6, 1.2 Hz, 1H)	134.7	7.74 (d, *J* = 6.7 Hz)	7.74 (d, *J* = 6.7 Hz)
7	7.66 (d, *J* = 8.0 Hz, 1H)	117.9	7.64 (d, *J* = 6.7 Hz, 1H)	7.64 (d, *J* = 6.7 Hz, 1H)
8	-	159.6	-	-
9	-	184.2	-	-
10	-	183.9	-	-
11	-	134.2	-	-
12	-	120.6	-	-
13	-	148.9	-	-
14	-	140.1	-	-
1-CH_3	1.55 (d, *J* = 6.4 Hz, 3H)	20.9	1.54 (d, *J* = 6.5 Hz, 3H)	1.53 (d, *J* = 6.7 Hz, 3H)
3-CH_3	1.38 (d, *J* = 6.3 Hz, 3H)	21.4	1.36 (d, *J* = 6.5 Hz, 3H)	1.34 (d, *J* = 6.1 Hz, 3H)
8-OCH_3	4.01 (s, 3H)	56.6	4.0 (s, 3H)	4.0 (s, 3H)

^{1}H and ^{13}C NMR: [a]$CDCl_3$ (400 and 100 MHz)

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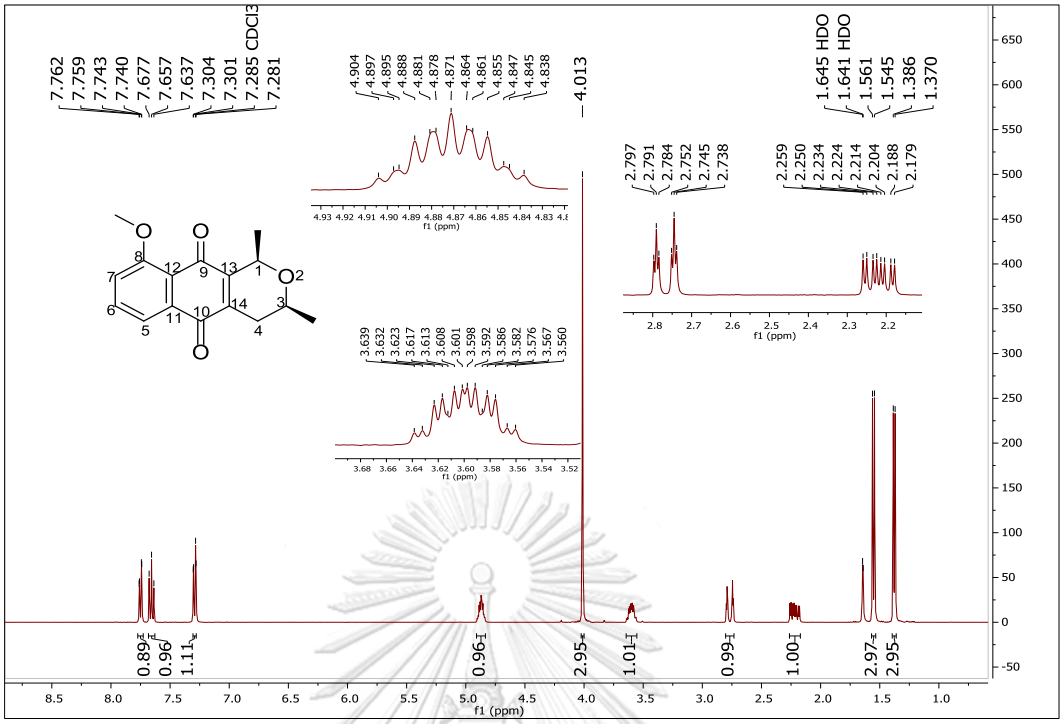

Figure 3.7 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **86**

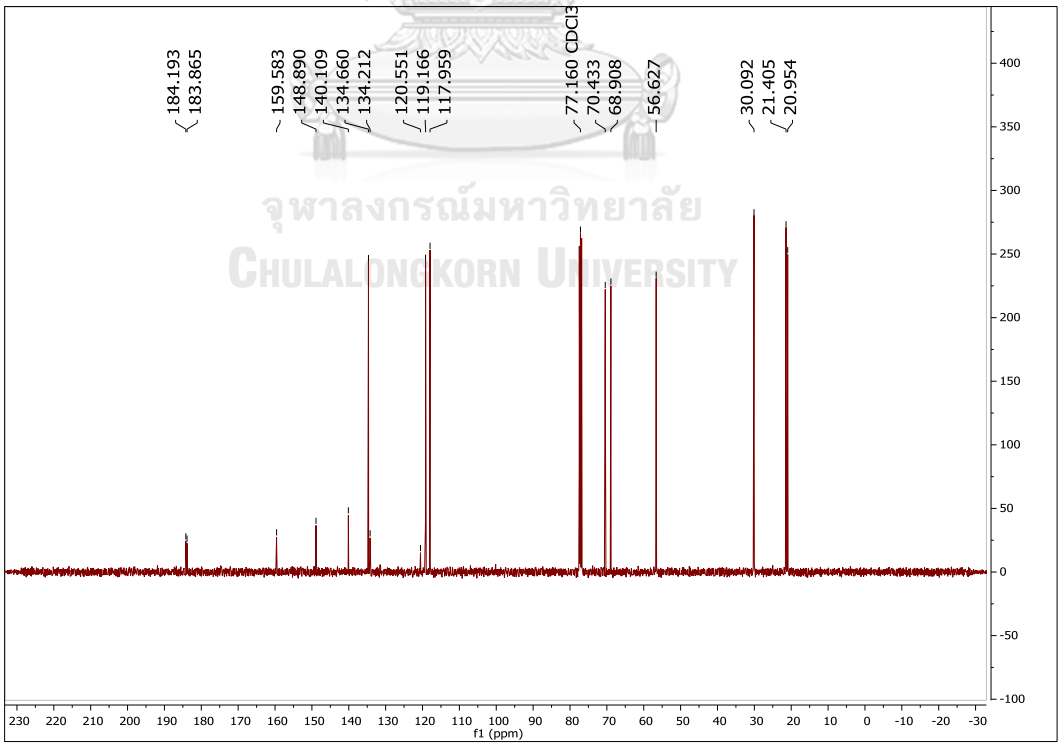

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Figure 3.8 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **86**

Based on previous studies, the ^{1}H NMR data of this compound was slightly similar to that of isoeletherin (**87**). The optical rotation of **86** could be determined and compared to that reported by Zhengxiong (1986) [64]. The specific rotations of **86** and **87** were $[\alpha]_D^{21}$ +203.73 (C 1.0, $CHCl_3$) and $[\alpha]_D^{23}$ -51.8 (C 0.965, $CHCl_3$), respectively. By single crystal X-ray diffraction, **86** was confirmed its structure and proposed ORTEP crystal is presented in **Figure 3.9**. Hence, **86** should be (+)-eleutherin.

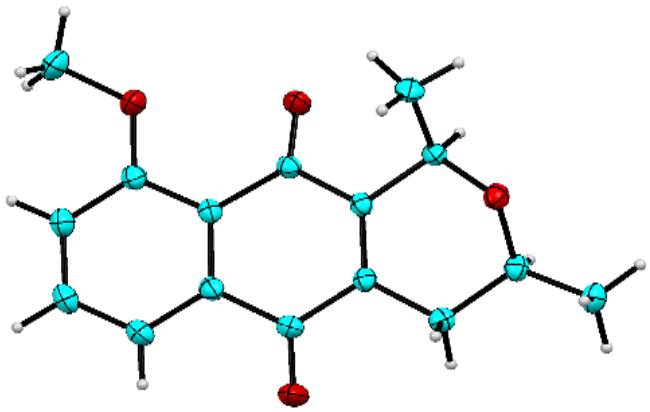

Figure 3.9 The proposed ORTEP of (+)-eleutherin (**86**)

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3.3.3.3 Anthraquinones (99-100, 117-119)

The NMR characteristics of anthraquinones normally exhibit the presence of fourteen carbon atoms in their basic skeleton with ABC aromatic rings. The ^{1}H NMR data (**Figure 3.10**) of **99** revealed the proton signals in downfield region of aromatic ring A for two doublet of doublets at δ_H 7.83 ppm (*J* = 7.6, 0.8 Hz, H-5) and at δ_H 7.33 ppm (*J* = 8.4, 0.8 Hz, H-7), respectively. One triplet was assigned at δ_H 7.66 ppm (*J* = 8.0 Hz, H-6). The singlet pattern of two hydroxyl groups adjacent to carbonyl could be

seen much downfield at δ_H 13.70 ppm (C-4) and 12.91 ppm (C-8), respectively. The presence of the methyl group signal was detected at δ_H 2.64 ppm while the methoxyl signal was recognized at δ_H 3.98 ppm (C-3). The methoxyl ester could be visualized at δ_H 4.09 ppm (C-2). According to the spectral data basis, it can be concluded that **99** was 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester. The ^{1}H NMR spectral data (**Figure 3.11**) of **100** exhibited the similar pattern to that of **99**. The presence of the methoxyl in **100** at C-3 position was assigned at δ_H 3.98 ppm which replaced the hydroxyl group in **99**. The comparison of the ^{1}H NMR spectral assignment of **99** and **100** is summarized in **Table 3.4**. Based on the spectral data, the structure of **100** was identified as 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester.

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Table 3.4 The ^{1}H NMR spectral assignment of **99-100**, 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid and 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester [78]

Position	Chemical shift (ppm)			
	99[a]	**4,8-dihydroxy-3-methoxy-1-methylanthroquinone-2-carboxylic** [78]	**100**[b]	**8-hydroxy-3,4-dimethoxy-1-methylanthroquinone-2-carboxylic acid methyl ester** [78]
5	7.83 (dd, *J* = 7.6, 0.8 Hz, 1H)	7.70 (dd, *J* = 7.6, 1.7 Hz, 1H)	7.67 (dd, *J* = 7.6, 1.2 Hz, 1H)	7.76 (dd, *J* = 7.1, 2.7 Hz, 1H)
6	7.66 (t, *J* = 8.0 Hz, 1H)	7.56 (t, *J* = 7.6 Hz, 1H)	7.60 (t, *J* = 8.0 Hz, 1H)	7.57 (t, *J* = 7.1 Hz, 1H)
7	7.33 (dd, *J* = 8.4, 0.8 1H)	7.24 (dd, *J* = 7.6, 1.7 Hz, 1H)	7.23 (dd, *J* = 8.4, 1.2 Hz, 1H)	7.22 (dd, *J* = 7.1, 2.7 Hz, 1H)
1-CH_3	2.64 (s, 3H)	2.57 (s, 3H)	2.67 (s, 3H)	2.68 (s, 3H)
2-CO_2CH_3	4.09 (s, 3H)	4.08 (s, 3H)	4.02 (s, 3H)	4.03 (s, 3H)
3-OCH_3	3.98 (s, 3H)	3.97 (s, 3H)	3.98 (s, 3H)	3.99 (s, 3H)
4-OCH_3	-	-	3.96 (s, 3H)	3.97 (s, 3H)
4-OH	13.70 (s, 1H)	13.60 (s, 1H)	-	-
8-OH	12.91 (s, 1H)	12.80 (s, 1H)	12.55 (s, 1H)	12.53 (s, 1H)

^{1}H NMR: [ab]$CDCl_3$ (400 MHz)

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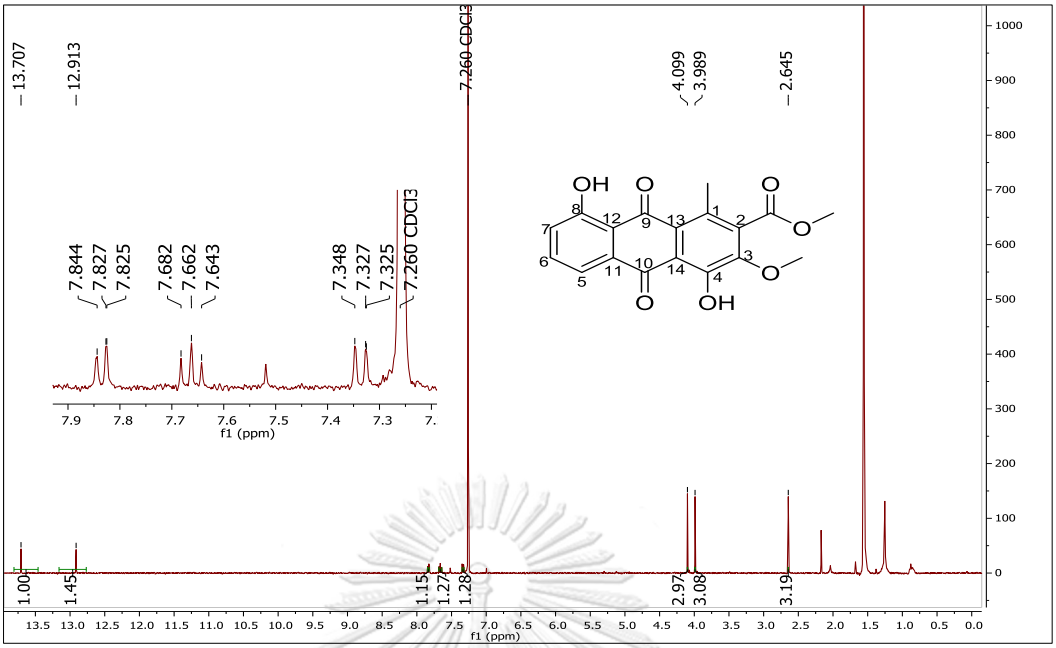

Figure 3.10 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **99**

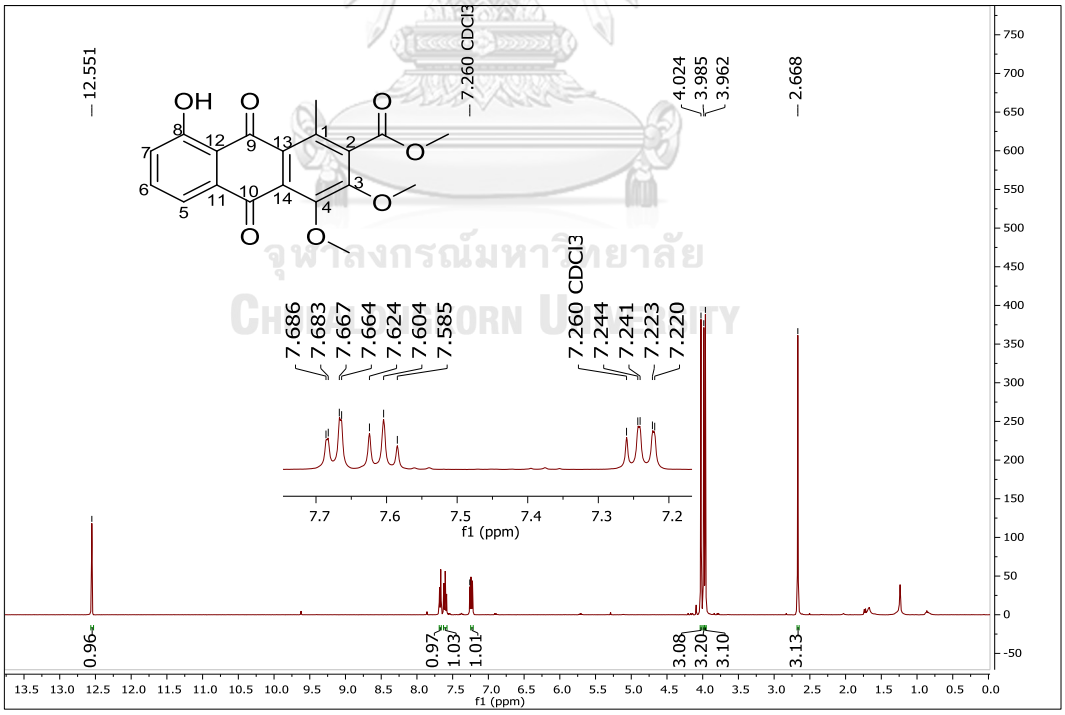

Figure 3.11 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **100**

1,8-Dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone (**117**) was yielded as orange powder. The ^{1}H NMR spectrum (**Figure 3.12**) of **117** displayed three signals in the downfield region of aromatic ring A. One triplet signal at **δ**H 7.66 ppm (C-5) with coupling constant of 8.0 Hz, one doublet signal at **δ**H 7.88 ppm (*J* = 6.8 Hz) and the other doublet signal at **δ**H 7.30 ppm (*J* = 8.4 Hz) were detected. In ring C, one aromatic singlet signal revealed at **δ**H 7.89 ppm (C-4), one singlet of methyl group at **δ**H 2.38 ppm and one signal was recognized as hydroxyl group at C-2 position (**δ**H 6.73 ppm). The presence of two methoxyl groups appeared at **δ**H 4.03 ppm (C-1) and **δ**H 4.02 ppm (C-8). The ^{13}C NMR spectrum (**Figure 3.13**) reveals fourteen aromatic carbons at **δ**C 117.0 to 182.9 ppm whereas the carbon signals of two methoxyl groups were shown at **δ**C 62.5 and 56.8 ppm and the signal of methyl group was assigned at 16.3 ppm. By comparison of the spectral data of the compound with previous study, it can be concluded that **117** was 1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone. This compound was reported as a synthesized compound [77] and thus was the first report as a natural constituent. The ^{1}H and ^{13}C NMR data of **117** is tabulated in **Table 3.5**.

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Table 3.5 The ^{1}H and ^{13}C NMR spectral assignment of **117** and 1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone [77]

Position	Chemical shift (ppm)			
	117[a]		**1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone** [77]	
	δH	δC	δH	δC
1	-	154.0	-	153.9
2	-	145.8	-	145.6
3	-	135.5	-	136.0
4	7.89 (s, 1H)	126.0	7.89 (s, 1H)	125.8
5	7.88 (d, *J* = 6.8 Hz, 1H)	122.7	7.88 (dd, *J* = 7.5, 1.2 Hz, 1H)	119.2
6	7.66 (t, *J* = 8.0 Hz, 1H)	134.5	7.66 (dd, *J* = 8.4, 7.5 Hz, 1H)	134.4
7	7.30 (d, *J* = 8.4 Hz, 1H)	117.9	7.30 (dd, *J* = 8.4, 1.2 Hz, 1H)	117.6
8	-	160.0	-	159.7
9	-	182.9	-	186.0
10	-	182.8	-	182.7
11	-	138.7	-	135.3
12	-	125.8	-	125.6
13	-	119.4	-	122.8
14	-	130.4	-	130.3
1-OCH_3	4.03 (s, 3H)	62.5	4.04 (s, 3H)	62.4
2-OH	6.73 (s, 1H)	-	6.73 (s, 1H)	-
3-CH_3	2.38 (s, 3H)	16.3	2.37 (d, *J* = 0.8 Hz, 3H)	16.2
8-OCH_3	4.02 (s, 3H)	56.8	4.03 (s, 3H)	56.7

^{1}H and ^{13}C NMR: [a]$CDCl_3$ (400 and 100 MHz)

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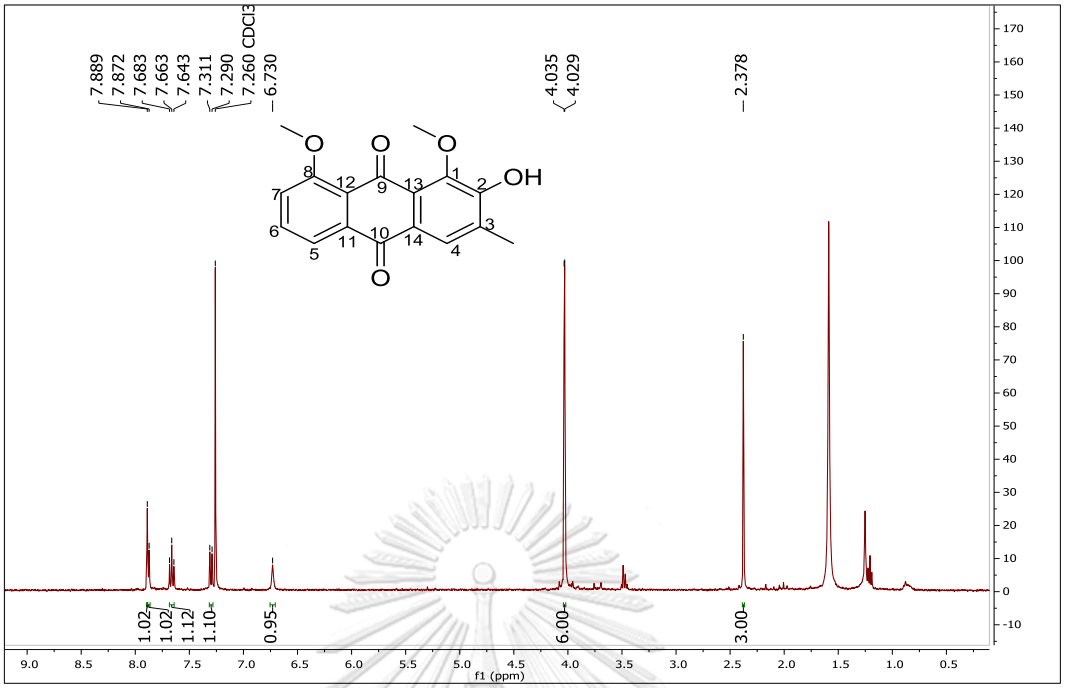

Figure 3.12 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **117**

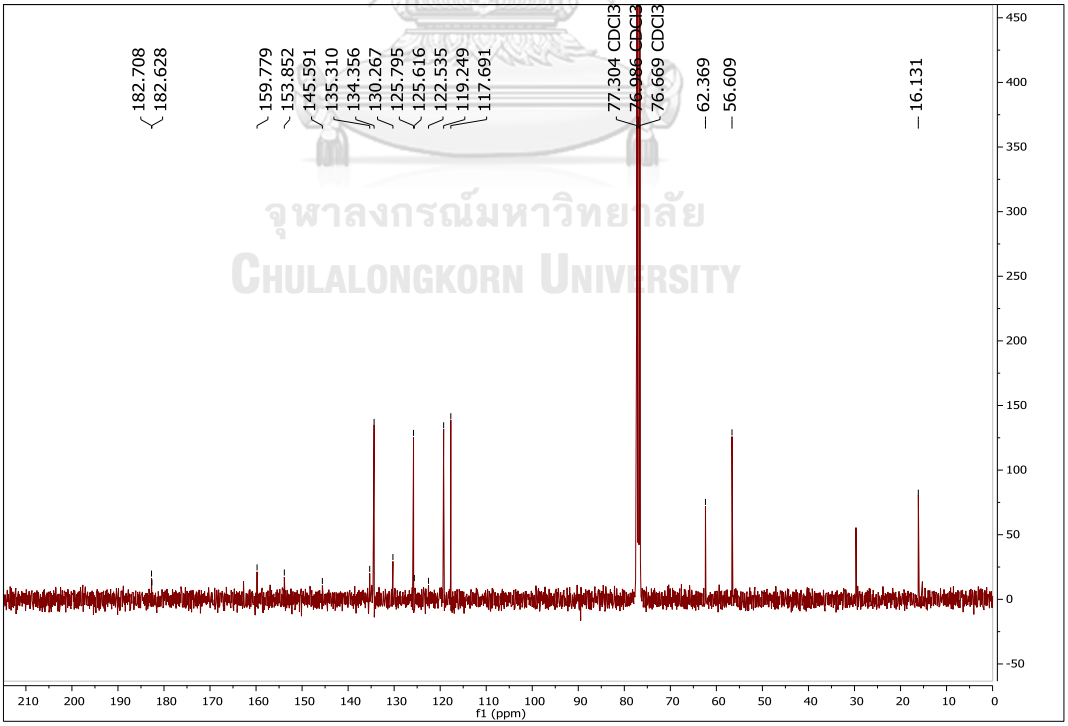

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Figure 3.13 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **117**

2,3-Dihydroxy-8-methoxy-1-methyl-9,10-anthraquinone (**118**) was isolated as orange powder. The ^{1}H NMR spectrum (**Figure 3.14**) exhibited three aromatic proton signals in ring A. Two-doublet signals (H-5 and H-7) were assigned at δ_H 7.79 ppm (J = 7.6 Hz) and 7.57 ppm (J = 8.0 Hz), respectively while the other double doublet signal was detected at δ_H 7.85 ppm (J = 7.6 Hz). The presence of the methoxyl group was assigned at δ_H 3.97 ppm. One singlet signal could be assigned for aromatic ring C at δ_H 7.51 ppm (H-4) and the methyl signal was visualized at δ_H 2.26 ppm (C-1). According to its ^{13}C NMR spectrum (**Figure 3.15**), fourteen aromatic carbons exhibited at δ_C 115.2 to 188.3 ppm. The methoxyl and methyl carbons could be assigned at δ_C 56.5 and 16.3 ppm, respectively. The other anthraquinone, 4-hydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthraquinone (**119**) displayed similar pattern of ^{1}H NMR spectrum to that of **118** by replacing the hydroxyl groups with methoxyl groups in **119** (**Figure 3.16**) at C-2 and C-3 positions. In addition, the presence of hydroxyl group at C-4 position as a chelated hydroxyl to the carbonyl group was assigned at δ_H 12.57 ppm. According to the spectral data, **119** was identified as 4-hydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthraquinone (**119**). The ^{1}H and ^{13}C NMR spectral assignment data of these compounds are presented in **Table 3.6**. It should be mentioned here that **118** and **119** were new compounds.

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Table 3.6 The ^{1}H and ^{13}C NMR spectral assignment of **118** and **119**

Position	Chemical shift (ppm)		
	118		**119**
	δ_H	δ_C	δ_H
1	-	150.5	-
2	-	149.5	-
3	-	135.5	-
4	7.51 (s, 1H)	121.6	-
5	7.85 (dd, *J* = 7.6 Hz, 1H)	119.1	7.70 (dd, *J* = 6.8, 1.2 Hz, 1H)
6	7.79 (d, *J* = 7.6 Hz, 1H)	130.9	7.63 (d, *J* = 8.0 Hz, 1H)
7	7.57 (d, *J* = 8.0 Hz, 1H)	115.2	7.26 (d, *J* = 7.2 Hz, 1H)
8	-	160.5	-
9	-	188.3	-
10	-	180.7	-
11	-	136.2	-
12	-	119.9	-
13	-	118.7	-
14	-	122.3	-
1-CH_3	2.26 (s, 3H)	16.3	2.69 (s, 3H)
2-OCH_3	-	-	3.97 (s, 3H)
3-OCH_3	-	-	3.99 (s, 3H)
8-OCH_3	3.97 (s, 3H)	56.5	4.03 (s, 3H)
4-OH	-	-	12.57 (s, 1H)

^{1}H and ^{13}C NMR: DMSO-d_6, 100 and 400 MHz)

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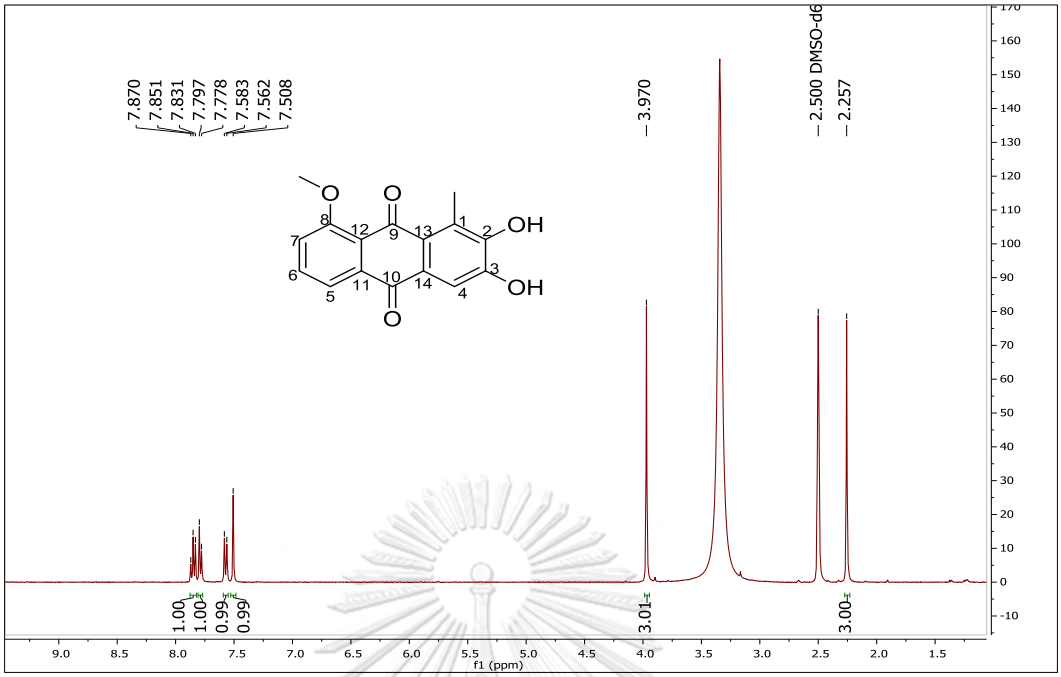

Figure 3.14 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **118**

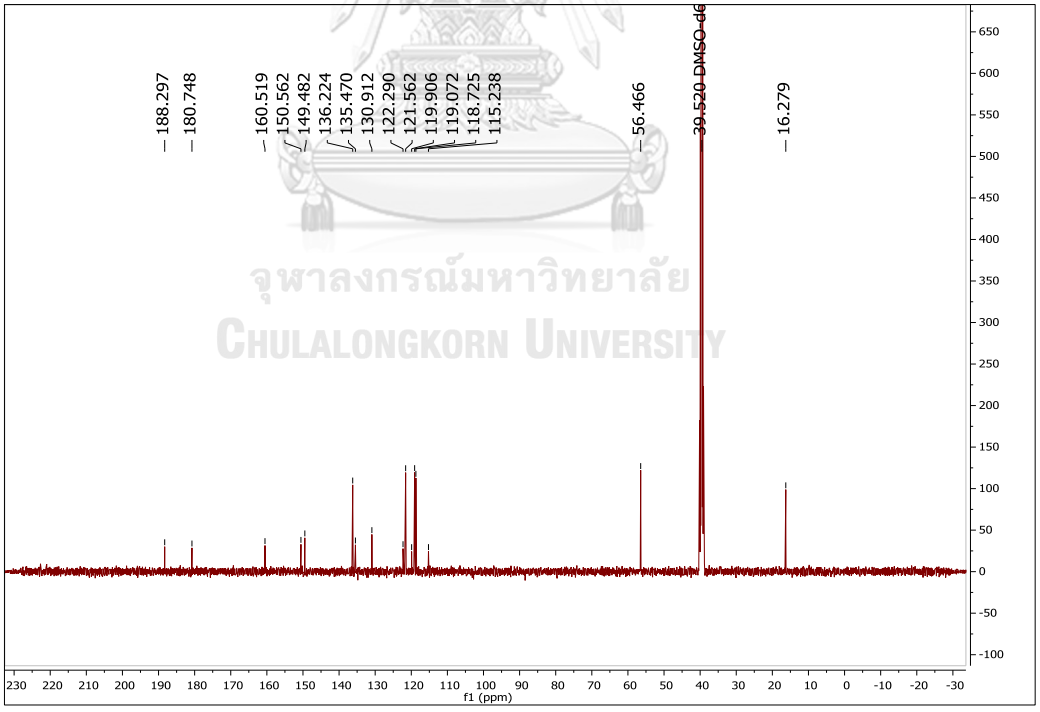

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Figure 3.15 The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of **118**

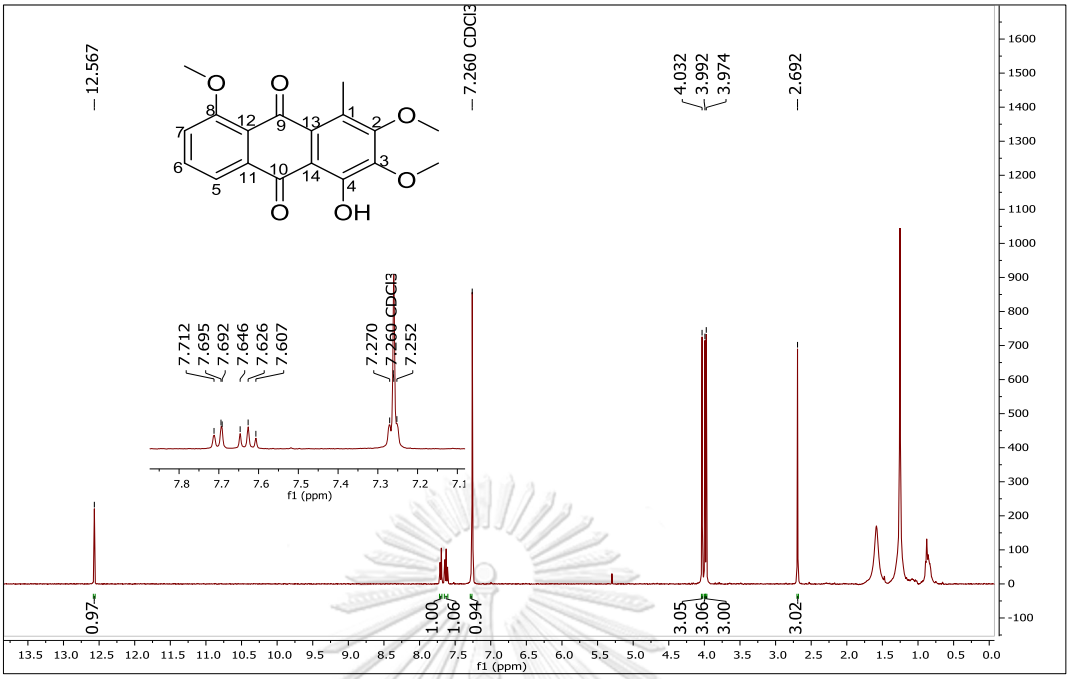

Figure 3.16 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **119**

3.3.4 Antibacterial Activity

3.3.4.1 Preliminary Screening of Antibacterial Activity

The antibacterial activity test of the crude extracts from *E. americana* was evaluated against pathogenic bacteria: *P. acnes* KCCM 41747, *S. aureus* ATCC 25923, *S. sobrinus* KCCM 11898, *S. mutans* ATCC 25175 (Gram-positive bacteria) and *S. typhi* ATCC 422 (Gram-negative bacteria) by agar diffusion method. By preparing those extracts in DMSO as 1 mM and CHL (0.5 mM) as positive control, the results presented as zone of inhibition (mm) are tabulated in **Table 3.7**.

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Table 3.7 Antibacterial activity of the crude extracts of *E. americana*

Entry	Extracts (1 mg/mL)	Inhibition zone (mm) ± SD				
		P. acnes KCCM 41747	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175	*S. typhi* ATCC 442
1	CH_2Cl_2	16.0±2.9	28.7±0.5	16.3±1.7	20.3±0.5	18.3±0.5
2	MeOH	13.0±2.5	8.3±0.9	6.0±0.0	8.7±0.5	17.3±1.7
3	CHL	25.0±0.0	26.0±0.8	26.0±0.0	29.3±0.5	20.0±0.0

The experiment was repeated three times and the inhibition zone is shown as average values of three independent determinations; Chloramphenicol (CHL) as positive control at 0.5 mM; Key to the inhibition zone activity (mm) >15 = Excellent, 13.1 – 15.0 = Very good, 10.1 – 13.0 = Good, 8.1 – 10.0 = Moderate, 6.1 – 8.0 = Weak, 6.0 = No activity

From Table 3.7, the CH2Cl2 extract possessed better antibacterial activity than the MeOH extract against all tested bacteria with the inhibition zone ranging from 16.0 to 28.7 mm. Thus, the CH2Cl2 extract was selected for further separation.

Based on TLC profile, ten fractions (**HD1** to **HD10**) were obtained from fractionation of the CH_2Cl_2 extract by quick column chromatography with hexane 100% and followed by increasing polarity with EtOAc in hexane (5-80%), 100% EtOAc and finally eluted with a mixture of MeOH in EtOAc (5-10%). The results of their antibacterial activity are summarized in **Table 3.8**. Six fractions (**HD5-HD10**) displayed better activity than others against all tested bacteria.

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Table 3.8 Antibacterial activity of subfractions from the CH_2Cl_2 extract of *E. americana*

Entry	Fractions (1 mg/mL)	Inhibition zone (mm) ± SD				
		P. acnes KCCM 41747	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175	*S. typhi* ATCC 442
1	**HD1**	7.0 ±0.0	7.3±0.5	7.3±0.5	7.0±0.0	7.0±0.5
2	**HD2**	7.0±0.0	7.3±0.5	7.7±0.9	7.0±0.0	7.3±0.5
3	**HD3**	7.0±0.0	8.3±0.5	9.3±0.9	7.3±0.5	10.7±0.9
4	**HD4**	7.0±0.0	7.3±0.5	8.3±0.9	7.3±0.5	8.0±0.82
5	**HD5**	7.0±0.0	11.0±0.8	12.7±0.5	10.0±0.8	11.7±0.5
6	**HD6**	7.0±0.00	14.3±0.5	16.3±2.6	14.3±0.5	13.0±2.2
7	**HD7**	7.7±0.5	11.7±0.5	13.0±0.8	8.3±0.5	11.0±0.8
8	**HD8**	7.3±0.5	12.0±1.6	12.3±0.5	12.7±0.9	11.7±0.5
9	**HD9**	7.0±0.0	11.3±0.5	11.7±0.5	10.0±0.0	10.0±0.0
10	**HD10**	11.3±2.1	16.7±1.2	17.7±1.2	19.0±1.4	15.3±0.5
11	CHL	25.0±0.0	26.0±0.0	26.0±0.0	29.3±0.5	20.0±0.0

The experiment was repeated three times and the inhibition zone is shown as average values of three independent determinations; Chloramphenicol (CHL) as positive control at 500 μM; Key to the inhibition zone activity (mm) >15 = Excellent, 13.1 – 15.0 = Very good, 10.1 – 13.0 = Good, 8.1 – 10.0 = Moderate, 6.1 – 8.0 = Weak, 6.0 = No activity

Active fractions **HD5** and **HD8**-**10** were further separated and eight compounds were isolated from those fractions which were (+)-eleutherol (**85**), (+)-eleutherin (**86**), 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**99**), 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**100**),

(+)-3-*epi*-hongconin (**116**), 1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone (**117**), 2,3-dihydroxy-8-methoxy-1-methyl-9,10-anthraquinone (**118**) and 4-hydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthraquinone (**119**). Their antibacterial activities were screened by agar diffusion method as mentioned in chapter II and the zones of inhibition were calculated as presented in **Table 3.9**.

Table 3.9 Antibacterial activity of isolated compounds from *E. americana*

Entry	Cpds	Inhibition zone (mm) ± SD				
		P. acnes KCCM 41747	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175	*S. typhi* ATCC 442
1	**85**	8.0±0.0	8.0±0.0	10.0±0.0	8.0±0.0	12.0±0.0
2	**86**	18.0±0.0	14.7±0.5	17.7±0.5	15.0±0.0	16.7±0.5
3	**99**	8.0±0.0	7.0±0.0	9.3±0.5	8.0±0.0	8.0±0.0
4	**100**	7.0±0.0	7.7±0.5	9.0±0.0	9.0±0.0	7.7±0.5
5	**116**	10.7±0.7	12.3±0.5	11.0±0.0	12.0±0.0	8.0±0.0
6	**117**	8.0±0.0	8.0±0.0	8.0±0.0	8.3±0.5	9.0±0.0
7	**118**	7.3±0.5	8.0±0.0	10.0±0.0	8.7±0.5	12.0±0.0
8	**119**	7.0±0.0	7.7±0.5	9.0±0.0	9.0±0.0	7.7±0.5

The concentration of compounds used for testing was 1 mM

The experiment was repeated three times and the concentration is shown as average values of three independent determinations

Criteria of inhibition zone activity (mm): inhibition zone >15.0: excellent, 13.1-15.0: very good, 10.1-13.0: good, 8.1-10.0: moderate, 6.1-8.0: weak, ≤6.0: no activity

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From the CH_2Cl_2 extract of *E. americana*, three types of compounds were isolated, *i.e.* naphthalene (**85** and **116**), 1,4-naphthoquinone (**86**) and anthraquinone (**99–100**, **117–119**). The antibacterial activity of isolated compounds was accumulated in **Table 3.9**. (+)-Eleutherin (**86**) revealed very good to excellent antibacterial activity against all tested bacteria with the diameter of inhibition zones ranging from 14.7 to 18.0 mm. This compound exhibited most potential antibacterial effect against all tested bacteria compared to naphthalene and anthraquinone derivatives. Previous studies reported that the quinone moiety in typical 1,4-naphthoquinones played role in pharmacological activities such as antimalarial, antifungal, antibacterial and anticancer agents [79]. Moreover, 1,4-naphthoquinone derivatives particularly showed antibacterial activity on several types of aerobic, facultative anaerobic and anaerobic bacteria, either Gram negative or Gram positive bacteria [80]. Many naturally occurring naphthoquinones performed potential antibacterial effect against several species of both aerobic and anaerobic bacteria. Moreover, naphthoquinones have been widely used as colorant for cosmetics, fabrics, foods and medicinal purposes including antitumor, anti-inflammatory and antimicrobial agents [81, 82]. These activities can be observed depending on the position(s) of substituents in the aromatic ring of naphthoquinones. (+)-Eleutherin (**86**) with methoxyl substituent at C-8 position and the appearance of aromatic ring C displayed broad spectrum in antibacterial effect due to its ability to inhibit all Gram positive and negative bacteria representative aerobic, facultative anaerobic and anaerobic bacteria.

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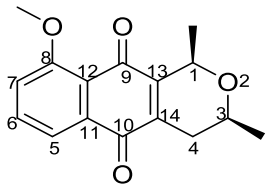
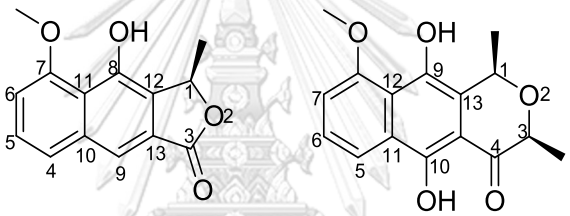


86

In addition, (+)-3-*epi*-hongconin (**116**) displayed good activity against *P. acnes*, *S. aureus*, *S. sobrinus* and *S. mutans* with inhibition zone ranging from 10.7 to 12.3 mm while (+)-eleutherol (**85**) showed good to moderate activity against *S. typhi* and *S. sobrinus* with the inhibition zones of 12.0 and 10.0 mm, respectively.



85 **116**

Compound **116** with the presence of hydroxyl group at C-10 and different skeleton of ring C showed more inhibitory effect against Gram positive bacteria than **85**, whereas **116** displayed less effective to inhibit Gram negative bacteria than **85**. Moreover, the antibacterial effect of **116** also displayed the importance of five membered lactone ring C that made this compound more active than **85** with furan ring C against Gram positive bacteria. The structure-antibacterial relationship of both naphthalenes are depicted in **Figure 3.17**.

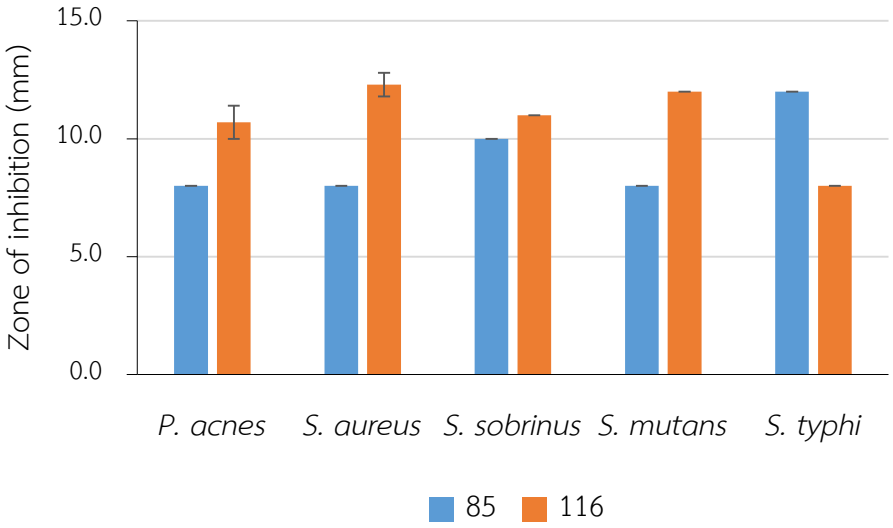
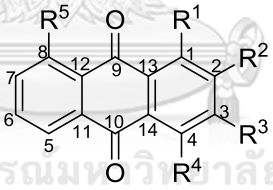


Figure 3.17 The structure-antibacterial relationship of naphthalenes (**85** and **116**)

In addition, the structure-antibacterial relationship of all isolated anthraquinones are presented in **Figure 3.18**.



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99: $R^1 = CH_3$, $R^2 = CO_2CH_3$, $R^3 = OCH_3$, $R^4 = OH$, $R^5 = OH$
100: $R^1 = CH_3$, $R^2 = CO_2CH_3$, $R^3 = OCH_3$, $R^4 = OCH_3$, $R^5 = OH$
117: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = CH_3$, $R^4 = H$, $R^5 = OCH_3$
118: $R^1 = CH_3$, $R^2 = R^3 = OH$, $R^4 = H$, $R^5 = OCH_3$
119: $R^1 = CH_3$, $R^2 = R^3 = OCH_3$, $R^4 = OH$, $R^5 = OCH_3$

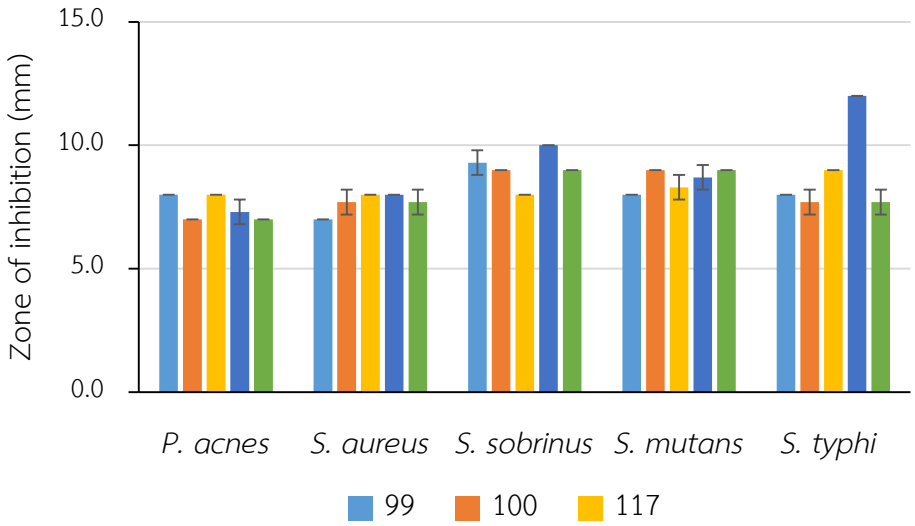

Figure 3.18 The structure-antibacterial relationship of anthraquinones (**99-100**, **117-119**)

As shown in **Figure 3.18**, the inhibitory effect of anthraquinone derivatives against tested bacteria decreased rather than naphthalenes and naphthoquinone. Compound **118** displayed good activity only against *S. typhi*, the others showed moderate and weak antibacterial effect against all tested bacteria. This indicated that **118** with hydroxyl substituent at C-2 and C-3, and methoxyl group at C-8 was more effective than other anthraquinones against Gram-negative bacteria. In addition, all anthraquinones showed slightly decreased the antibacterial effect against Gram-positive bacteria. In particular, **118** exhibited specific antibacterial effect on the growth of Gram-negative bacteria but all compounds had little inhibitory effect against Gram-positive bacteria.

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Based on literature reviews, Yang and co-workers reported that anthraquinones possessing aromatic ring C displayed lower antibacterial effect than those with cycloaliphatic ring [83]. Another study was reported by Lu and co-workers in 2011 involving anthraquinones bearing polar functional group such as hydroxyl, hydroxyl-methyl and carboxyl could enhance the antibacterial activity whereas the methyl and methoxyl might decrease their antibacterial activity [84].

3.3.4.2 MIC, MBC Values and MIC Index of Isolated Compounds

Determination of MIC values of isolated compounds were evaluated by broth micro-dilution method, and resazurin was used as an indicator of bacteria growth. Further evaluation is to determine MBC and MIC index as previously reported in chapter II. The results are presented in **Table 3.10**.

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Table 3.10 MIC, MBC values and MIC index of isolated compounds from *E. americana*

Entry	Cpd	*P. acnes* KCCM41747			*S. aureus* ATCC25923			*S. sobrinus* KCCM11898			*S. mutans* ATCC25175			*S. typhi* ATCC 422		
		MIC	MBC	MICI	MIC	MBC	MICI	MIC	MBC	MICI	MIC	MBC	MICI	MIC	MBC	MICI
1	**85**	125	250	2	125	250	2	62.5	250	4	125	250	2	31.25	125	4
2	**86**	0.24	1.95	8	7.8	15.6	2	0.24	0.98	4	3.9	15.6	4	0.98	3.9	4
3	**99**	250	>1,000	>4	250	>1,000	>4	250	>1,000	>4	250	>1,000	>4	250	>1,000	>4
4	**100**	250	>1,000	>4	250	>1,000	>4	125	>1,000	>4	125	>1,000	>4	250	>1,000	>4
5	**116**	62.5	250	4	31.25	250	8	31.25	125	4	31.25	125	4	125	125	1
6	**117**	250	500	4	250	500	4	250	250	2	250	250	2	250	500	2
7	**118**	250	500	2	125	500	4	62.5	125	2	125	125	1	31.25	125	4
8	**119**	250	>1,000	>4	250	>1,000	>4	125	>1,000	>4	250	>1,000	>4	250	>1,000	>4
9	CHL	15.6	250	16	62.5	500	8	15.6	250	16	15.6	250	16	15.6	250	16

CHl (Chloramphenicol). Serial concentration of compounds (1,000 – 0.122 μM) by two fold serial dilution.
The experiment was repeated three times and the concentration is shown as average values of three independent determinations
MICI (MIC index). MICI ≤ 4 (bactericidal), MICI > 4 (bacteriostatic)

Based on the results in **Table 3.10**, all tested bacteria were suppressed by **86** with the lowest MIC values. Compound **86** exhibited MIC value of 0.24 μM against *P. acnes* and *S. sobrinus* while with MIC value of 7.8 μM of the compound inhibited the growth of *S. aureus*. This compound also suppressed the growth of *S. mutans* and *S. typhi* at 3.9 and 0.98 μM, respectively. This indicated that **86** had potential as antibacterial agent that could offer protection against infection of Gram negative and positive pathogenic bacteria especially for skin and oral cavity diseases as well as typhoid fever. On the other hand, this compound was may give a way for drug discovery to suppress the growth of bacteria resistant.

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The MIC values of naphthalenes (**85** and **116**) showed higher MICs than **86**. With MIC value of 31.25 μM, **116** inhibited the growth of *S. aureus*, *S. sobrinus* and *S. mutans* whereas *P. acnes* and *S. typhi* were inhibited with the MIC values of 62.5 and 125 μM, respectively. However, **85** displayed MIC values of 125 μM against *P. acnes*, *S. aureus* and *S. mutans* while *S. sobrinus* was inhibited with MIC of 62.5 μM. Moreover, **85** exhibited lower MIC than **116** against Gram negative bacteria (*S. typhi*) with the MIC of 31.25 μM.

All anthraquinone exhibited MIC values ranging from 62.5 – 250 μM against Gram positive bacteria. The growth of *S. typhi* was suppressed by **118** with low MIC value of 31.25 μM among other anthraquinones.

According to the MBC and MIC index presented in **Table 3.10**, CHL as a positive control, **99**, **100** and **119** were bacteriostatic agents against all tested bacteria with the MIC index > 4. Bactericidal agents were performed by **85**, **117** and **118** against all bacteria with the MIC index ≤4 while **86** and **116** showed MIC index of 8 (bacteriostatic agents) in the lower concentration against *P. acnes* and *S. aureus*, respectively. Moreover, both **86** and **116** displayed their ability as bactericidal agents with the MIC index of ≤4 against other bacteria.

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To the best knowledge, the antibacterial activity of all compounds against *P. acnes*, *S. sobrinus* and *S. mutans* are reported for the first time.

3.4 Conclusions

Eight naturally occurring compounds have been isolated from the CH_2Cl_2 extract of *E. americana* rhizomes and confirmed their structures by spectroscopic analysis. They are identified as two naphthalenes ((+)-eleutherol, **85** and (+)-3-*epi*-hongconin, **116**), one naphthoquinone ((+)-eleutherin, **86**) and five anthraquinones (8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**99**), 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**100**), 1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone (**117**), 2,3-dihydroxy-8-methoxy-1-methyl-9,10-anthraquinone (**118**), and 4-hydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthraquinone (**119**)). Amongst these compounds, **118** and **119** are reported for the first time as new compounds, whereas **116** and **117** are addressed as new naturally occurring compounds for the first time.

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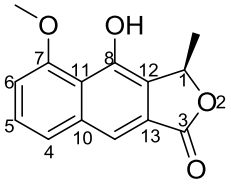

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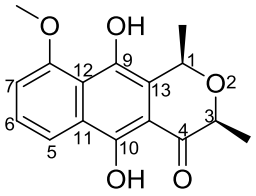

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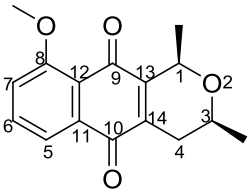

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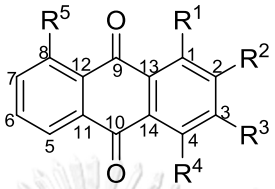

99: $R^1 = CH_3$, $R^2 = CO_2CH_3$, $R^3 = OCH_3$, $R^4 = OH$, $R^5 = OH$
100: $R^1 = CH_3$, $R^2 = CO_2CH_3$, $R^3 = OCH_3$, $R^4 = OCH_3$, $R^5 = OH$
117: $R^1 = OCH_3$, $R^2 = OH$, $R^3 = CH_3$, $R^4 = H$, $R^5 = OCH_3$
118: $R^1 = CH_3$, $R^2 = R^3 = OH$, $R^4 = H$, $R^5 = OCH_3$
119: $R^1 = CH_3$, $R^2 = R^3 = OCH_3$, $R^4 = OH$, $R^5 = OCH_3$

According to the antibacterial activity of isolated compounds, (+)-eleutherin (**86**) was the most active compound against all tested bacteria comparing to the others.

By broth micro-dilution method, this compound also revealed the lowest MIC values ranging from 0.24 – 7.8 μM against all tested bacteria. The suppression of Gram-positive bacteria was displayed by **116** with the MIC values of 31.25 to 62.5 μM, whereas its ability to inhibit Gram -negative bacteria was shown at 125 μM of MIC value. For anthraquinone series, the antibacterial effect of **118** revealed the MICs slightly lower than other anthraquinones especially its ability to suppress the growth of *S. typhi* at 31.25 μM.

Bactericidal agent was performed by **85**, **117** and **118** against all tested bacteria while **86** and **116** showed their bactericidal effect against the tested bacteria except

P. acnes and *S. aureus*, respectively. To the best knowledge, the antibacterial activity of all compounds against *P. acnes*, *S. sobrinus* and *S. mutans* was addressed for the first time in chemical literature.

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CHAPTER IV

FURTHER STUDY ON ANTIBACTERIAL, ANTI-ALLERGIC AND ANTICANCER ACTIVITIES OF ISOLATED COMPOUNDS FROM *D. cochinchinensis* and *E. americana*

4.1 Introduction

In recent years, approximately 80% of the world population depends on traditional herbal medicines as a part of standard healthcare [85]. Healing potential of plants as traditional medicines against many kinds of diseases have been known for thousands of years. Plants produce series of organic compounds generally represent of secondary metabolites showing the medicinal properties against several diseases such as cancer, tumour, inflammatory and infectious diseases [86]. Therefore, "plant" is one of natural sources which played important roles in development of sophisticated traditional medicine systems particularly for drug discovery as antibacterial, anticancer, anti-inflammatory, antitumor, *etc*. Fifteen isolated compounds from *D. cochinchinensis* and *E. americana* as reported in previous chapters exhibited potential for curing various types of diseases. Hence, further studies of those isolated compounds were carried out to discover other biological activities particularly as antibacterial, anti-allergic and anticancer agents. Due to the limited amount of some compounds, fourteen compounds were further evaluated their antibacterial activity in

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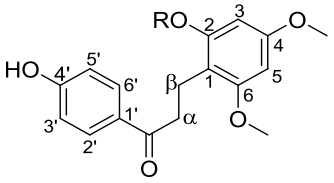
combination with CHL and only nine compounds were selected for further study on anti-allergic and anticancer activities as figured out below.



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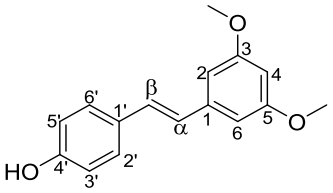
23: R = H



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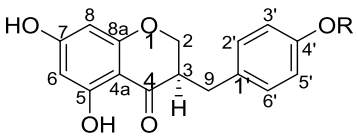
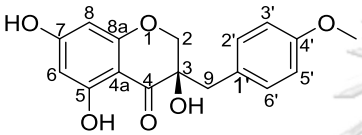


82: R = CH_3
84: R = H



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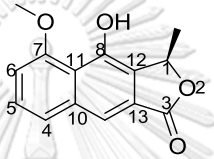

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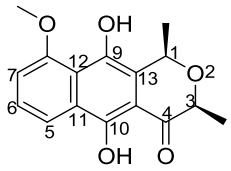

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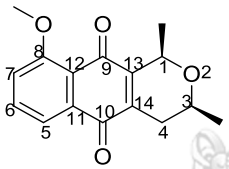

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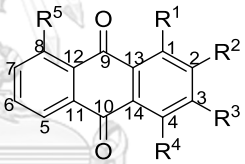

99: R^1 = CH_3, R^2 = CO_2CH_3, R^3 = OCH_3, R^4 = OH, R^5 = OH
100: R^1 = CH_3, R^2 = CO_2CH_3, R^3 = OCH_3, R^4 = OCH_3, R^5 = OH
117: R^1 = OCH_3, R^2 = OH, R^3 = CH_3, R^4 = H, R^5 = OCH_3
118: R^1 = CH_3, R^2 = R^3 = OH, R^4 = H, R^5 = OCH_3
119: R^1 = CH_3, R^2 = R^3 = OCH_3, R^4 = OH, R^5 = OCH_3

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4.2 Literature Reviews

4.2.1 Antibacterial Activity

Antibiotics are important drugs in curing and preventing infectious diseases caused by pathogenic bacteria. However, the treatment of infectious diseases has become less effective due to the increasing of resistant pathogenic bacteria to many commercial antibiotics. It is a rapid growing problem leading to an urgent need for

novel antibacterial agents. In many cases, the use of single antibacterial agent sometimes did not show the effective inhibitory effect to solve this problem. One of the methods to find the new antibacterial agents is the combination of two or more compounds revealing as antibacterial agents. The combination effect has become practical due to the synergistic effect commonly displaying higher effect compared to the activity of compounds and/or antibiotics alone even at lower concentration. It also could not only enhance the activity but also hinder the development of resistance mechanism of pathogenic bacteria [87].

Several reports have been recorded antibacterial activity from combination of two or more compounds with antibiotics. For instance, in 2012, Tran and co-workers [88] reported the synergistic effect between chalcone analogues with non-beta lactam antibiotics namely ciprofloxacin, CHL, erythromycin, vancomycin, doxycycline and gentamicin against methicillin-sensitive *Staphylococcus aureus* (MSSA) and methicillin-resistant *Staphylococcus aureus* (MRSA). The results showed that the combination between 2′,2-dihydroxychalcone (**122**) and doxycycline displayed synergistic effect against MSSA and MRSA in rates of increasing susceptibility of bacteria with doxycycline with MIC values of 0.125 and 0.25 μg/mL, respectively. The other combination between ciprofloxacin with **122** and 4-hydroxychalcone (**123**) exhibited synergistic effect against MRSA with MICs of 0.0625 μg/mL in both combinations.

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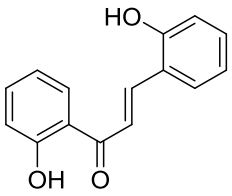

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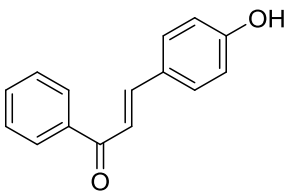
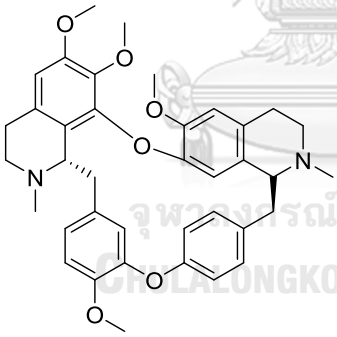


123

Zuo and co-workers in 2011 [89] conducted the antibacterial effect of tetrandrine (**124**) and demethyltetrandrine (**125**) in combination with ampicillin, axithromycin, cefazolin and levofloxacin against ten isolated of staphylococcal chromosomal cassette mec (SCCmec) III type MRSA. The significant synergies of **124** with cefazolin against 90% of the isolates were demonstrated with their MIC values ranged from 74 – 94%.



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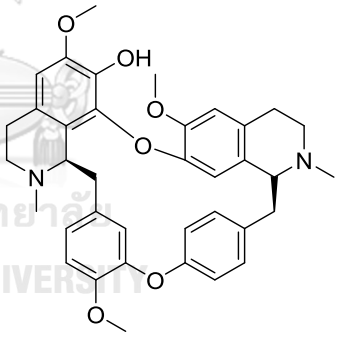

125

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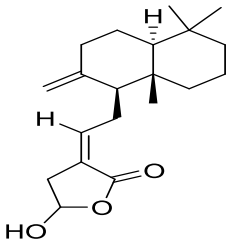
The combination activity also reported by Reuk-Ngam and co-workers in 2014 [90]. They tested the combination effect of coronarin D (**126**) with nine classical antibiotics against four Gram positive bacteria. The result showed that in combination,

126 could decrease the MIC values of oxacillin, ciprofloxacin and gentamicin with a range of sixteen to two-hundred sixty folds.



126

Joray and co-workers in 2011 [91] revealed the combination effect of 23-methyl-6-odesmethylauricepyrone (**127**) with erythromycin and gentamicin against MSSA and MRSA. The result showed that the combination between **127** with erythromycin and gentamycin against MSSA displayed synergism by decreasing the antibiotics concentration at 300 and 260 times, respectively.

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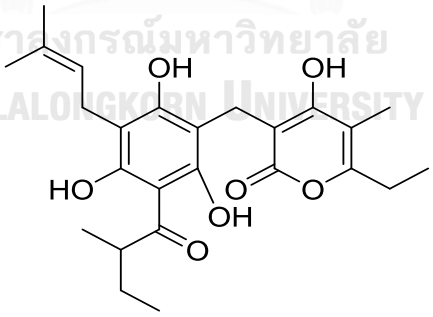
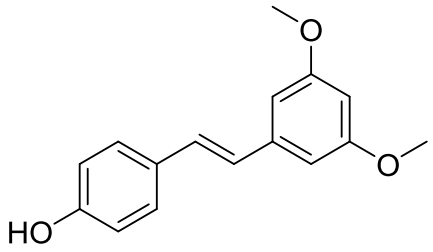


127

In 2017, Lee and co-workers [56] reported antibacterial activity of the combination between pterostilbene (**27**) with gentamicin. The results showed the

synergistic effect of the combination with the fractional inhibitory concentration index (FICI) less than 0.5 against *S. aureus* ATCC 25923, *E. coli* O157 and *P. aeruginosa* 15442.



27

4.2.2 Anti-Allergic activity

Type I of allergic disorders are caused by an exacerbated immune response to several kinds of antigens including foods, medicines, cosmetics, mold spores, dust mites and pollen which can induce the production of antigen-specific IgE antibodies that bind to receptors on mast cells or basophils [92]. The interaction of antigen-bound IgE in surface membrane receptors produce various inflammatory mediators such as cytokines, histamines, prostaglandins and leukotrienes which can induces reaction cause allergic diseases including asthma, sinusitis, food allergy, atopic dermatitis, vasodilation, mucous secretion and bronchoconstriction [93-95]. β–Hexosaminidase is also stored in the secretory granules of mast cells and basophils**.** When the cells are activated, the enzymes with histamine are released. Hence, this enzyme activity is considered to be marker of mast cells or basophil degranulations and also used for screening the anti-allergic agents [96]. Moreover, many allergies related conditions

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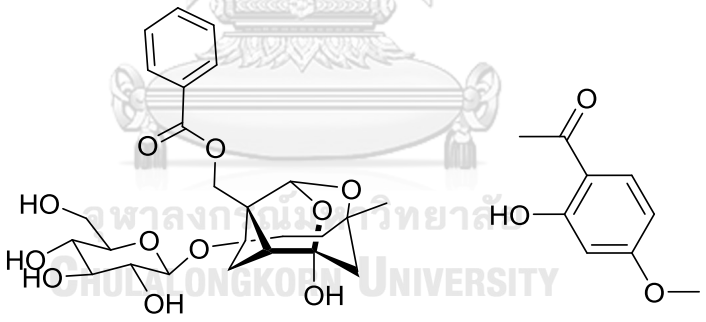
considerably have improved and can reduce the effect of diseases. However, new allergic diseases arise concurrent with changing environment and lifestyles have generated rapidly [97].

Lee and co-workers in 2008 [14] isolated paeoniflorin (**128**) and paeonol (**129**) from the roots of *Paeonia lactiflora* and evaluated their anti-allergic effect against passive cutaneous anaphylaxis (PCA) reaction induced by IgE-antigen complex and scratching behaviors induced by compound 48/80. The results revealed that **128** exhibited the most potent inhibition against scratching behaviors and the acetic acid-induced writhing syndrome in mice while **129** most potently inhibited PCA reaction and mast cells degranulation.

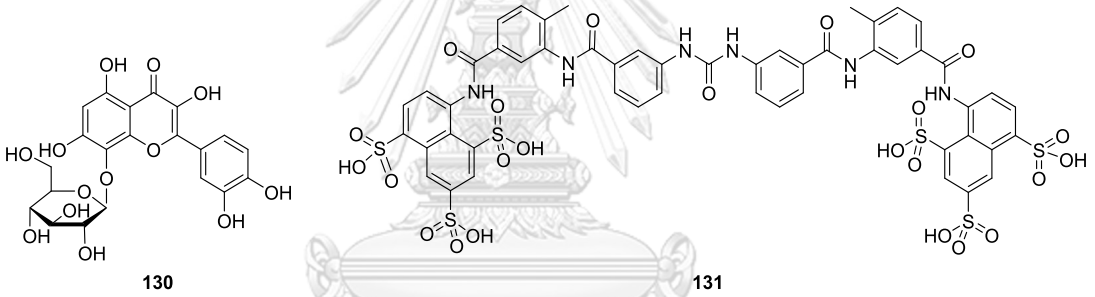


128 **129**

In 2011, Madaka and Tewtrakul [98] evaluated anti-allergic activities of the EtOH and water extracts from the rhizomes of eight selected Zingiberaceous plants using the rat basophil leukemia strain 2H3 (RBL-2H3) cells. Both EtOH and water extracts of *Boesenbergia thorelii* displayed the most potent anti-allergic effects against antigen-

induced β-hexosaminidase release as a marker of degranulation in RBL-2H3 cells with IC_{50} values of 23 and 26 μg/mL, respectively.

Ganapaty and co-workers in 2010 [13] reported the anti-allergic effect of gossypin (**130**) and suramin (**131**) in mast cells-mediated allergy model. Both **130** and **131** inhibited compound 48/80-induced systematic anaphylaxis reaction, antiprurities and reduced the histamine release in rat. For further investigation, both compounds performed significant protection against rat peritoneal mast cells activated by compound 48/80.



130 **131**

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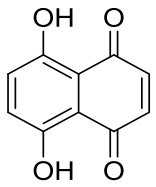
Anti-allergic activity was also conducted by Pinho and co-workers in 2014. They evaluated the anti-allergic activity of naphthazarin (**132**) and diospyrin (**133**), the naphthoquinones from the root barks of *Diospyros chamaethamnus* by inhibition of RBL-2H3 rat basophil degranulation using two complementary stimuli: IgE/antigen and calcium ionophore A23187. The results showed that with 0.1 mM, **132** reduced degranulation induced by IgE/antigen but not A23187, unlike **133** at 10 mM decreased degranulation in A23187-stimulated cells [99].



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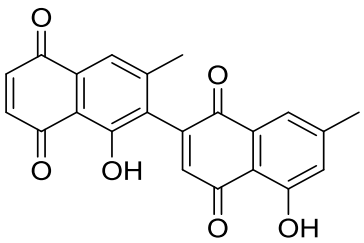

133

4.2.3 Anticancer Activity

Cancer is a group of diseases characterized by uncontrolled proliferation of abnormal cells that can spread rapidly to other tissues and organs [100]. In 2009, World Health Organization (WHO) reported that around 7.4 people worldwide died of cancer in 2004 and this number as projected will increase to 11.5 million in 2023 [101].

In recent years, though chemotherapy is still being used as a standard treatment in cancer therapy, searching and identifying for potent anticancer agents from natural products has also been increased. Natural products such as plants, marine organisms, microorganisms, lichens and animals are very important sources of promising leads for development of novel cancer therapeutics. Approximately, 60% of currently used anticancer agent are derived from the natural sources, particularly plants with more than 3000 species have been recorded as a source of potent anticancer [102].

There are significant number of compounds from synthesis and nature that have been already reported and involved in treatment of cancer.

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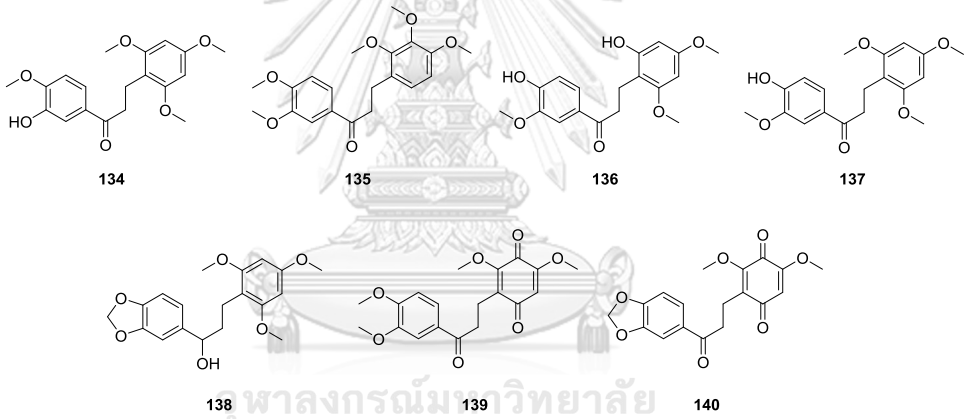
Seven retro-dihydrochalcones including taccabulins A-E (**134-138**) and evelynin A-B (**139-140**) were isolated from *Tacca* sp and investigated their antiproliferative activities against HeLa, A549 and PC-3 cancer cell lines. The results showed that the most potent antiproliferative agent was **134** followed by **139** and **140** with IC_{50} values of 0.58, 6.3 and 8.8 μM, respectively while **137** revealed its ability at 28.8 μM. Other compounds showed IC_{50} values higher than 50 μM. In addition, **140** exhibited antiproliferative against A549 and PC-3 with IC_{50} values of 8.3 and 5.0 μM, respectively followed by **137** (IC_{50} = 40.3 μM) [103].



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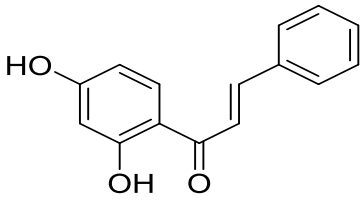
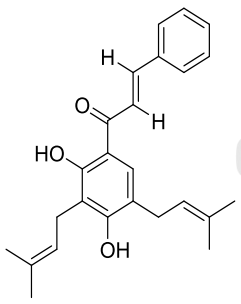
In 2009, Lou and co-workers [104] reported that 2′,4′-dihydroxychalcone (**141**) from herbal oxytropis was able to induce cytotoxicity in MGC-803. This compound could suppress the growth of gastric cancer (MCG-803) cells and induce apoptosis.



141

Caamal-fuentes and co-workers in 2015 [105] isolated four compounds from *Aeschynomene fascicularis* root bark including spinochalcone A (**142**), spinochalcone C (**143**), isocordoin (**144**) and secundiflorol G (**145**) and evaluated their potential as anticancer agents. The results showed that **142** displayed cytotoxic activity against DU-145 cell line and antiproliferative activity against KB cell line. Compound **145** exhibited strong cytotoxic effect against KB and Hep-2 cell lines. In addition, **144** revealed moderate activity against KB, Hep-2 and DU-145 cell lines.



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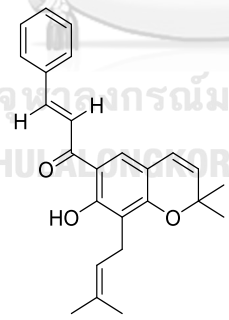

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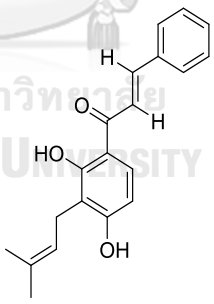

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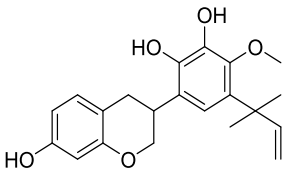

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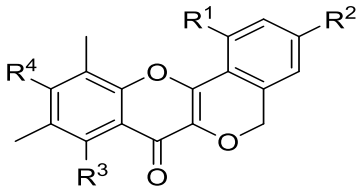
Eight new peltogynoids (bougainvinones A-H, **146-153**) were isolated from the stem barks of *Bougainvillea spectabilis* by Do and co-workers in 2016 and investigated their cytotoxicity effect against KB, HeLa S-3, HT-29, MCF-7 and HepG2 cancer cell lines.

The results revealed that **152** exhibited cytotoxicity against all tested cancer cell lines and moderate activity was shown by **147** and **148** against KB cancer cell lines [106].



146: R^1 = OH, R^2 = H, R^3 = OCH_3, R^4 = OCH_3
147: R^1 = OH, R^2 = H, R^3 = OH, R^4 = OCH_3
148: R^1 = OH, R^2 = H, R^3 = OH, R^4 = OH
149: R^1 = H, R^2 = H, R^3 = OH, R^4 = OH
150: R^1 = H, R^2 = H, R^3 = OCH_3, R^4 = OH
151: R^1 = H, R^2 = H, R^3 = OCH_3, R^4 = OCH_3
152: R^1 = H, R^2 = OCH_3, R^3 = OH, R^4 = OH
153: R^1 = H, R^2 = OCH_3, R^3 = OCH_3, R^4 = OH

In 2017, Do and co-workers addressed five new flavones (bougainvinones I-M, **154-158**) and three known congeners (**159-161**) from the stem barks of *Bougainvillea spectabilis*. Compound **158** displayed promising cytotoxicity effect against KB and HeLa S-3 cell lines while other compounds showed moderate activity against KB cell line [107].

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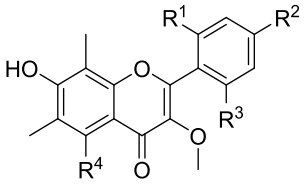

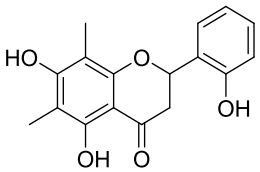

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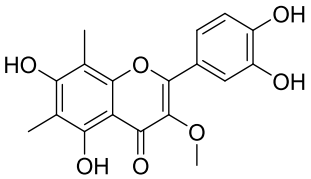

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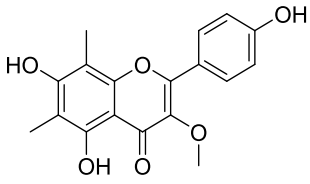
154: R^1 = OH, R^2 = H, R^3 = H, R^4 = OH
155: R^1 = OH, R^2 = H, R^3 = CH_2OH, R^4 = OH
156: R^1 = OH, R^2 = H, R^3 = CH_2OH, R^4 = OCH_3
157: R^1 = H, R^2 = OH, R^3 = H, R^4 = OCH_3
158: R^1 = H, R^2 = H, R^3 = H, R^4 = OCH_3



161

4.3 Experimental

4.3.1 Media and Chemicals

NB and commercial agar media were used for antibacterial activity assay. CHL ≥ 98% (Aldrich Sigma) was used as positive control and resazurin sodium salt (Aldrich Sigma) was used as oxidation-reduction reaction and bacterial growth indicator. All organic solvents were commercial grade purified prior to use by standard methodology except for those which were reagent grades. Eagle's minimal essential medium (EMEM) (Aldrich Sigma) and quercetin hydrate (Sigma) as positive control were used for anti-allergic assay. EMEM, McCoy's 5A medium (Sigma-Aldrich), Dulbecco's modified Eagle's medium (DMEM) (Aldrich Sigma) were used on anticancer assay and 5-fluorourasil (Sigma) was used as positive control. All commercial grade organic solvents were purified prior to use by standard methodology except for those which were reagent grades.

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4.3.2 Bacterial Strains and Cell Lines Cultures

4.3.2.1 Bacterial Strains

The microorganisms were used in this research were reported in previous chapter. The bacteria were routinely sub-cultured and maintained in nutrient agar medium.

4.3.2.2 Cell Lines Cultures

The rat basophil leukemia (RBL-2H3) cells was obtained from Riken Bioresource Center, Japan and used for anti-allergic assay, and routinely maintained in 10% FBS and 1% antibiotic (penicillin) in Eagle's minimal essential medium (EMEM). The cultures were maintained at 37°C in humidified atmosphere containing 5% CO_2 in air.

HeLa (human cervical), HCT116 (human colon carcinoma) and HepG2 (human liver carcinoma) cancer cell lines were obtained from Riken Bioresource Center, Japan. Those cells were routinely maintained in 10% FBS and 1% antibiotic (penicillin) in Eagle's minimal essential medium (EMEM) for HeLa and McCoy's for HCT116 cells while HepG2 cells were supplemented in Dulbecco's modified Eagle's medium (DMEM). All the cultivation was conducted at 37°C with constant 5% CO_2.

4.3.3 Bioassays

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4.3.3.1 The Evaluation of Synergistic Effect on Antibacterial Activity

4.3.3.1.1 Agar Well Diffusion Method

Solutions containing the combination between compound and CHL, compound alone, and CHL alone were assayed using agar well diffusion method. At first, CHL was prepared at 50 μM (1/10 preliminary screening). Two fold serial dilution for each compound was prepared from 1/20 of preliminary screening (50 to 0.781 μM). CHL was combined with each concentration of compound. These concentration ranges

were chosen based on previously obtained preliminary screening for compound and antibiotic alone. After incubation at 37°C for 24 h, antibacterial activity was observed by measuring the diameter of inhibition zone around the well and following the criteria of inhibition zone activity (mm) to determine their activity.

The experiment was carried out in triplicate and the average zone of inhibition was calculated. The criteria of inhibition zone activity (mm) were as follows: inhibition zone >15.0: excellent, 13.1-15.0: very good, 10.1-13.0: good, 8.1-10.0: moderate, 6.1-8.0: weak, ≤6.0: no activity.

4.3.3.1.2 The Broth Micro-dilution Method for Determination of MIC in Combination

To all wells were added 50 µL of NB and the serial dilution was performed using a multichannel pipette. The first compound of the combination was serially diluted along the ordinate, while the second compound was dilute along the abscissa. After that, each well was bacterial suspension 10 µL (1 to 2x10^8 CFU/mL obtained from the 0.5 McFarland standards) and 40 µL of NB. The plates were incubated at 37°C for 18-24 h. The synergistic effect has been defined as the MIC of both compounds in combination compared with each use alone, measuring the fractional inhibitory concentration index (FICI) using the equation below

$$\text{FICI} = \frac{\text{MICcompound (combination)}}{\text{MICcompound (alone)}} + \frac{\text{MICantibiotic (combination)}}{\text{MICantibiotic (alone)}}$$

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The FICI was interpreted as follows: Synergy, FICI≤0.5; additive, 0.5<FICI 1; indifference, 1<FICI ≤2; antagonism, FICI>2.

4.3.3.2 The Evaluation of Anti-Allergic Activity

RBL-2H3 cells monolayer in the culture dish was detached using trypsin and then suspended in EMEM medium (10% FBS). The cells were diluted using the culture media to $5X10^5$/mL and routinely maintained in petridish and incubated at 37°C supplemented with constant 5% CO_2.

Two 96 well plates were prepared in this assay. One for A23187 allergic reaction test and the other for cell viability assay.

4.3.3.2.1 Anti-Allergic Reaction on β-Hexosaminidase Release

100 µL of the cells suspension ($5X10^4$/well) were seeded into 96 well plate and incubated at 37°C, in 5% CO_2 for 48 h. After the cells grown to confluence for 40 h, the old medium was removed and washed with 100 µL Tydore's buffer/well. Then, after discarding the wash solution, another 100 µL of the buffer was added to each well. In order to investigate the effect of crude extracts and isolated compounds, the cells were treated with 0.5 µL of all samples (dissolved in DMSO) and control (quercetin hydrate as positive control and the buffer as blank) by varying concentrations in three replicates. The cells were incubated at 37°C with 5% CO_2 for

1 h. After incubation, all samples were removed and washed by 100 µL Tydore's buffer. Then, 100 µL of 1% A23187 in Tydore's buffer was added into the plate (for blank was only added Tydore's buffer) and incubated for 1 h at 37°C with 5% CO_2. After incubation, 50 µL of supernatant was transferred of each well to a new 96 well plate and 50 µL substrate (*p*-nitrophenyl-*N*-acetyl-β-glucosaminide dissolved in citric acid buffer) was added to it. The plates were then covered by using aluminium foil and incubated at room temperature for 3 h at 40 rpm. Then, 100 µL of the stop solution ($NaHCO_3$ buffer) was added to stop the enzymatic reaction. The colour of the reactions mixture changed to yellow indicating the release of the β-hexosaminidase. The absorbance was measured at 405 nm using the microplate reader.

4.3.3.2.2 Cell Viability

The cells were seeded in 96 well plate, the same as for the anti-allergic reaction and incubated at 37°C in 5% CO_2 incubator for 40 h. Then, old media was removed and washed by 100 µL Tydore's buffer. After discarding the wash solution, 0.5 µL of control and samples were added into the well in the same order for anti-allergic reaction and incubated at 37°C in 5% CO_2 incubator for 1 h. Then, the old media was discarded from the plate and the wells were washed by PBS. MTT reagent was prepared at concentration of 5 mg/mL in PBS. This solution was diluted 10 times using EMEM medium (10% FBS). Then, 100 µL of the solution was added to each well and incubated at 37°C with 5% CO_2 for 4 h. After incubation, the medium was discarded

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from the wells and 100 µL of stop solution (HCl-isopropanol) was added in to the wells. After that, the plate was incubated at room temperature for 4 h. Finally, the absorbance was measured at 570 nm using microplate reader.

4.3.3.3 The Evaluation of Anticancer Activity by in Vitro Cytotoxicity Assay

Day 1: Cells Preparation

The cells on the plate were washed by PBS and added 1 to 3 mL of trypsin and incubated for 3 min. After that, 1 to 3 mL of media was added with ratio of trypsin and media (1:3) and then removed the cells into the falcon tube. Centrifuged for 3 min. After centrifugation, the supernatant was removed. Added new media into the falcon tube (± 5 mL) and the cells were counted by using haemocytometer. After that the cells suspension ($5x10^5$ cells/well) were seeded into 96 well plate and incubated at 37°C, in 5% CO_2 for 24 h.

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Day 2: Treatment with Samples:

After the cells grown to confluence for 24 h, the media was removed by section and added 100 µL new media. In order to evaluate the effect of crude extracts and isolated compounds on the growth of cancer cell lines, each compound/extract with various concentrations in three replications was tested by adding 1 µL/well into the plate. The cells were treated by commercial anticancer (5-fluorourasil) as standard or positive control and 100 µL/well DMSO as negative control while for blank was only

added 100 µL/well media. Then, the cells were incubated at 37°C with 5% CO_2 for 72 h.

Day 5: MTT (3-[4,5-dimethylthiazol-2-y-2,5-diphenyl tetrazodium bromide) Assay:

After incubation for 72 h from day 2, the cells were added 10 µL MTT and incubated at 37°C with constant 5% CO_2 for 4h. After incubation, the media was removed and then added 100 µL/well of HCl-isopropanol into the plate. The plate was incubated at room temperature for another 4 h. By using microplate reader (Biotek-ELX800, BioTek), the absorbance was measured at 570 nm after 4 h of the treatment.

4.4 Results and Discussion

4.4.1 Synergistic Effect on Antibacterial Activity

Nowadays, the treatment for infectious diseases has become less effective due to the increasing pathogenic bacteria become resistant to many commercial antibiotics. Commonly, the combination between antibiotic and compound exhibited higher activity compared to the activity of antibiotics and/or compound alone. Kubo and co-workers in 1994 recorded that the synergistic effect of two or more compounds or antibiotics not only could enhance the antibacterial activity but also could hinder the increasing of resistance mechanisms of pathogenic bacteria.

In this study, the combination between CHL and isolated compounds was conducted by agar well diffusion and checkerboard method in order to increase their

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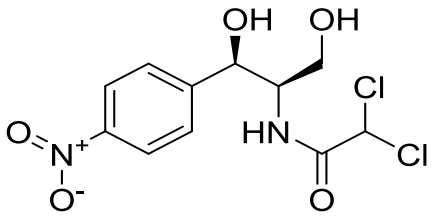
individual antibacterial activity, overcome the resistance and also minimize toxicity of CHL as well as the compounds.



Chloramphenicol

4.4.1.1 Synergistic Effect by Agar Well Diffusion Method

In synergistic study, the combination between each compound with CHL was conducted by agar well diffusion method against five pathogenic bacteria. 50 μM of CHL was combined with various concentrations of each compound by two fold serial dilution. The antibacterial activity of all compounds at 50 μM against all tested bacteria was no active with the inhibition zone less than 6 mm while the inhibitory effect of CHL at 50 μM also decreased below 10 mm against all tested bacteria.

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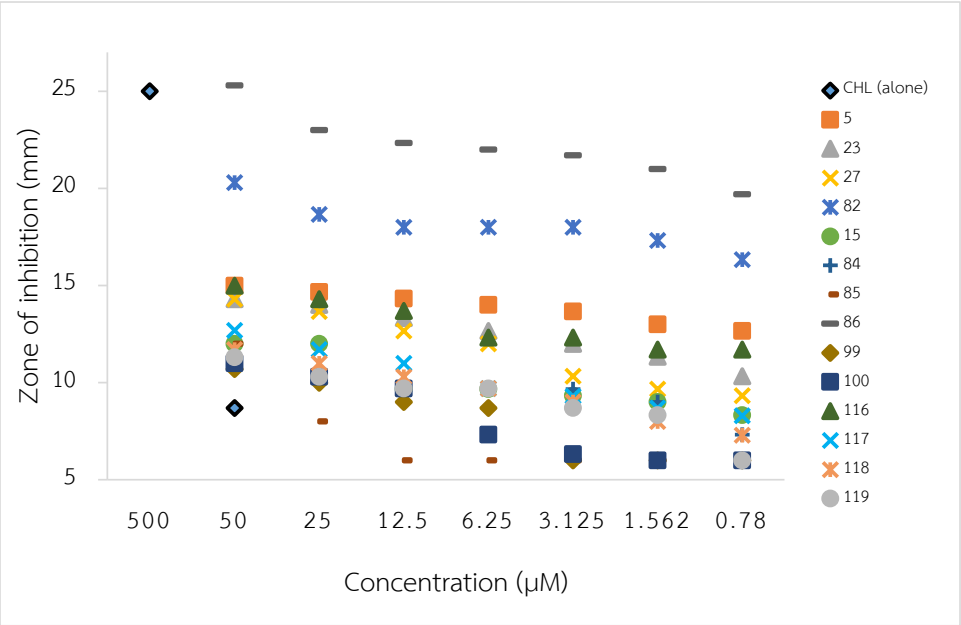

Figure 4.1 Synergistic effect of CHL and isolated compounds in combination against *P. acnes*

The synergistic effect of compounds and CHL against *P. acnes* as shown in **Figure 4.1** revealed that the most synergistic effect was observed from the combination between **86** and CHL. The existence of **86** from 50 to 0.78 μM enhanced the antibacterial activity of CHL at 50 μM. The antibacterial effect of CHL was one thousand two hundred eighty fold times higher than its ability alone while antibacterial activity of **86** in combination also increased its activity with excellent effect by showing an inhibition zone of 19.7 mm compared to its activity alone. The combination of **86** at 50 μM and CHL at 50 μM exhibited slightly high activity with inhibition zone of 25.3 mm compared to activity of CHL alone at 500 μM.

The combination also influenced the activity of **82** at lowest concentration (0.78 μM) and CHL in combination against *P. acnes*. The antibacterial effect of the

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combination revealed excellent activity with an inhibition zone of 16.3 mm higher than their activity alone while **5**, **23** and **116** in combination with CHL showed good activity at 0.78 µm and **27** in combination showed decreasing of inhibition zone of below 10 mm at 1.56 µM. However, other compounds displayed antibacterial activity below 10 mm when the concentration of those compounds in combination decreased at 6.25 µM.

The combination effect of the compounds and CHL against *S. aureus* was also revealed as presented in **Figure 4.2**.

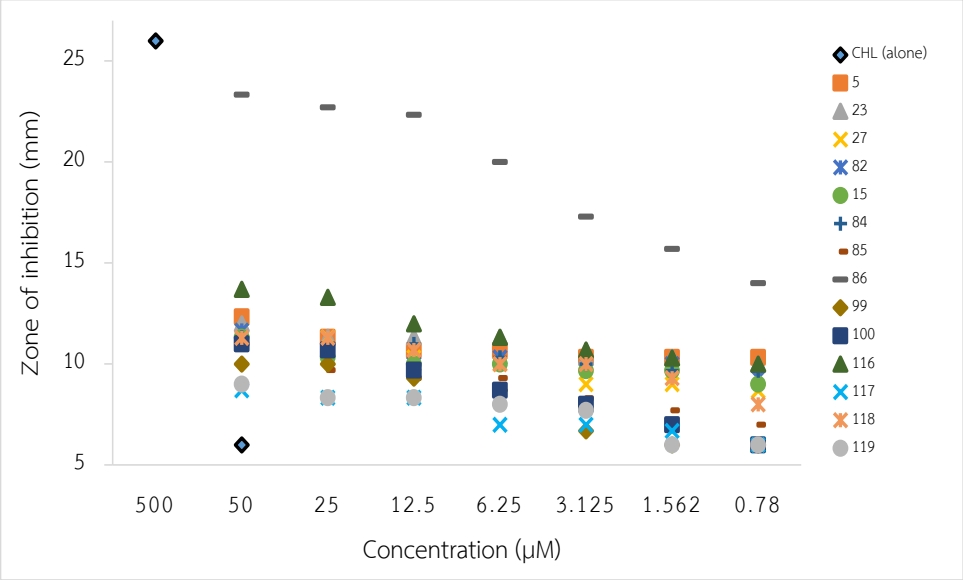

Figure 4.2 Synergistic effect of CHL and isolated compounds in combination against *S. aureus*

As shown in **Figure 4.2**, the combination of **86** and CHL also performed excellent antibacterial activity against *S. aureus* at concentration from 1.56 to 50 µM with inhibition zone ranging from 15.7 to 20.3 mm while the effect of the combination

at 0.78 µM showed very good activity with inhibition zone of 14.0 mm. However, the antibacterial activity of the combination still recorded higher activity than inhibitory effect of the compound and CHL alone. Other combinations with their concentration of 0.78 µM also displayed high activity compared to their activity alone (50 µM).

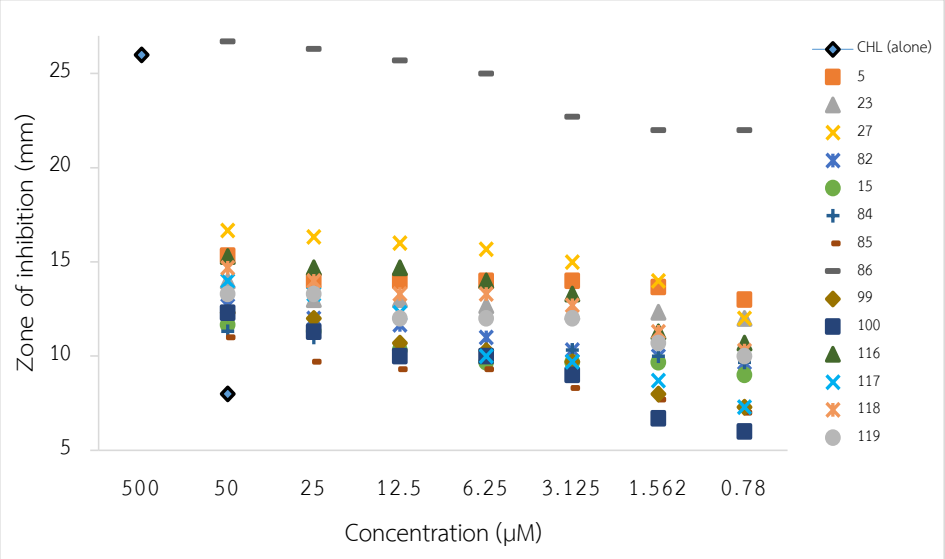

Figure 4.3 Synergistic effect of CHL and isolated compounds in combination against *S. sobrinus*

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The combination effect of the compounds and CHL against *S. sobrinus* was recorded in **Figure 4.3**. The results showed that **86** and CHL in combination performed synergistic effect at lowest concentration (0.78 µM) with inhibition zone more than 20 mm. The combination displayed excellent activity at 25 to 50 µM of **86** with inhibition zones of 26.3 and 26.7 mm respectively higher than inhibitory effect of CHL alone at 500 µM (inhibition zone = 26 mm). in addition, **27** at 3.13 to 50 µM in combination with CHL (50 µM) also exhibited excellent effect with inhibition zones ranging from 15

to 16.7 mm while other combinations also increased the activity of CHL when the concentration of the compounds at 3.125 to 50 μM. However, **85** and **100** at 1.56 μM when combined with CHL decreased their activity compared to the activity of CHL and compounds alone.

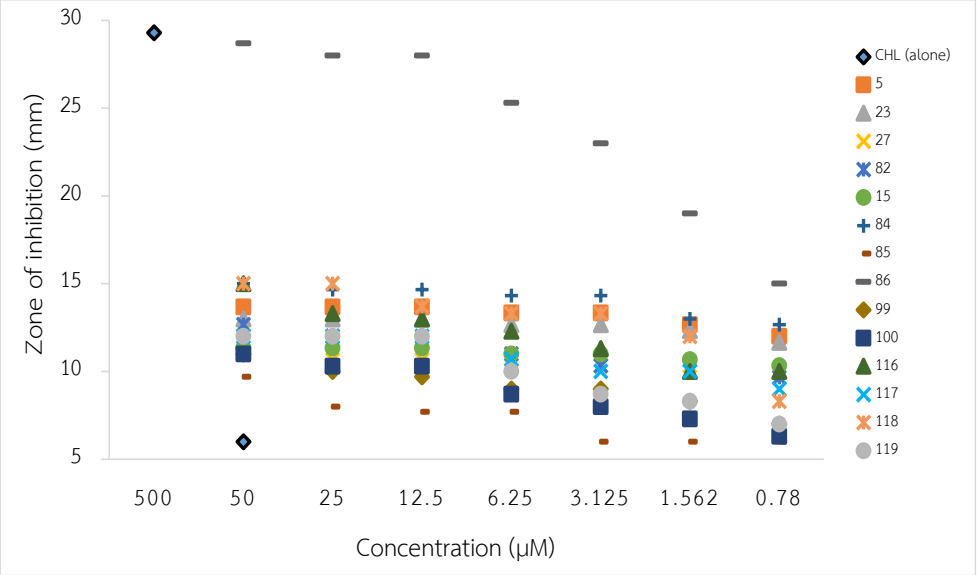

Figure 4.4 Synergistic effect of CHL and isolated compounds in combination against *S. mutans*

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The synergistic effect was shown by the combination of **86** and CHL against *S. mutans* as presented in **Figure 4.4**. The combination revealed excellent inhibitory effect when **86** at 0.78 to 50 μM combined with CHL at 50 μM with inhibition zone ranging from 15 to 28.7 mm. Furthermore, the other combinations also increased the activity of the compounds and CHL with inhibitory effect higher than their activity alone.

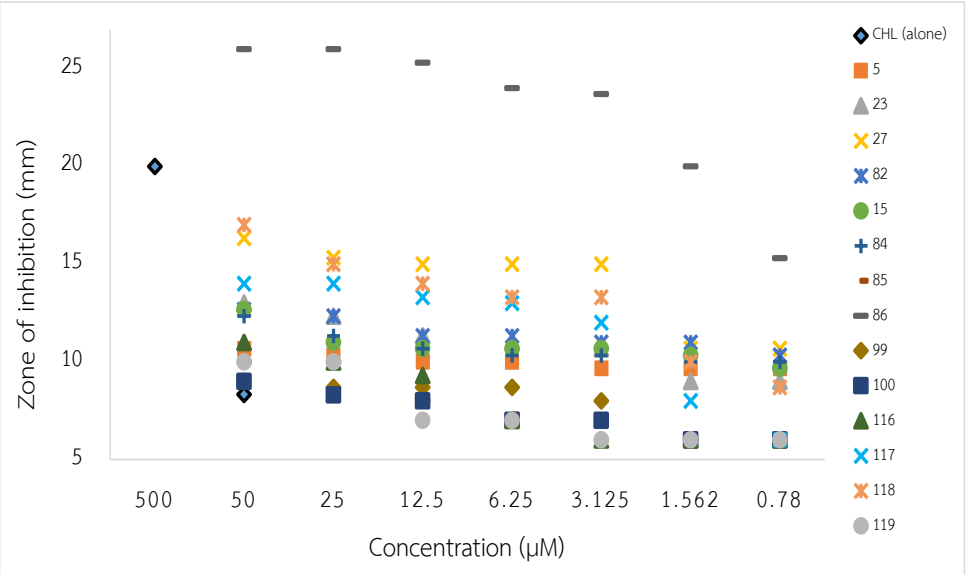

Figure 4.5 Synergistic effect of CHL and isolated compounds in combination against *S. typhi*

The antibacterial activity of CHL combined with each compound against *S. typhi* is revealed in **Figure 4.5**. The results showed that the effect of antibacterial activity of CHL in combination with **86** at 1.56 μM revealed the same inhibition zone (20 mm) with the ability of CHL alone at 500 μM. When the concentration of **86** increased from 3.13 to 50 μM, the activity of the combination also increased, higher than activity of CHL (500 μM) alone. Moreover, the increasing antibacterial activity with excellent effect was also performed by CHL and **27** in combination compared to their antibacterial effect alone, followed by the combination between CHL and **118** with inhibition zones ranging from 8.7 to 17.0 mm higher than activity of CHL and **118** alone.

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4.4.1.2 Synergistic Effect by Broth Micro-dilution Method (Checkerboard Method)

In order to improve the effect of both antibiotic and compounds, the synergism between CHL and compounds in combination on susceptibility of five pathogenic bacteria were evaluated by checkerboard method and the activity was evaluated by mean MIC in combination. The results are presented in **Table 4.1**.

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Table 4.1 Synergistic effect of Combination between Isolated Compounds and Chloramphenicol (µL)

Mix	*P. acnes*				*S. aureus*				*S. sobrinus*				*S. mutans*				*S. typhi*			
	MIC		FICI	Activity	MIC		FICI	Activity	MIC		FICI	Activity	MIC		FICI	Activity	MIC		FIC I	Activity
	Alone	Mix			Alone	Mix			Alone	Mix			Alone	Mix			Alone	Mix		
5	62.5	3.91	0.31	S	62.5	7.81	0.25	S	62.5	3.91	0.19	S	62.5	3.91	0.19	S	62.5	7.81	0.1	S
CHL	15.63	3.91			62.5	7.81			15.63	1.953			15.63	1.95			15.63	0.98	9	
23	62.5	15.63	0.75	A	62.5	7.81	0.25	S	62.5	3.91	0.31	S	62.5	3.91	0.31	S	-	-	ND	ND
CHL	15.63	7.81			62.5	7.81			15.63	3.91			15.63	3.91			-	-		
27	62.5	3.91	0.19	S	62.5	3.91	0.09	S	62.5	1.95	0.04	S	62.5	1.95	0.04	S	62.5	0.98	0.0 2	S
CHL	15.63	1.95			62.5	1.95			15.63	0.12			15.63	0.06			15.63	0.12		
82	62.5	1.95	0.09	S	62.5	3.91	0.13	S	62.5	3.91	0.07	S	62.5	3.91	0.07	S	62.5	7.81	0.1 6	S
CHL	15.6	0.98			62.5	3.91			15.63	0.12			15.63	0.12			15.63	0.49		
15	-	-	ND	ND	62.5	15.63	0.31	S	-	-	ND	ND	62.5	7.81	0.13	S	-	-	ND	ND
CHL	-	-			62.5	3.91			-	-			15.63	0.12			-	-		
84	62.5	7.81	0.37	S	62.5	15.91	0.38	S	-	-	ND	ND	-	-	ND	ND	-	-	ND	ND
CHL	15.63	3.91			62.5	7.81			-	-			-	-			-	-		
85	-	-	ND	ND	-	-	ND	ND	62.5	7.81	0.25	S	-	-	ND	ND	31.25	3.91	0.1 4	S
CHL	-	-			-	-			15.63	1.95			-	-			15.63	0.24		
86	0.24	0.007	0.04	S	7.81	0.12	0.02	S	0.24	0.03	0.12	S	3.91	0.06	0.02	S	0.98	0.12	0.1 3	S
CHL	15.63	0.12			62.5	0.06			15.63	0.06	9		15.63	0.03			15.63	0.06		
116	62.5	7.81	0.25	S	31.25	1.95	0.02	S	31.25	0.49	0.024	S	31.25	0.98	0.04	S	-	-	ND	ND
CHL	15.63	1.95			62.5	0.49			15.63	0.12			15.63	0.06			-	-		
118	-	-	ND	ND	-	-	ND	ND	62.5	3.91	0.125	S	-	-	ND	ND	31.25	1.95	0.0 8	S
CHL	-	-			-	-			15.63	0.98			-	-			15.63	0.24		

ND: not determined, S: Synergy, A: Additive

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From **Table 4.1**, the most synergistic effect against *P. acnes* was revealed by the combination between CHL and **86** with MIC values in combination of 0.12 and 0.007 μM, respectively. The increasing antibacterial activity of CHL and **86** in combination was one hundred thirty and thirty-five fold times more than their activity alone, respectively. Other synergistic effect was noticed by the combination of CHL and **82** with decreasing the MIC values of 0.98 and 1.95 μM, respectively with the rates in increasing of activity both CHL and **82** were sixteen and thirty-two folds compared to their activity alone. In addition, the synergism against *S. aureus* was shown by the combination of CHL and **86** with the rate of antibacterial activity of one thousand and sixty-five fold times than their ability alone, respectively and followed by combination of CHL and **116** with increasing the inhibitory effect both CHL and **116** as one hundred twenty and sixteen, respectively. Compound **86** in combination with CHL also showed the most active combination against *S. sobrinus*, *S. mutans* and *S. typhi* by increasing the activity of CHL in combination as 260, 521 and 260 fold times, respectively while the inhibitory effect of **86** increased higher than its ability alone.

According to FICI, all the combinations showed synergistic effect against *S. aureus*, *S. sobrinus*, *S mutans* and *S. typhi* with FICI less than 0.5. Furthermore, additive effect was shown by the combination between CHL and **23** against *P. acnes* with the FICI value of 0.75 while other combinations displayed synergistic effect.

This study documented that antibacterial activity of all studied compounds with CHL in combination were reported for the first time.

4.4.2 Anti-allergic Activity by A23187-Induced Stimulation in RBL-2H3- Cells

Inhibitory effect of two crude extracts (the CH2Cl2 extracts of *D. cochinchinensis* and *E. americana*) and nine isolated compounds on the release of β-hexosaminidase in rat basophil leukemia (RBL-2H3) cells by calcium ionophore A23178-induced stimulation were investigated by the modified method [92]. β-Hexosaminidase is an enzyme used as a degranulation biomarker for antigen induced degranulation of both RBL-2H3 cells and rat mast cells protease (RMCP) since this enzyme is released in parallel with histamine when the cells are immunologically activated [108]. This enzyme shows the optimal activity at low pH (pH = 4.5), which is a typical condition during inflammatory processes [109]. This assay was performed by a colorimetric assay using β-hexosaminidase activity to determine the amount of granules released from RBL-2H3 cells [110]. The results are expressed as the percentage of β-hexosaminidase release into the medium are shown in **Table 4.2**.

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Table 4.2 The percentages of **β**-hexosaminidase release and cell viability of crude extracts on anti-allergic activity by A23187-induced stimulation

Entry	Extracts (50 μg/mL)	Cell viability (%)	**β**-hexosaminidase release (%)
1	CH_2Cl_2-DC	99.5	9.7
2	CH_2Cl_2-EM	99.7	21.7
3	Quercetin dihydrate[a]	98.3	29.4

[a]Positive control (20 μM)

The CH_2Cl_2 extracts of *D. cochinchinensis* (CH_2Cl_2-DC) and *E. americana* (CH_2Cl_2-EM) were investigated for anti-allergic activities on **β**-hexosaminidase release using calcium ionophore A23187-induced stimulation in RBL-2H3 cells. According to Table **4.2**, both CH_2Cl_2-DC and CH_2Cl_2-EM extracts at 50 μg/mL showed anti-allergic activities with **β**-hexosaminidase release of 9.7 and 21.7%, respectively while quercetin (positive control) at 20 μM released 29.4%. Nevertheless, those two extracts and positive control at the mentioned concentration did not adversely affect the viability of RBL-2H3 cells with the percentage of cell viability of 99.5, 99.7 and 98.3%, respectively. Since CH_2Cl_2-DC and CH_2Cl_2-EM extracts displayed potent anti-allergic, isolated compounds from those two extracts were further investigated for their anti-allergic activities. The results are presented in **Table 4.3**.

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Table 4.3 The percentages of β-hexosaminidase release and cell viability of isolated compounds on anti-allergic activity by A23187-induced stimulation

Entry	Compound (100 μM)	Cell viability (%)	β-hexosaminidase release (%)
1	**5**	98.3	6.8
2	**23**	99.5	28.5
3	**27**	98.8	27.3
4	**82**	99.4	16.4
5	**15**	100.1	23.5
6	**84**	100.2	34.5
7	**85**	99.7	29.5
8	**86**	99.8	15.9
9	**110**	99.6	11.9

In the preliminary screening on anti-allergic are presented in **Table 4.3**, nine compounds with 100 μM showed low percentage of β-hexosaminidase release from the cells with approaching 100% viability of the cells indicating that all isolated compounds inhibited the release of the enzyme without giving negative affect to the viability of the cells. Therefore, all these compounds had potential to be effective treatments for allergies. Compound **5** showed better activity than the others with 6.8% of β-hexosaminidase release followed by **110**, **86** and **82** with the percentage of the enzyme release of 11.9, 15.9 and 16.4%, respectively. Good inhibitory effect of **15**, **27**, **23** and **85** was exhibited by the presence of β–hexosaminidase release ranged from 23.5 to 19.5% while **84** showed moderate effect with 34.6% of the enzyme release

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from RBL-2H3 cells. In addition, all compounds were not toxic to RBL-2H3 cells which are showed viability of the cells more than 90%.

Due to the percentage of β–hexosaminidase release of all compounds were lower than 50%, further investigation of those compounds on anti-allergic activity was conducted. By dose dependent manner with varying concentration started from 100 μM, those compounds were evaluated using the same method with the preliminary screening. The % inhibition of the β–hexosaminidase release by all compounds was calculated by the linier equation of each compound and IC_{50} values were graphically determined. The results of IC_{50} of each compound could be obtained as presented in **Table 4.4**.

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Table 4.4 The IC_{50} values of isolated compounds on anti-allergic activity

Entry	Compound	IC_{50} (μM)
1	**5**	7.8
2	**23**	15.8
3	**27**	17.5
4	**82**	20.2
5	**15**	36.1
6	**84**	46.0
7	**86**	27.4
8	**85**	40.5
9	**100**	34.1
10	Quercetin dihydrate[a]	2.9

[a]Positive control

As shown in **Table 4.4**, **5** possessed the highest activity with IC_{50} value of 7.8 μM which was two folds lower than **23** (IC_{50} 15. 8 μM). This indicated that changing methoxyl group to hydroxyl slightly decreased anti-allergic activity of **23**. Previous study reported that **5** decreased the Ca^{2+} influx in Jurkat T cells and suppressed cytokine by inhibiting phytohemagglutanin (PHA)-induced IL-2 release which was one of the inflammatory mediators [111].

Compound **27** displayed anti-allergic activity by releasing β–hexosaminidase from RBL-2H3 cells in dose-dependent manner with IC_{50} value of 17.5 μM. Investigation

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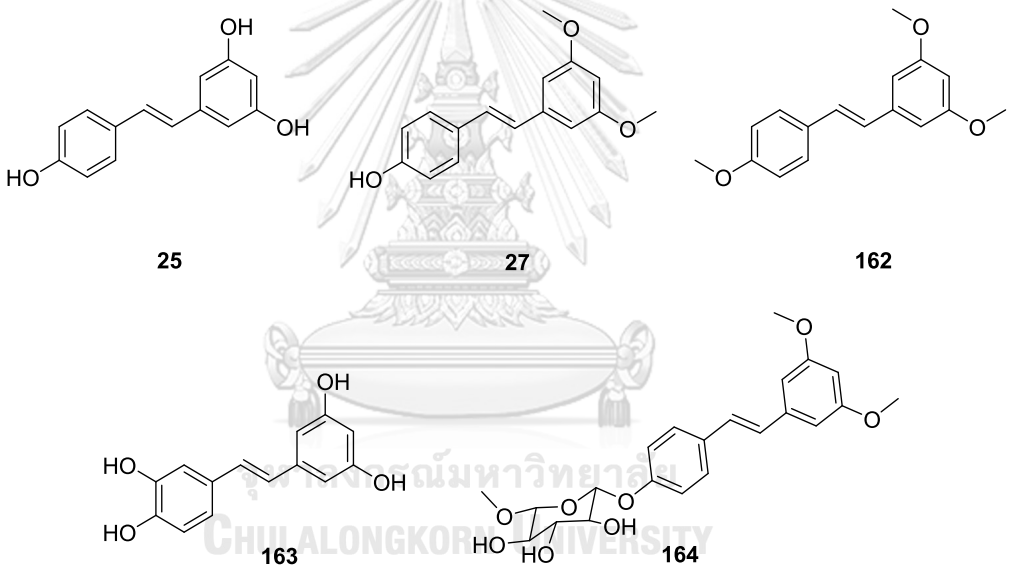
of anti-allergic activity on β–hexosaminidase release by Cheong in 1999 expressed that replacing hydroxyl groups in aromatic rings of resveratrol (**25**) to methoxyl (trimethylresveratrol, **162**) decreased the anti-allergic activity with the IC_{50} values of 15.3 and 56.2 μM, respectively [108]. In comparison between resveratrol (**25**) and its analogues (**27** and **162**) on β–hexosaminidase release revealed that anti-allergic activity of **27** bearing two methoxyl groups at atom carbon 3′ and 4′ positions slightly decreased comparing with **25** whereas **27** was three times more active than **162**.



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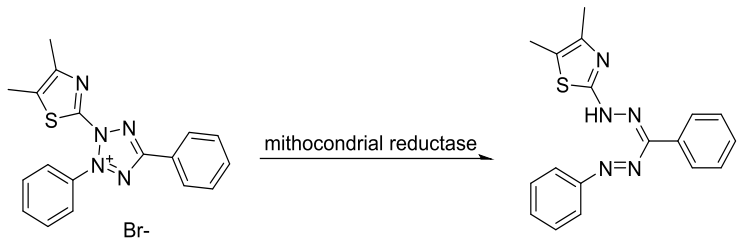
In 2014, Hamada and co-workers reported that anti-allergic activities of **25** and **27** showed little inhibitory effect with the percentage of histamine release (41 and 43%) from the rat peritoneal mast cells (RPMS), respectively. On the other hand, the anti-allergic activity of **163** significantly decreased the histamine release at 5% from the cells and **164** slightly higher than **25** and **27** on the histamine release (35%) [112].

This indicated that potential of anti-allergic on β–hexosaminidase and histamine release with lower IC_{50} values were observed for stilbenoids bearing most hydrophilic group.

Compound **82** also showed potent anti-allergic on β–hexosaminidase release and followed by **86** with IC_{50} values of 20.2 and 27.4 μM, respectively. Moderate activity was performed by **100** and **15** with IC_{50} values of 34.1 and 36.1 μM whereas **85** and **84** revealed IC_{50} values slightly high at 40.5 and 46.0 μM, respectively.

4.4.3 Anticancer Activity by *in Vitro* Cytotoxicity Assay

The anticancer activity of extracts and isolated compounds mediated cytotoxicity against HeLa, HCT116 and HepG2 cell lines were conducted by enzyme-based method using MTT to assign cell viability with colorimetric method. The yellow MTT is reduced by mitochondrial succinate dehydrogenase to an insoluble purple formazan. This method is safe, can be widely and easily used in both cell viability and cytotoxicity tests with high reproducibility [113, 114].



3-(4,5-dimethilthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

(E,Z)-5(4,5-dimethylthiazol-2-yl)-1,3-diphenylformazan (formazan)

The CH_2Cl_2-DC and CH_2Cl_2-EM were preliminary tested for their cytotoxicity against HeLa, HCT116 and HepG2 cancer cell lines as presented in **Table 4.5**. With 100 µg/mL, CH_2Cl_2-DC suppressed the growth of HeLa, HCT116 and HepG2 cell lines with the percentages of cell viability of 13.5, 29.9 and 18.3%, respectively while the percentages of cell viability of CH_2Cl_2-EM were 8.5, 13.3 and 6.1, respectively. Moreover, the CH_2Cl_2-EM against tested cancer cells showed significant cytotoxicity with IC_{50} values of 2.0, 19.9 and 5.1 µM, respectively. CH_2Cl_2-DM also showed potential to inhibit the growth of HeLa and HepG2 cell lines with IC_{50} values of 2.4 and 12.3 µM, respectively whereas its cytotoxicity against HCT116 was moderate. However, those extracts sill active in the growth of inhibition assay.

Table 4.5 The cell viability and IC_{50} values of CH_2Cl_2 extracts

Entry	Extracts (100 µg/mL)	Cell viability (%)			IC_{50} (µM)		
		HeLa	HCT116	HepG2	HeLa	HCT 116	HepG2
1	CH_2Cl_2-DC	13.5	29.9	18.3	2.4	50.4	12.3
2	CH_2Cl_2-EM	8.5	13.3	6.1	2.0	19.9	5.1
3	5-Fluorouracil[a]	7.4	8.9	5.8	0.6	3.2	0.7

[a]Positive control (10 µM)

DC (*D. cochinchinensis*), EM (*E. americana*)

Based on the preliminary screening of those extracts on cytotoxicity assay. Nine isolated compounds from both extracts were further evaluated their cytotoxicity

against CCD841 (normal cells) and cancer cell lines using the same method with preliminary test. The cytotoxicity of the compounds against CCD841 cells by showing the percentage of cell viability was shown in **Table 4.6**.

Table 4.6 The cytotoxicity of isolated compounds against CCD 841 cells

Entry	Compound (50 μM)	Cell viability (%)
1	**5**	98.8
2	**23**	94.4
3	**27**	94.8
4	**82**	92.8
5	**15**	92.9
6	**84**	96.4
7	**85**	102.8
8	**86**	17.5
9	**100**	101.9
10	5-Fluorouracil*	84.6

*Positive control

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The cytotoxicity of the compounds (IC_{50} values) could be obtained by calculating the linier equation of each compound as presented in **Table 4.7**.

Table 4.7 The IC_{50} values of isolated compounds on anticancer activity

Entry	Compounds	IC_{50} value (μM)		
		HeLa	HCT116	HepG2
1	**5**	8.3	22.9	45.3
2	**23**	1.4	34.1	17.5
3	**27**	1.4	65.4	22.8
4	**82**	18.4	15.5	14.0
5	**15**	14.6	49.2	82.1
6	**84**	>100	>100	20.3
7	**86**	0.3	4.6	6.7
8	**85**	>100	50.5	58.5
9	**100**	>100	34.1	15.7
10	5-Fluorouracil*	0.6	3.2	0.7

*Positive control

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As seen in **Table 4.7**, **86** was found to be the most active among all the compounds against HeLa, HCT116 and HepG2 cell lines with IC_{50} values of 0.3, 4.6 and 6.7 μM, respectively. It was also reported that **86** showed twice more potent than the standard agent 5-fluorouracil (commercial anticancer drug) which demonstrated IC_{50} value of 0.6 μM against HeLa cell lines. Moreover, **23** and **27** also showed significant cytotoxicity activity against HeLa cell lines (IC_{50} values of 1.4 μM) followed by **5**, **15** and **82** with IC_{50} values of 8.3, 14.6 and 18.4 μM. However, **84**, **85** and **100** could be

regarded as inactive against the cells (IC_{50} value >100 μM). On the other hand, the cytotoxicity effect of **82** and **5** against HCT116 cell lines exhibited high activity with IC_{50} values of 15.5 and 22.9 μM, respectively and followed by **23** and **100** showed moderate activity (IC_{50} value of 34.1 μM) whereas **15**, **85** and **27** were weakly cytotoxicity with IC_{50} ranging from 49.5–65.4 μM and **84** was inactive with IC_{50} value more than 100 μM. The most cytotoxicity effect against HepG2 cell lines was demonstrated by **86** with IC_{50} value of 6.7 μM and followed by **82**, **100** and **23** (IC_{50} values of 14.0, 15.7 and 17.5 μM), respectively while **84** and **27** showed IC_{50} values of 20.3 and 22.8 μM, respectively.

From the results, **86** was a potential anticancer agent with highest cytotoxicity against tested cancer cell lines. A literature search recorded few reports regarding to biological activities of quinones. Previous study reported that quinonoid moiety has been shown multiple anti-proliferative activity against a broad spectrum of organisms and cancer cells, particularly to confer *in vitro* anti-topoisomerase activity [115]. Compound **86** has been identified as catalytic inhibitor of specific topoisomerase II which is known as highly active in cells which are rapidly proliferating [68, 116]. Sperry and co-workers also reported that **86** was more active than **165** to inhibit human topoisomerase IIα (hTopIIα) whereas other natural pyranonapthoquinones (**166** and **167**) exhibited weak hTopIIα inhibition [115]. This indicated that the substitution of oxygenated at C-8 could be important for inhibitory activity.

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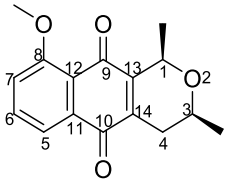

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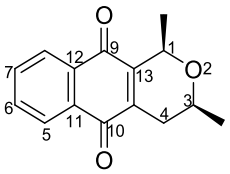

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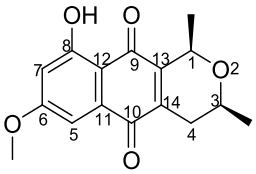

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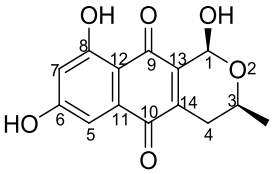

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In addition, Naysmith and co-workers in 2017 documented that either **86** or **87** were particularly active against glioma and breast cancer cell lines. However, **86** generally performing higher activity than **87** [117].

In addition, dihydrochalcones (**5** and **23**) were demonstrated anticancer activity against HeLa, HCT116 and HepG2 cell lines. Compound **23** displayed six times more potent anticancer activity than **5** against HeLa cell lines. It also showed higher activity than **5** against HepG2 cell lines. The presence of hydroxyl group at C-2 position (**23**) enhanced anticancer activity against those both cell lines. It slightly different from bearing methoxyl group at the same position of **5** with lessen activity. However, the cytotoxicity effect against HCT116 cell line of **5** was more active than **23**.

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Compound **27** also displayed similar significant cytotoxicity effect with **23** against HeLa cells. It also showed high anticancer activity against HepG2 cells whereas weak inhibitory effect was revealed by this compound. The previous study reported structural anticancer activity of **27** and its series against HeLa cells. The introduction of smaller methoxyl or dimethylaminomethyl side chains into the C-3 position of **27** was to induce anticancer activity against HeLa cells while pterostilbene derivative containing a pyrrolidin-1-ylmethyl group substituent at C-3′ position showed similar

potent to **27**, and the presence of larger dialkylaminomethyl side chain into C-3′ position of **27** was to decrease the anticancer effect. In this case, methoxyl and dimethylaminomethyl groups were suitable substituents at the C-3′ position of **27**. The presence of all dialkylaminomethyl side chain into C-5′ position of 3′-methoxypterostilbene decreased the activity. Investigation by Liu and co-workers in 2015 revealed that the inhibitory effect among these compounds against HeLa cells could be related to the hydrophobicity and permeability changes resulting from different substituents and position of side chains in **27** [118]. The anticancer activity of **27** was also reported by the proteomic approaching in HepG2 cells. The results showed that **27** induced apoptosis by altering the expression of apoptotic genes and the G2/M phase of cell cycle arrest [119]. These previous studies supported the finding results in this research that **27** was a potent anticancer agent against both HeLa and HepG2 cell lines. However, the anticancer effect of **27** showed weak activity against HCT116 cell. In contrast, **27** decreased the protein levels of β-catenin, cyclin D1 and c-MYC, altered the cellular localization of β-catenin and inhibited the phosphorylation of p65 in HT-29 cell lines which is one of the types of colon cancer cells [120]. Compound **27** was a more potent chemo preventive agent than resveratrol (**25**) for the prevention of colon cancer [121].

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Another interesting observation for cytotoxicity effect against HeLa, HTC116 and HepG2 cell lines could also be found from homoisoflavanone series. Compound **15** was slightly more active than **82** against HeLa cell line whereas **15** was less potent

than **82** against HCT116 and HepG2 cell lines and **84** was inactive against HeLa and HCT116 cell lines whereas this compound increased anticancer activity against HepG2 more than almost four folds than **85** but slightly lower than **82**.

4.5 Conclusions

The biological activities including synergistic effect on antibacterial, anti-allergic and anticancer of fourteen compounds from *D. cochinchinensis* and *E. americana* have been further evaluated.

The further study on antibacterial activities of the isolated compounds in combination with CHL revealed that all combinations increased the activity of CHL as well as the compounds against all tested bacteria. The most synergistic effect against all tested bacteria was displayed by the combination between CHL and **86** by increasing the antibacterial activity of both CHL and **86** compared to their activity individually.

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For anti-allergic activity, **5** exhibited the strongest activity with IC_{50} value of 7.8 μM followed by **23** and **27** with IC_{50} values of 15.8 and 17.5 μM, respectively while other compounds showed their anti-allergic effect with IC_{50} values more than 20 μM but less than 50 μM.

Among isolated compounds, **86** possessed significant cytotoxicity against HeLa, HCT116 and HepG2 cancer cell lines with IC_{50} values of 0.3, 4.6 and 6.7 μM, respectively. However, the cytotoxic potential of **86** against HeLa twice more active

than positive control (5-fluorouracil) with IC_{50} value of 0.6 μM and its activity slightly different comparing with that of 5-fluorouracil that could suppressed the growth of HCT116 cell line. Compound **23** together with **27** also showed strongly cytotoxicity against HeLa cell line at 1.4 μM while **5**, 1**5** and **82** could suppress the growth of the cells with IC_{50} values less than 20 μM. In addition, **82** displayed cytotoxicity effect against HCT and HepG2 cell lines at 15.5 and 14 μM, respectively while **23** and **100** performed IC_{50} values of 17.5 and 15.7 μM, respectively.

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CHAPTER V

CONCLUSION

The isolation and purification of the CH_2Cl_2 extracts of *Dracaena cochinchinensis* and *Eleutherine americana* afforded fifteen compounds including two new (**118-119**), two naturally occurring natural compounds (**116-117**) and eleven known compounds. By means of spectroscopic data and X-ray analysis (in certain compounds), the structures of isolated compounds were identified as loureirin B (**5**), (3*R*)-eucomol (**15**), 2′,2-dihydroxy-4,6-dimethoxydihydrochalcone (**23**), pterostilbene (**27**), (3*R*)-5,7-dihydroxy-3-(4-methoxybenzyl) chroman-4-one (**82**), 7-*O*-methyl-eucomol (**83**), (3*R*)-5,7-dihydroxy-3-(4-hydroxybenzyl)chroman-4-one (**84**), (+)-eleutherol (**85**), (+)-eleutherin (**86**), 4,8-dihydroxy-3-methoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**99**), 8-hydroxy-3,4-dimethoxy-1-methylanthraquinone-2-carboxylic acid methyl ester (**100**), (+)-3-*epi*-hongconin (**116**), 1,8-dimethoxy-2-hydroxy-1-methyl-9,10-anthraquinone (**117**), 2,3-dihydroxy-8-methoxy-1-methyl-9,10-anthraquinone (**118**) and 4-dihydroxy-2,3,8-trimethoxy-1-methyl-9,10-anthra-quinone (**119**).

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For antibacterial activity by diffusion method, **86** showed the most active compound against all tested bacteria with inhibition zones ranging from 14.7 to 18.0 mm, followed by **82** (12.0 to 17.3 mm) while others compound showed good to weak activity. By the broth micro-dilution method, **86** displayed the lowest MIC values

against all tested bacteria ranging from 0.24 to 7.8 μM. Moreover, the lower MIC values of **86** than those for CHL (MIC = 15.6 to 62.5 μM) were observed. In addition, other compounds revealed their antibacterial effect of to 31.25 μM against all tested bacteria. According to MBC and MIC index, the bactericidal agent against all test bacteria was showed by **5**, **15**, **23**, **27**, **82**, **85** and **117-119** while **84** revealed bactericidal effect against *P. acnes* and *S. aureus* only with MIC index ≤4. Moreover, **86** and **116** displayed bactericidal effect against four bacteria except *P. acnes* and *S. aureus*, respectively and other compounds performed bacteriostatic effect against all tested bacteria with MIC index of ≥4.

The further study on antibacterial activities by combination between isolated compounds and CHL using diffusion and checkerboard methods revealed that the most synergistic effect against all tested bacteria was observed by the combination between CHL and **86** by promoting the activity of both CHL and **86** comparing with their activity alone.

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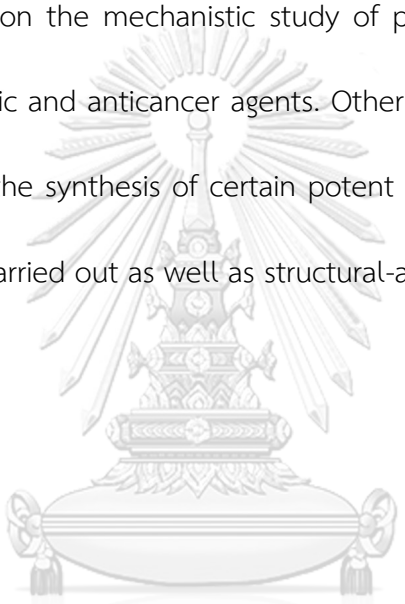
For anti-allergic activity, all isolated compounds displayed anti-allergic effect with IC_{50} less than 50 μM. The strongest activity was observed for **5** with IC_{50} value of 7.8 μM. By changing methoxyl to hydroxyl group at C-2 position of **5**, the anti-allergic activity of **23** decreased twice compared to **5**, followed by **27**. Moreover, other compounds exhibited anti-allergic with IC_{50} values ranging from 20.2 to 46.0 μM.

For anticancer activity, the most potent anticancer agent against HeLa cells was revealed by **86** with IC_{50} value of 0.3 μM followed by **23** and **27** with IC_{50} value of 1.4

μM. However, **86** displayed more active than 5-fluorourasil as commercial drug for cancer treatment. Compound **86** also suppressed the growth of HCT116 and HepG2 cancer cell lines with IC_{50} values of 4.6 and 6.7 μM, respectively.

Propose for the Future Work

To get more insight the biological activities of isolated compounds, the further research should focus on the mechanistic study of potent isolated compounds as antibacterial, anti-allergic and anticancer agents. Other biological activities should be scrutinized. Moreover, the synthesis of certain potent isolated compounds and their derivatives should be carried out as well as structural-activity relationship studies.



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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย
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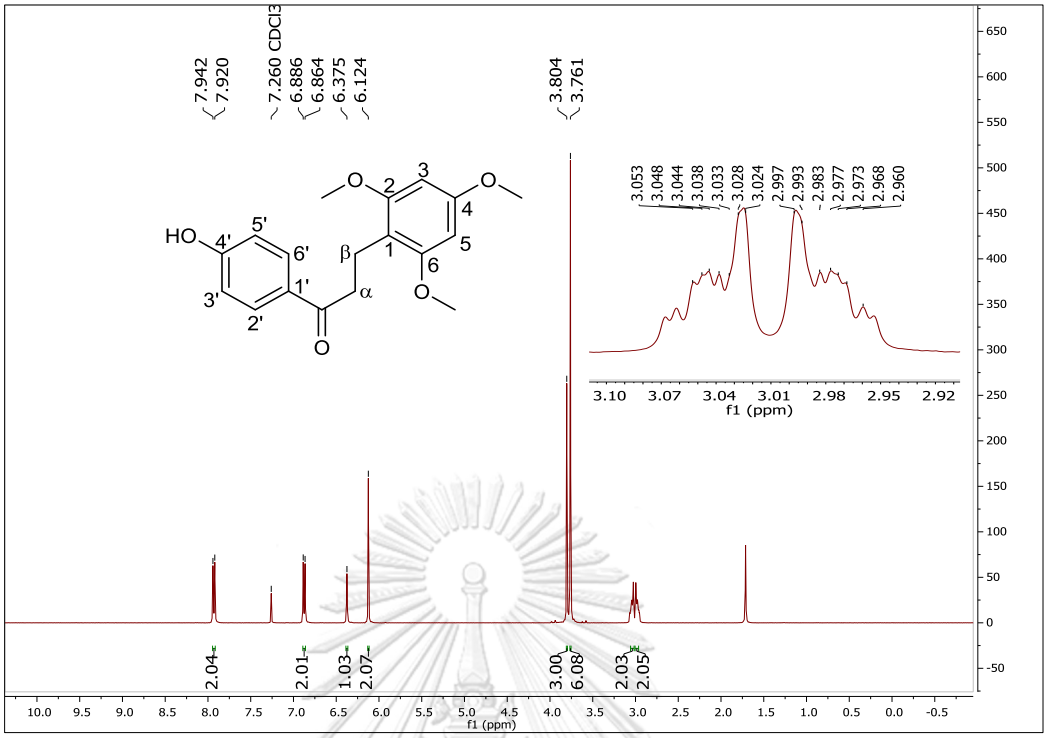

Figure A-1 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **5**

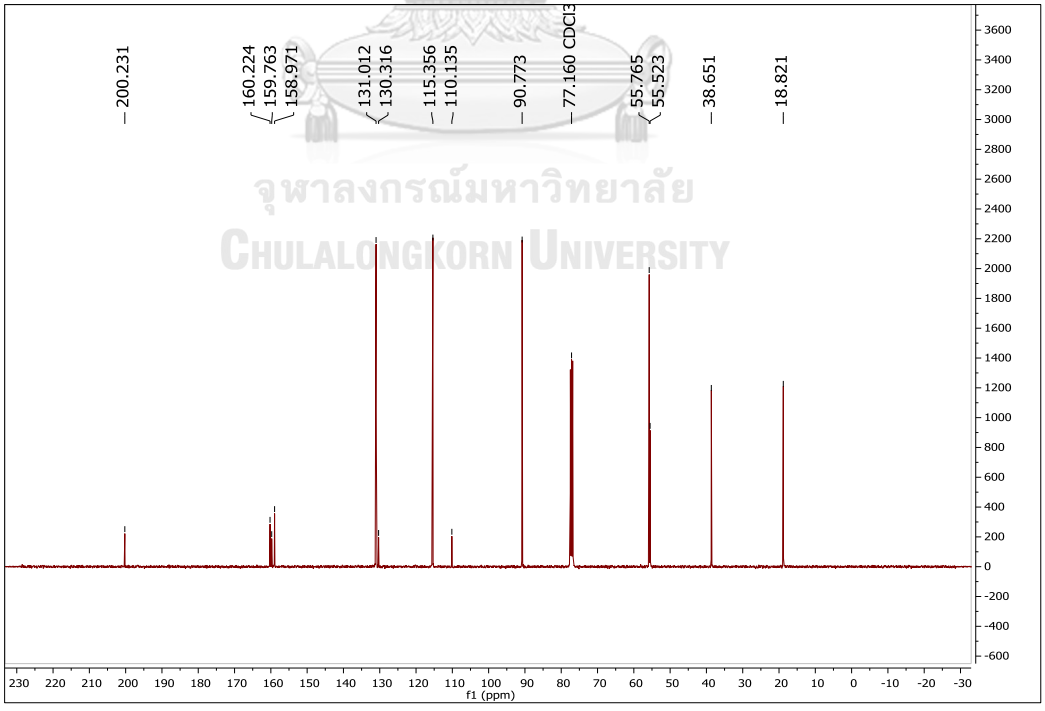

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Figure A-2 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **5**

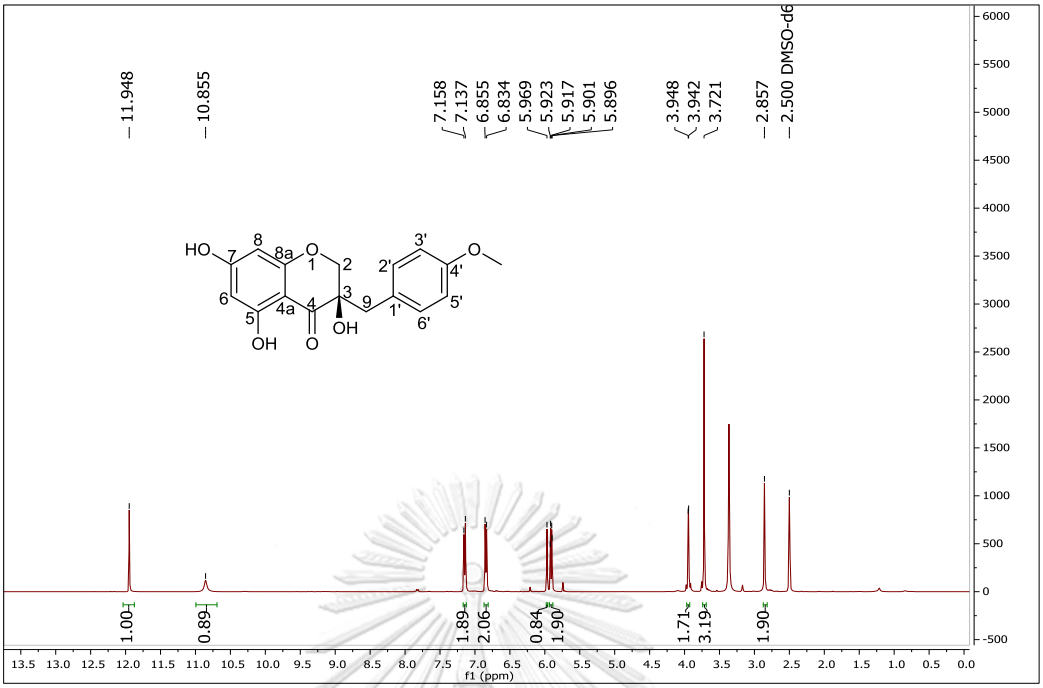

Figure A-3 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **15**

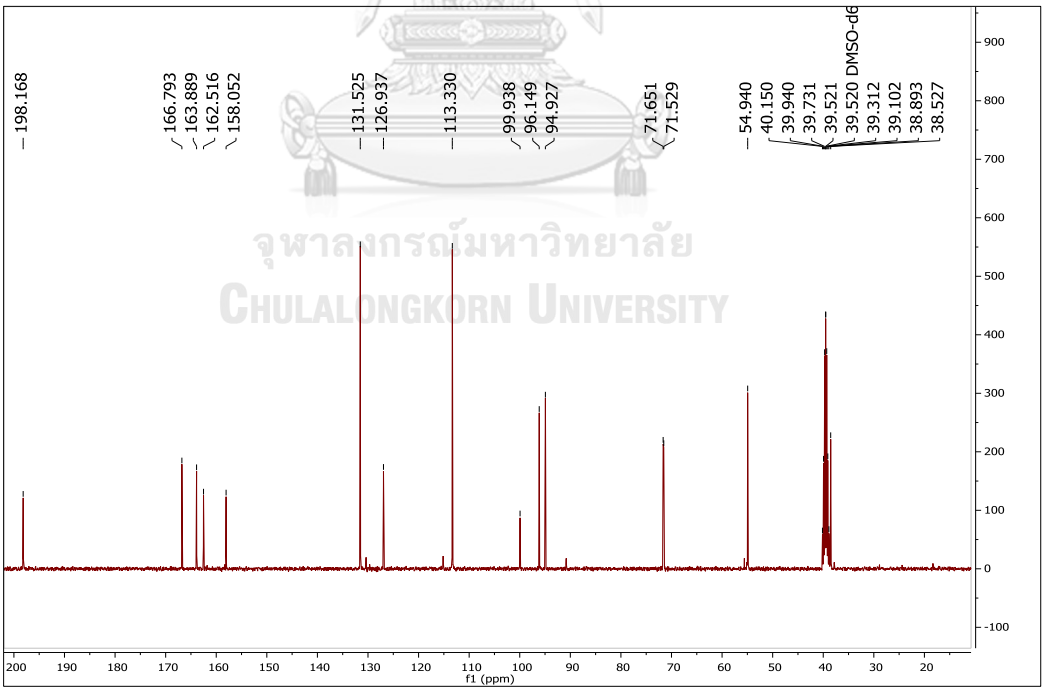

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Figure A-4 The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of **15**

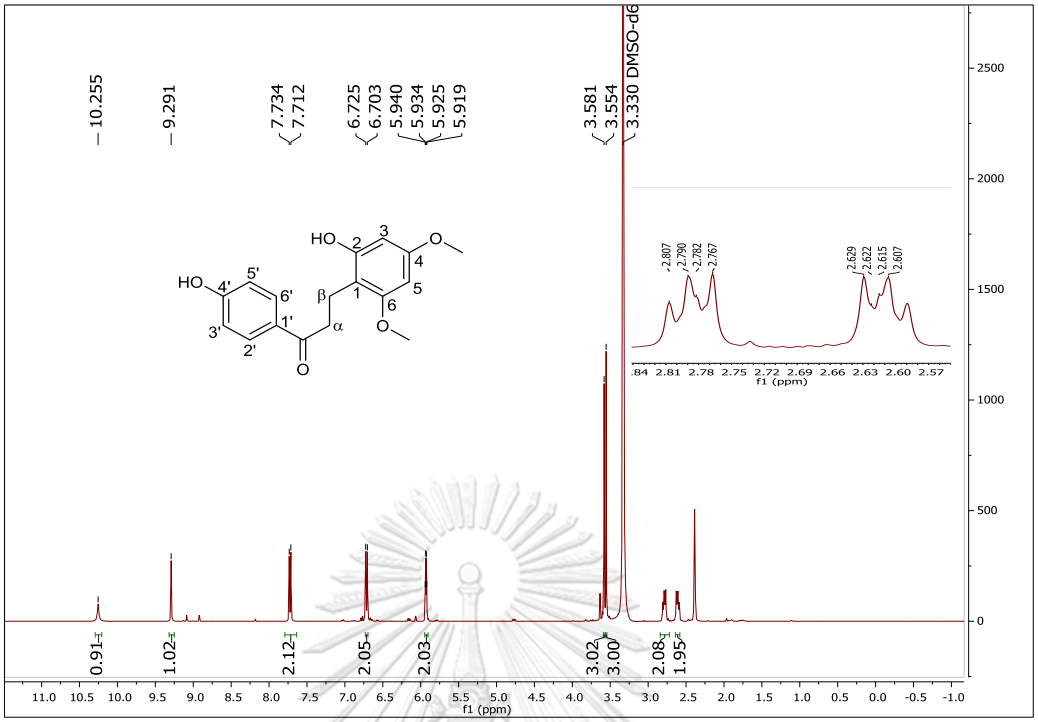

Figure A-5 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **23**

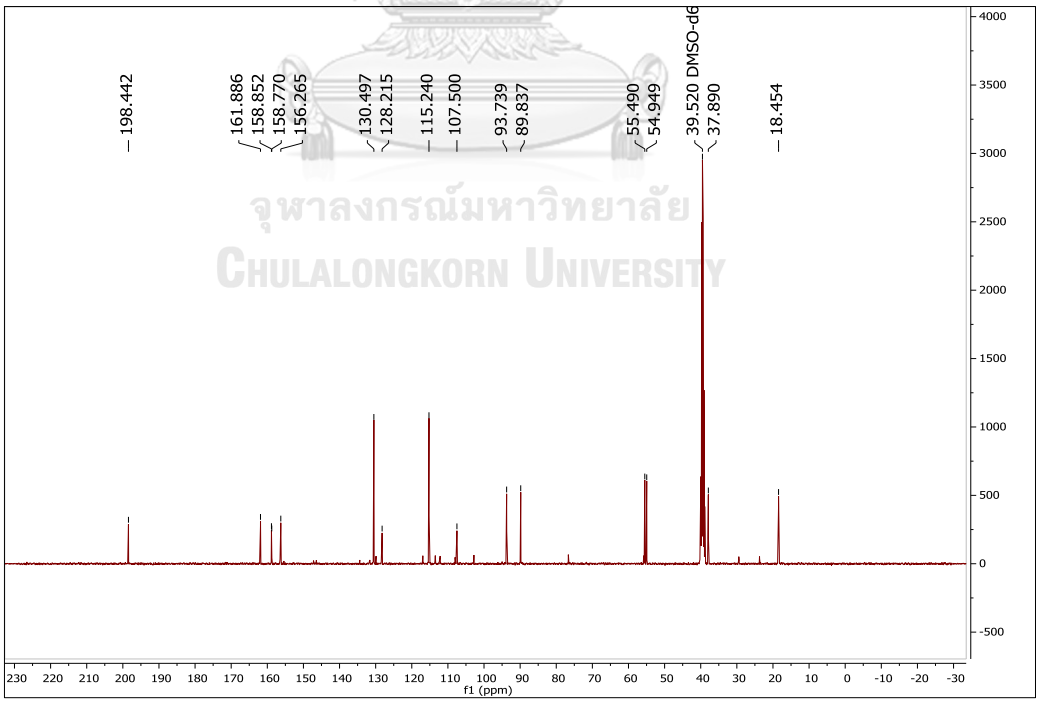

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Figure A-6 The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of **23**

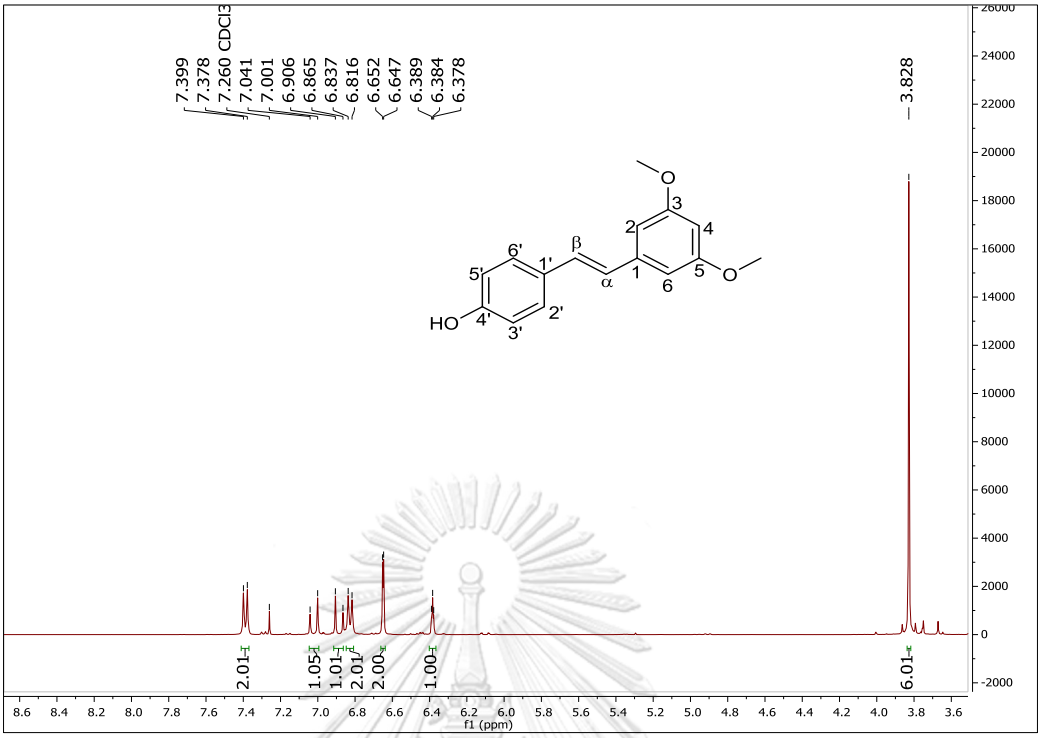

Figure A-7 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **27**

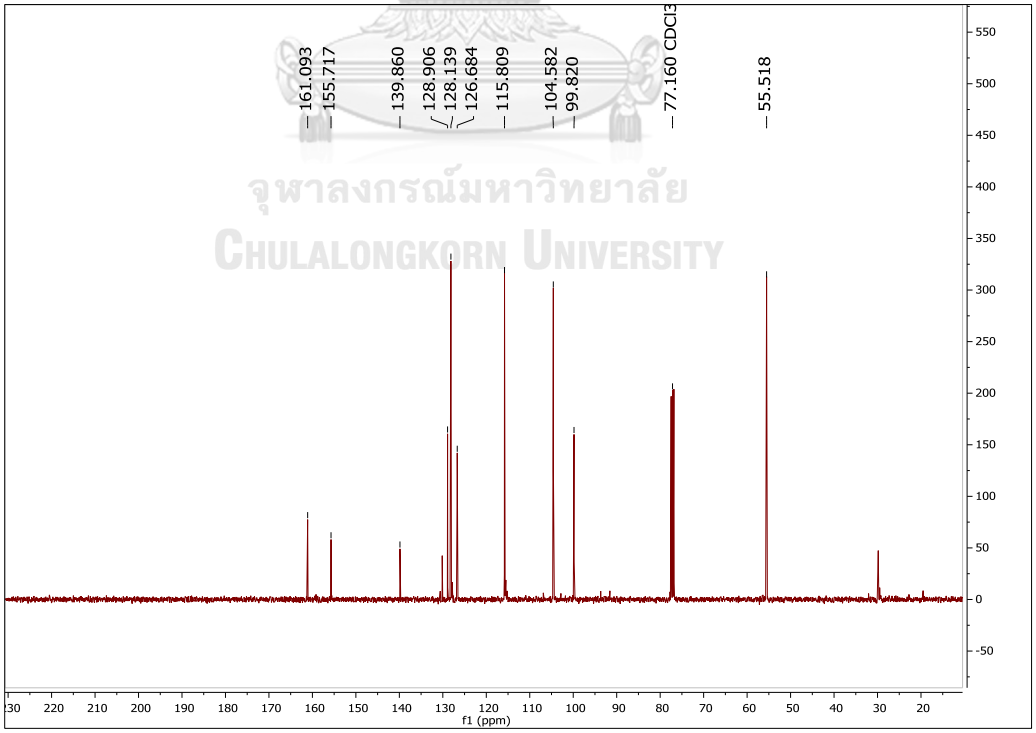

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Figure A-8 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **27**

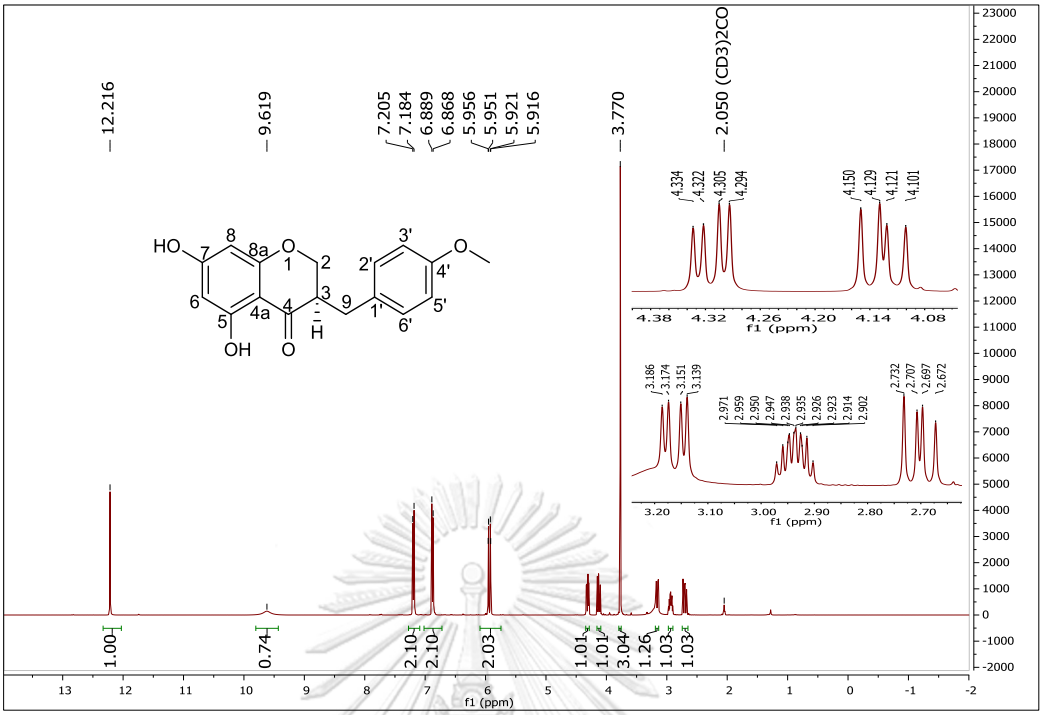

Figure A-9 The ^{1}H NMR spectrum (acetone-d_6, 400 MHz) of **82**

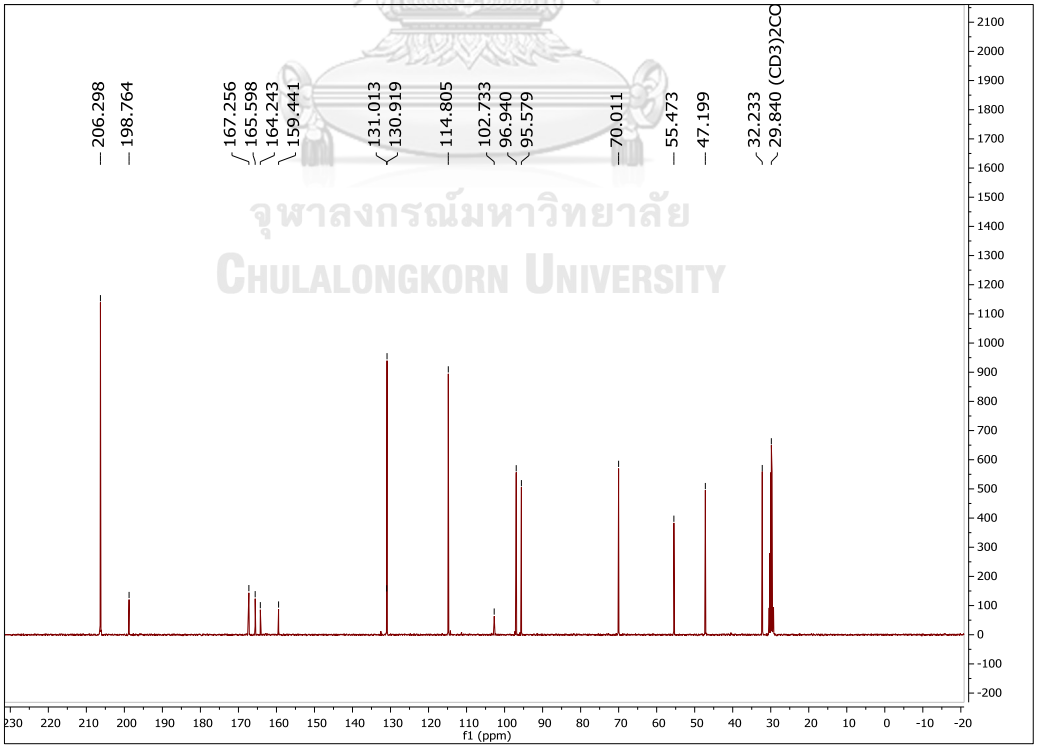

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Figure A-10 The ^{13}C NMR spectrum (acetone-d_6, 100 MHz) of **82**

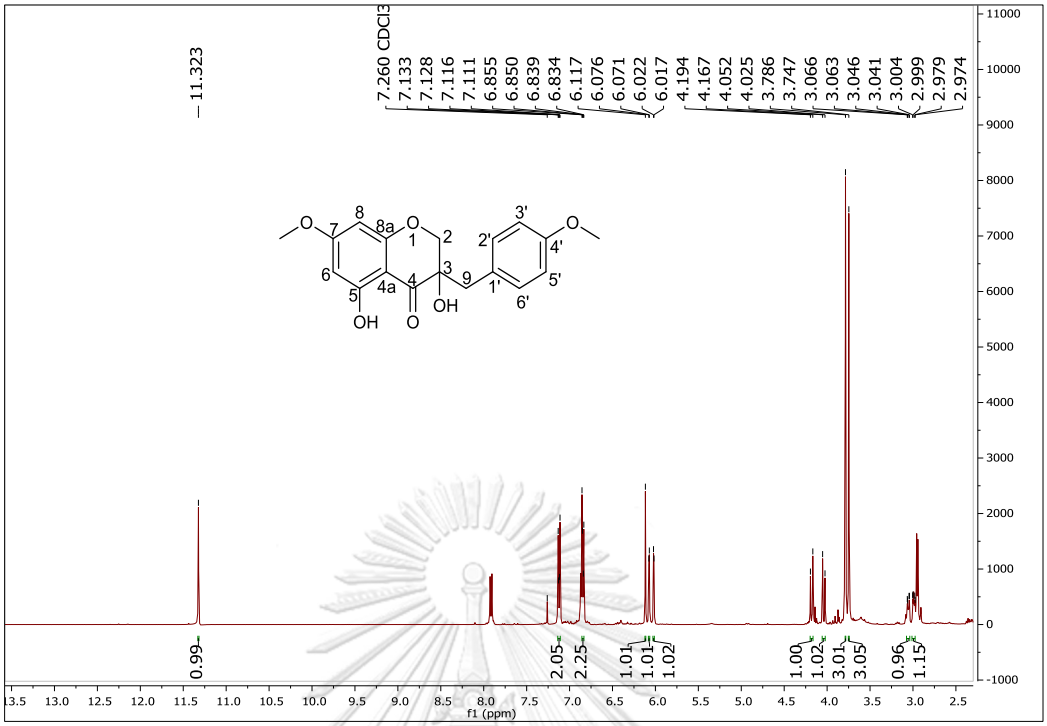

Figure A-11 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **83**

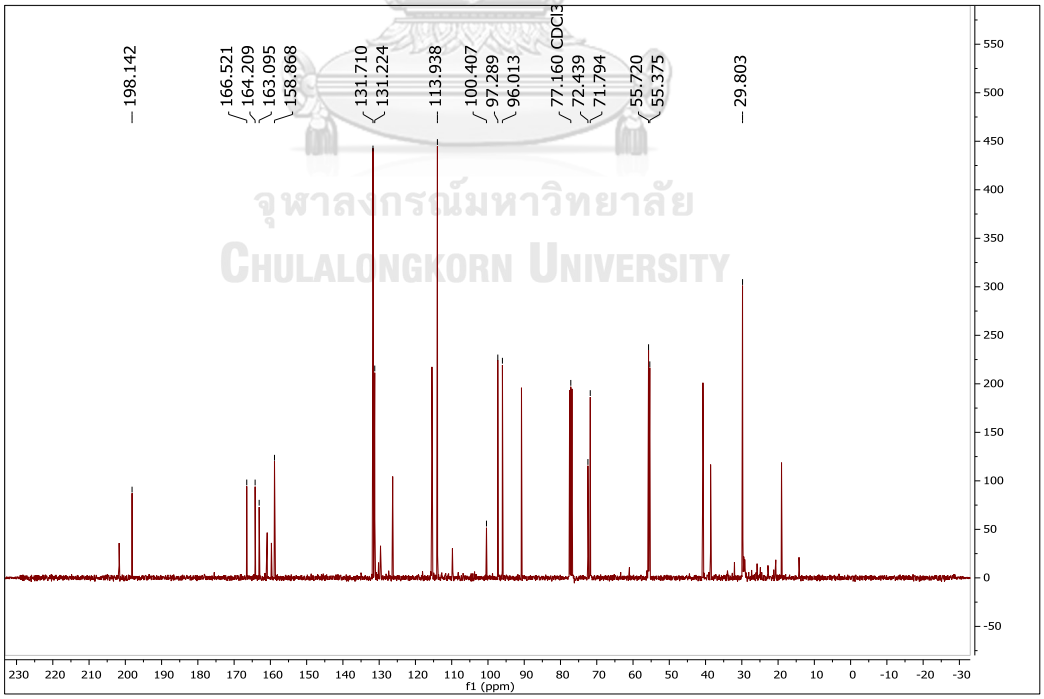

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Figure A-12 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **83**

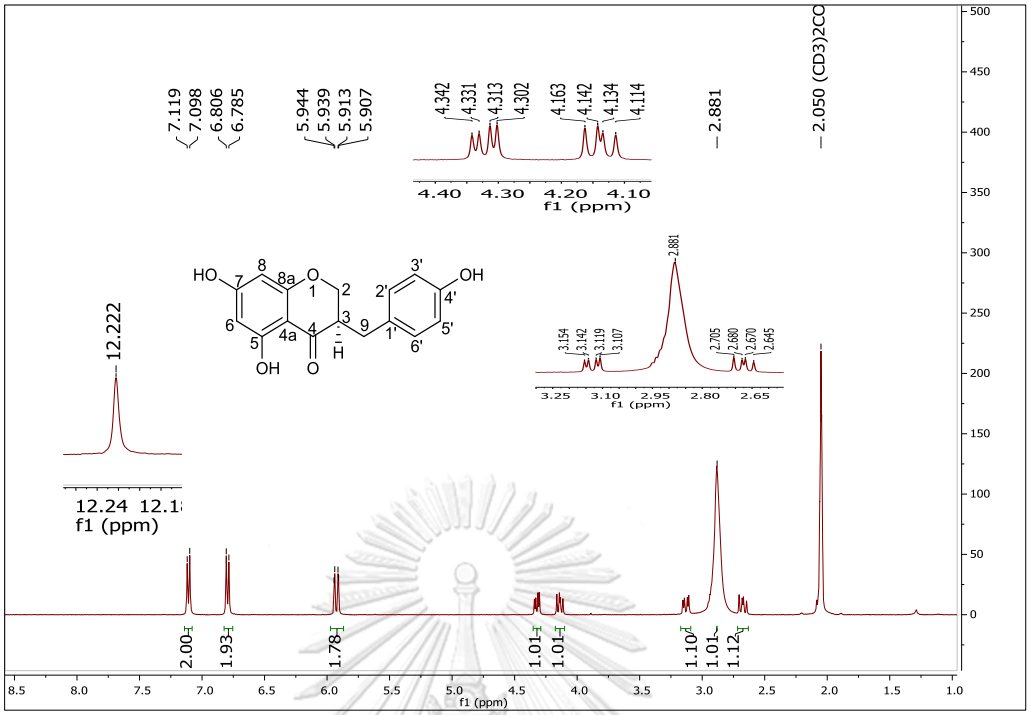

Figure A-13 The ^{1}H NMR spectrum (acetone-d_6, 400 MHz) of **84**

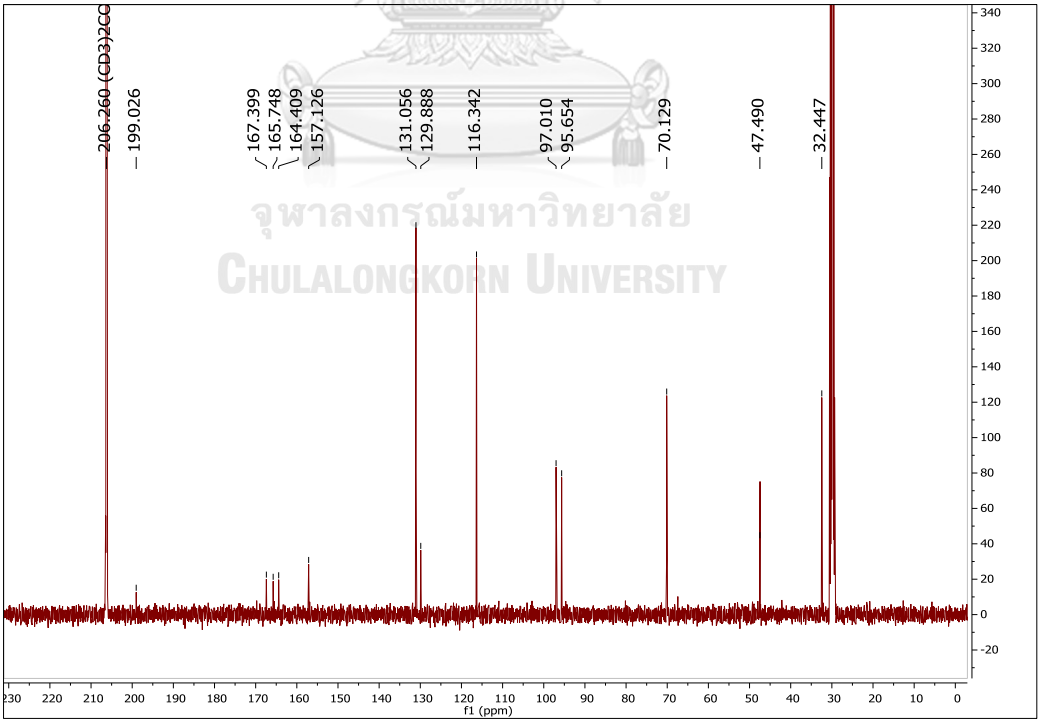

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Figure A-14 The ^{13}C NMR spectrum (acetone-d_6, 100 MHz) of **84**

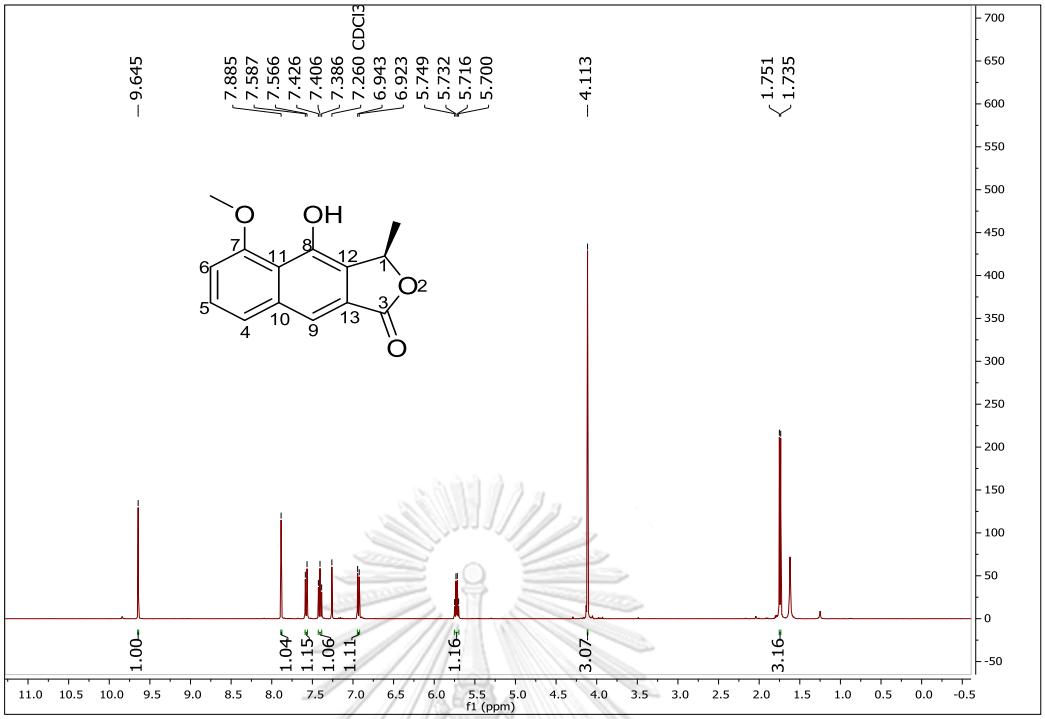

Figure A-15 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **85**

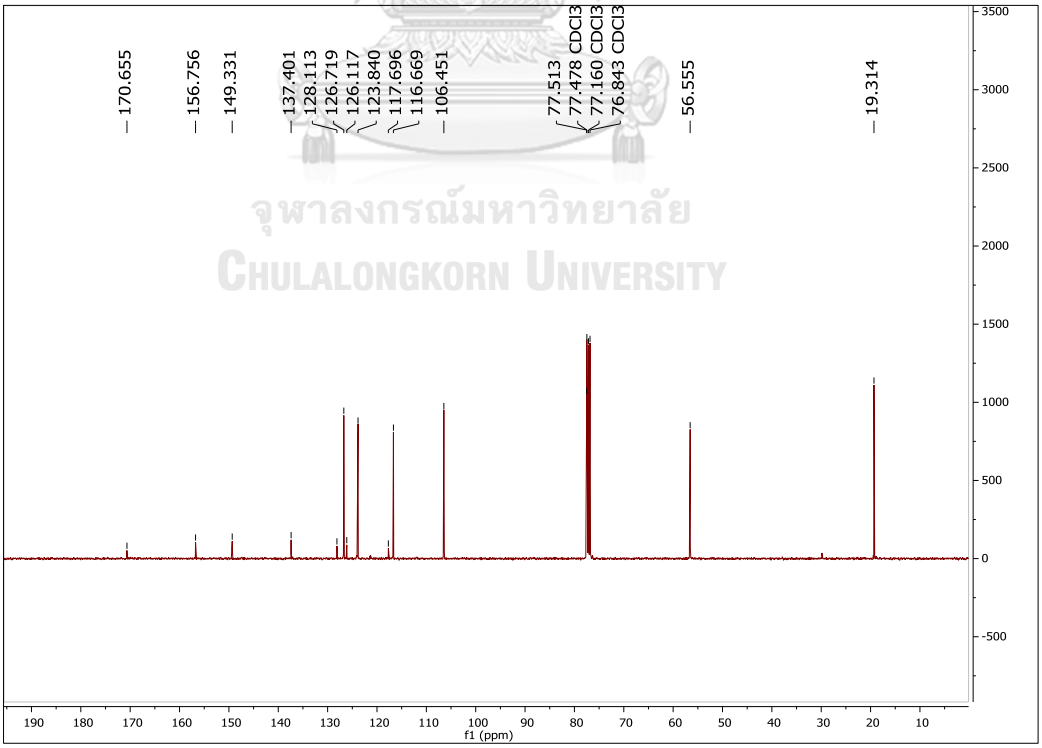

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Figure A-16 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **85**

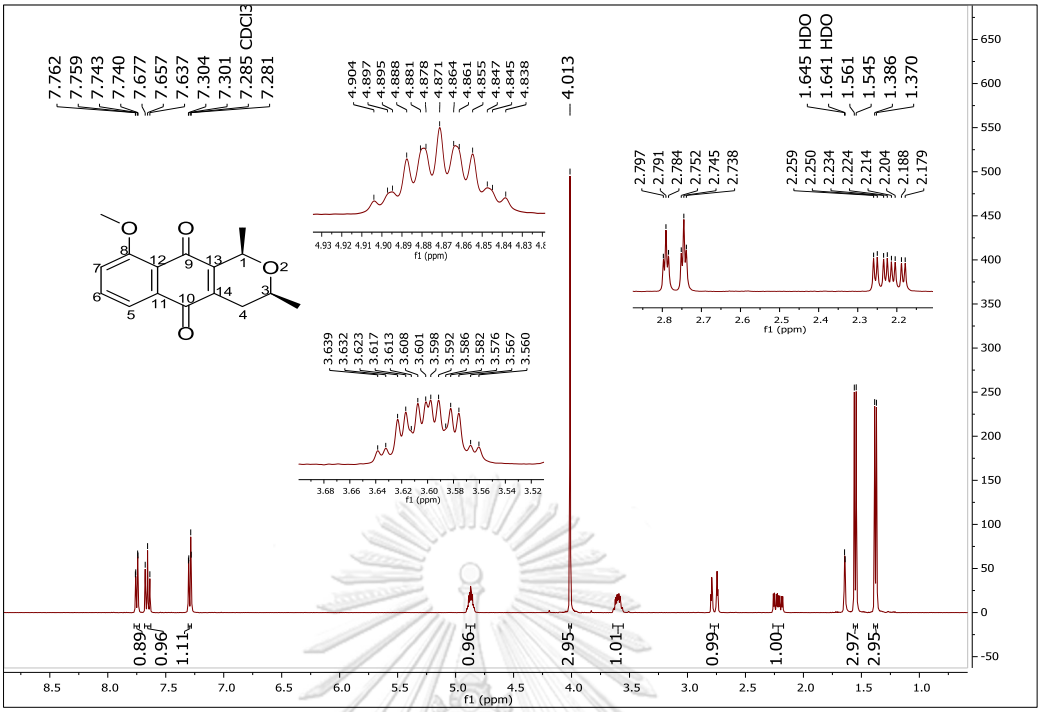

Figure A-17 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **86**

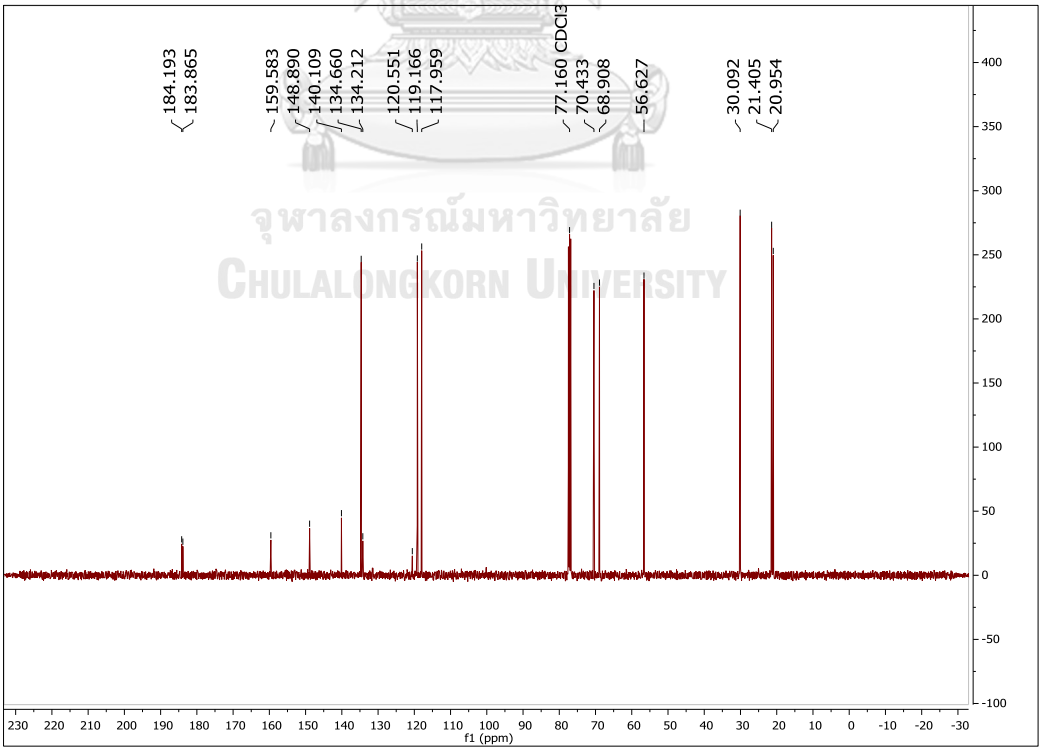

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Figure A-18 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **86**

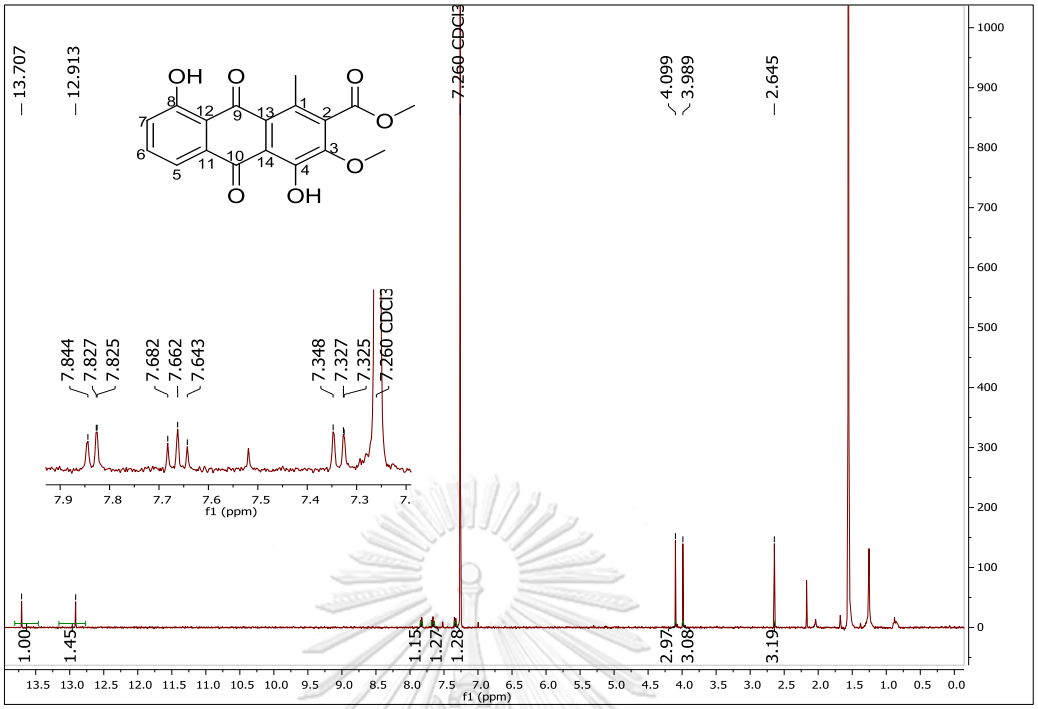

Figure A-19 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **99**

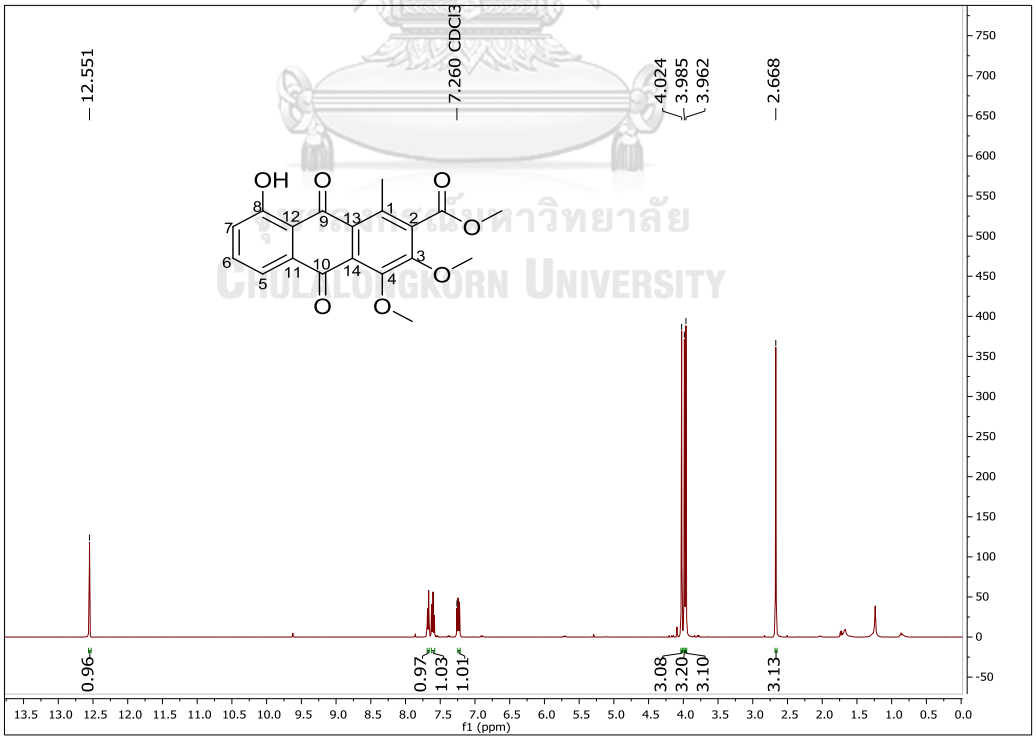

Figure A-20 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **100**

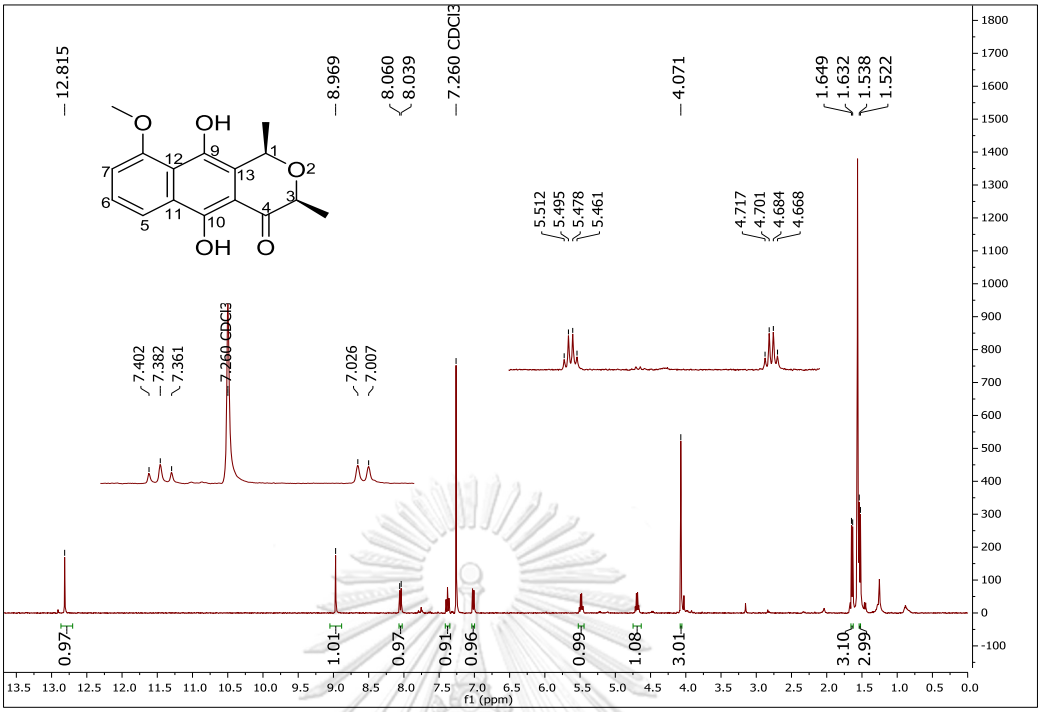

Figure A-21 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **116**

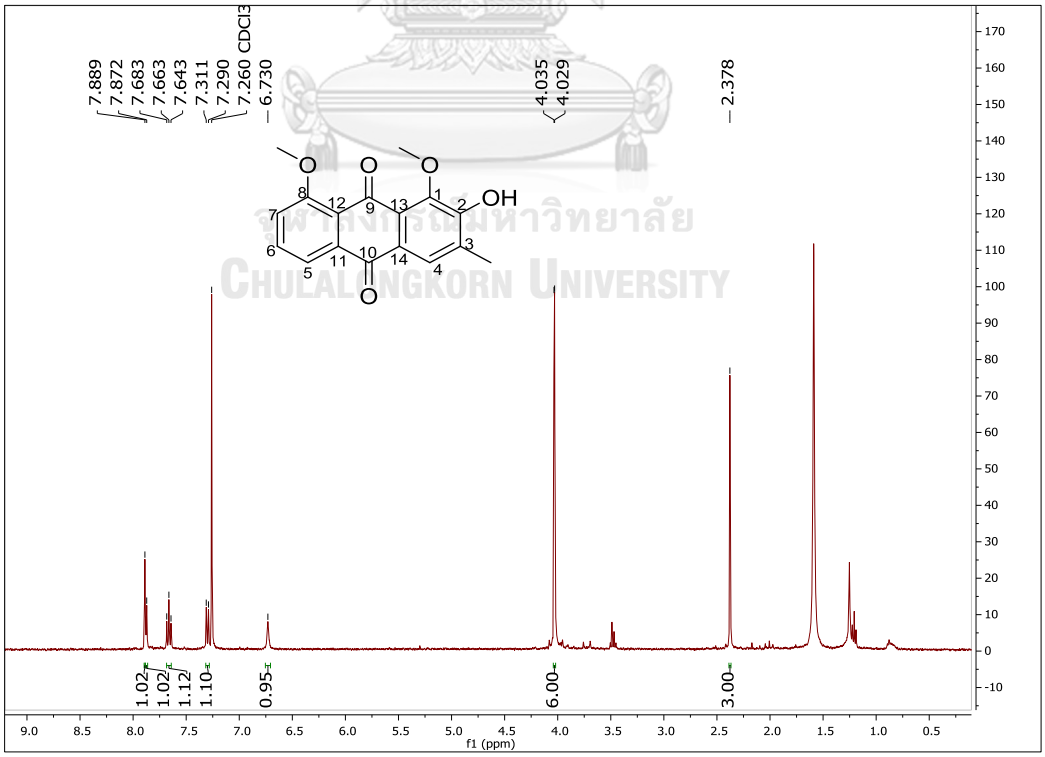

Figure A-22 The ^{1}H NMR spectrum ($CDCl_3$, 400 MHz) of **117**

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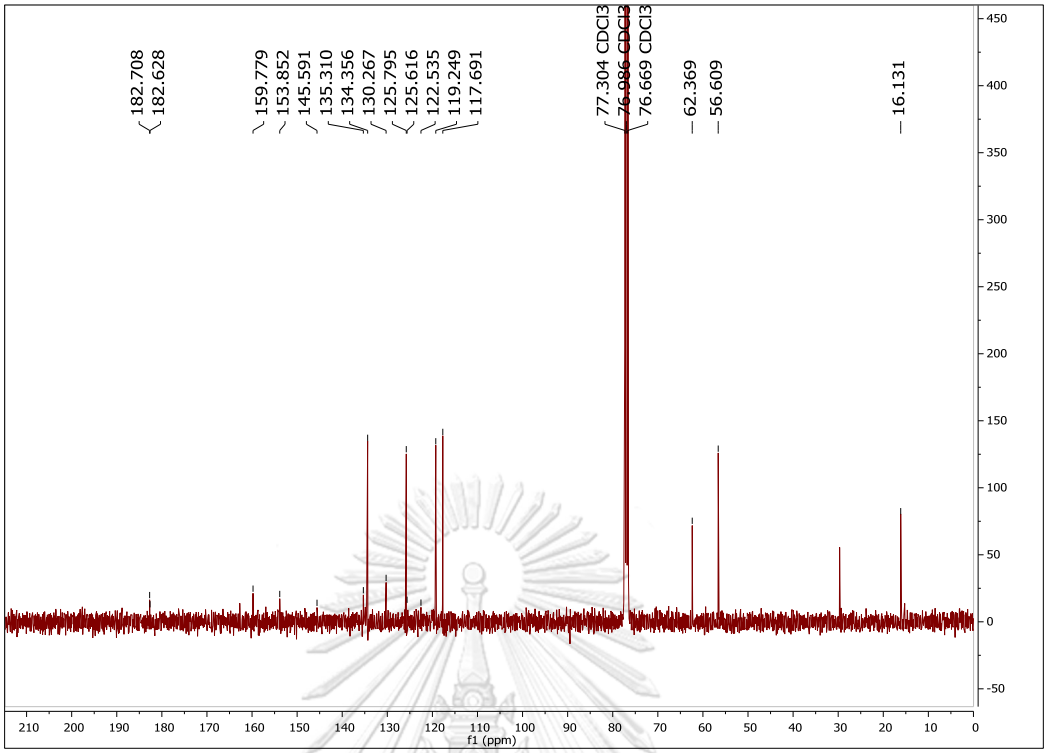

Figure A-23 The ^{13}C NMR spectrum ($CDCl_3$, 100 MHz) of **117**

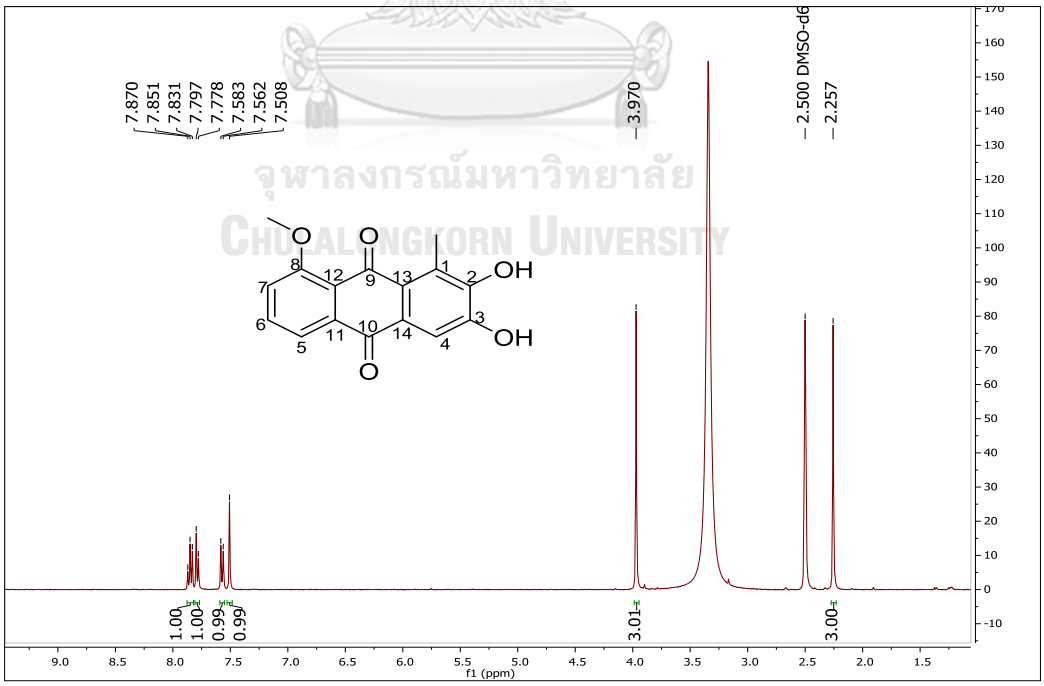

Figure A-24 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **118**

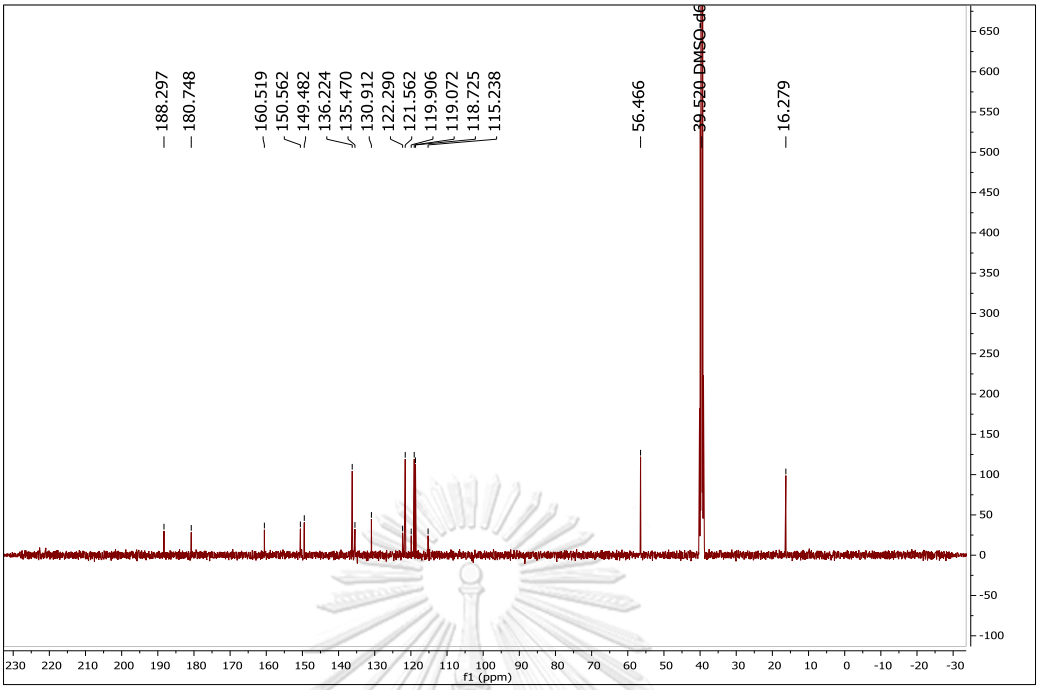

Figure A-25 The ^{13}C NMR spectrum (DMSO-d_6, 100 MHz) of **118**

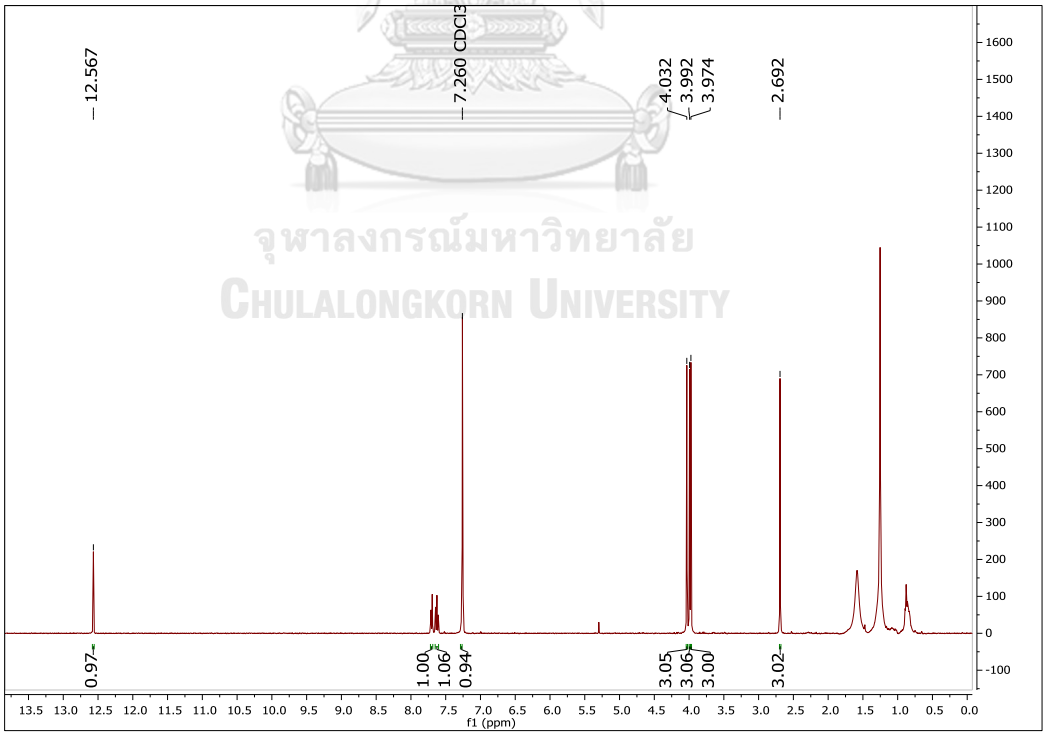

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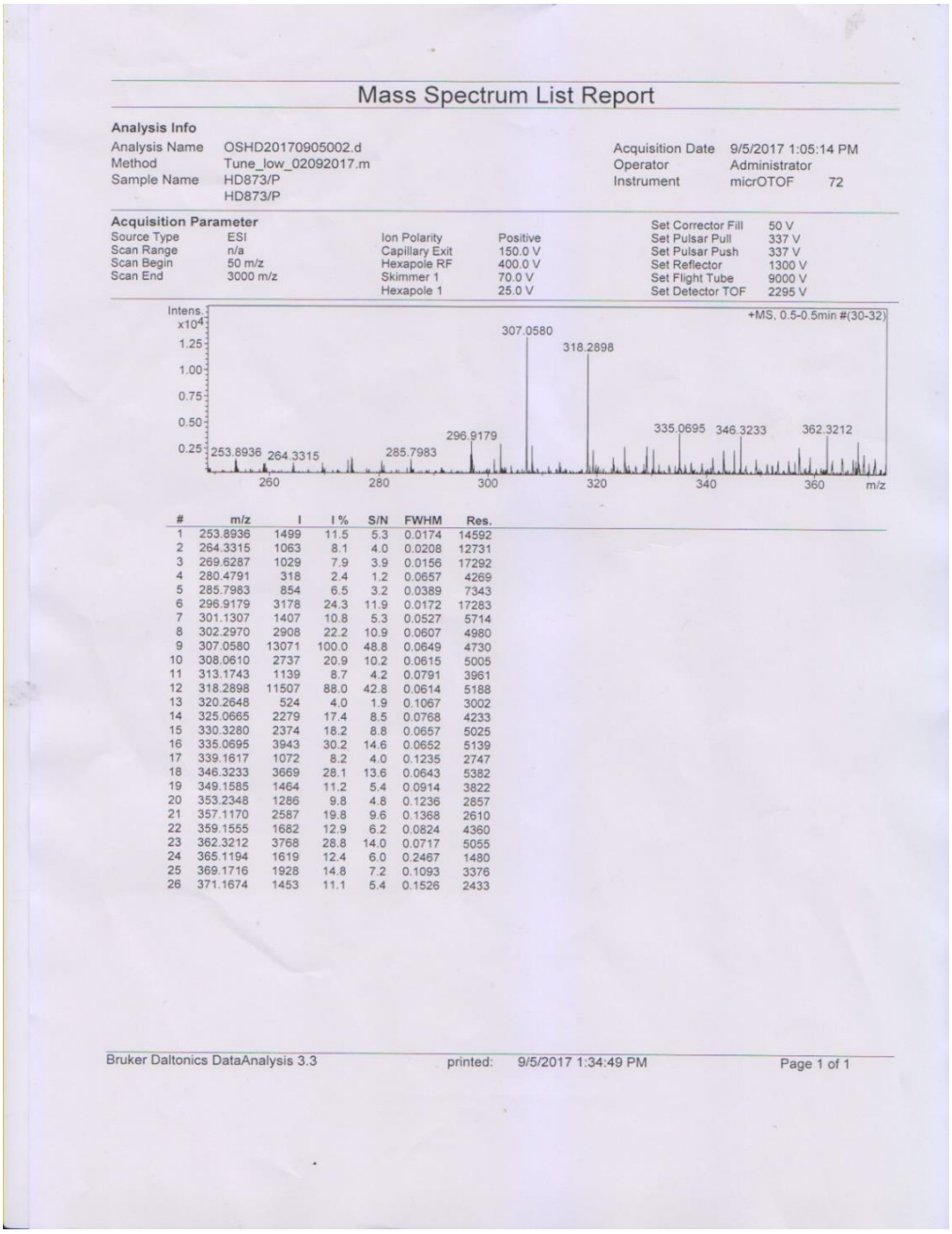
Figure A-26 The ^{1}H NMR spectrum (DMSO-d_6, 400 MHz) of **119**



Mass Spectrum List Report

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Acquisition Parameter

Source Type	ESI	Ion Polarity	Positive	Set Corrector Fill	50 V
Scan Range	n/a	Capillary Exit	150.0 V	Set Pulsar Pull	337 V
Scan Begin	50 m/z	Hexapole RF	400.0 V	Set Pulsar Push	337 V
Scan End	3000 m/z	Skimmer 1	70.0 V	Set Reflector	1300 V
		Hexapole 1	25.0 V	Set Flight Tube	9000 V
				Set Detector TOF	2295 V

#	m/z	I	I %	S/N	FWHM	Res.
1	253.8936	1499	11.5	5.3	0.0174	14592
2	264.3315	1063	8.1	4.0	0.0208	12731
3	269.6287	1029	7.9	3.9	0.0156	17292
4	280.4791	318	2.4	1.2	0.0657	4269
5	285.7983	854	6.5	3.2	0.0389	7343
6	296.9179	3178	24.3	11.9	0.0172	17283
7	301.1307	1407	10.8	5.3	0.0527	5714
8	302.2970	2908	22.2	10.9	0.0607	4980
9	307.0580	13071	100.0	48.8	0.0649	4730
10	308.0610	2737	20.9	10.2	0.0615	5005
11	313.1743	1139	8.7	4.2	0.0791	3961
12	318.2898	11507	88.0	42.8	0.0614	5188
13	320.2648	524	4.0	1.9	0.1067	3002
14	325.0665	2279	17.4	8.5	0.0768	4233
15	330.3280	2374	18.2	8.8	0.0657	5025
16	335.0695	3943	30.2	14.6	0.0652	5139
17	339.1617	1072	8.2	4.0	0.1235	2747
18	346.3233	3669	28.1	13.6	0.0643	5382
19	349.1585	1464	11.2	5.4	0.0914	3822
20	353.2348	1286	9.8	4.8	0.1236	2857
21	357.1170	2587	19.8	9.6	0.1368	2610
22	359.1555	1682	12.9	6.2	0.0824	4360
23	362.3212	3768	28.8	14.0	0.0717	5055
24	365.1194	1619	12.4	6.0	0.2467	1480
25	369.1716	1928	14.8	7.2	0.1093	3376
26	371.1674	1453	11.1	5.4	0.1526	2433

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Figure A-27 HRMS (ESI) of **118**

VITA

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