ANTIOXIDANT AND ANTIBACTERIAL AGENTS FROM *Parmotrema dilatatum* (Vainio) Hale, *Knema angustifolia* (Roxb.) Warb. AND *Persicaria odorata* Lour.

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สารต้านออกซิเดชันและสารต้านแบคทีเรียจากไลเคน *Parmotrema dilatatum* (Vainio) Hale.
กำลังเลือดม้า *Knema angustifolia* (Roxb.) Warb. และผักแพว *Persicaria odorata* Lour.

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น.ส.อัสฮาอิมา ปาระมิตะ เดฟิ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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อัสฮาอิมา ปาระมิตะ เดฟิ : สารต้านออกซิเดชันและสารต้านแบคทีเรียจากไลเคน *Parmotrema dilatatum* (Vainio) Hale. *กำลังเลือดม้า Knema angustifolia* (Roxb.) Warb. และผักแพว *Persicaria odorata* Lour.. (ANTIOXIDANT AND ANTIBACTERIAL AGENTS FROM *Parmotrema dilatatum* (Vainio) Hale, *Knema angustifolia* (Roxb.) Warb. AND *Persicaria odorata* Lour.) อ.ที่ปรึกษาหลัก : วรินทร ชวศิริ

ในการเสาะหาสารต้านอนุมูลอิสระและต้านแบคทีเรีย ได้ศึกษาสิ่งสกัดและสารที่แยกได้จากไลเคน *Parmotrema dilatatum* ต้นกำลังเลือดม้า แพว และต้นจันทน์ชะมด ได้แยกสารเก้าตัวจากไลเคน *P. dilatatum* ได้แก่ (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl)benzoate (PD.1), hopane-16β,22-diol (PD.2), methyl orsellinate (PD.3), methyl haemmatomate (PD.4), methyl β-orcinolcarboxylate (PD.5), 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (PD.6), atranol (PD.7), atranorin (PD.8) และ lecanorin (PD.9) พบว่าสิ่งสกัดไดคลอโรมีเทนและแอซีโทนและ atranol (PD.7) แสดงฤทธิ์ต้านอนุมูลอิสระที่ดีมาก ในส่วนของฤทธิ์ต้านไทโรซิเนสพบว่าสิ่งสกัดไดคลอโรมีเทนและ atranorin (PD.8) แสดงฤทธิ์ที่ดีที่สุด สำหรับการศึกษาฤทธิ์ยับยั้งแอลฟากลูโคซิเดสพบว่าสิ่งสกัดเมทานอลและ methyl haemmatommate (PD.4) แสดงฤทธิ์ที่ดีที่สุด นอกจากนี้ได้ศึกษาสิ่งสกัดและองค์ประกอบหลักจากต้นกำลังเลือดม้า แพวและต้นจันทน์ชะมด พบว่าสิ่งสกัดของต้นกำลังเลือดม้าแสดงศักยภาพที่ดีต่อ DPPH, ABTS, ฤทธิ์ต้านแบคทีเรีย, ไทโรซิเนสและแอลฟากลูโคซิเดส quercetin ที่แยกได้จากต้นกำลังเลือดม้าแสดงฤทธิ์ต้านอนุมูลอิสระ ไทโรซิเนสและเบาหวาน อนุพันธ์ของ mansonone G, MG1 และ MG3 แสดงการเสริมฤทธิ์ต้านแบคทีเรีย *S. aureus* เมื่อใช้ร่วมกับ streptomycin mansonone G และอนุพันธ์ทั้งหมดแสดงการเสริมฤทธิ์ต้าน *S. aureus* และ *S. mutans* เมื่อใช้ร่วมกับ tetracycline

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Asshaima Paramita Devi : ANTIOXIDANT AND ANTIBACTERIAL AGENTS FROM *Parmotrema dilatatum* (Vainio) Hale, *Knema angustifolia* (Roxb.) Warb. AND *Persicaria odorata* Lour.. Advisor: Asst. Prof. WARINTHORN CHAVASIRI, Ph.D.

In the search of antioxidant and antibacterial agents, the extracts and isolated compounds from lichen *Parmotrema dilatatum*, *Knema angustifolia full name*, *Persicaria odorata* and *Mansonia gagei*. were examined. Nine compounds were isolated from lichen *P. dilatatum* including (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl)benzoate (PD.1), hopane-16ß,22-diol (PD.2), methyl orsellinate (PD.3), methyl haemmatomate (PD.4), methyl β-orcinolcarboxylate (PD.5), 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (PD.6), atranol (PD.7), atranorin (PD.8) and lecanorin (PD.9). The dichloromethane and acetone fractions, and atranol (PD.7) gave excellent activities as antioxidants. While in tyrosinase activity the dichloromethane fraction and atranorin (PD.8) showed the highest inhibition. For α-glucosidase inhibition activity, the methanol fraction and methyl haemmatommate (PD.4) revealed the highest activity. The extracts and major constituents from *K. angustifolia*, *P. odorata* and *M. gagei* were determined for their biological activities. Amongst plants *K. angustifolia* was showed potential activity in DPPH, ABTS, antibacterial, tyrosinase assay and anti α-glucosidase assay. Meanwhile, quercetin isolated from *K. angustifolia* exhibited prospective antioxidants, anti-tyrosinase and anti-diabetic activities. MG1 and MG3 derivatives of mansonone G showed synergistic effect against *S. aureus* when combined with streptomycin. Mansonone G and all derivatives showed synergistic effect against *S. aureus* and *S. mutans* when combined with tetracycline.

Field of Study:	Biotechnology	Student's Signature ....
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LIST OF ABBREVIATIONS

μg	microgram
μL	microliter
μM	micromolar
δ	chemical shift
°C	degree Celsius
^{13}C-NMR	carbon-13 nuclear magnetic resonance
^{1}H-NMR	proton nuclear magnetic resonance
ABTS	2,2'-azino-bis (3-ethylbenzthiazoline-6-sulphonic acid)
acetone-d_6	deuterated acetone
ATCC	american type culture collection
BHT	Butylated hydroxytoluene
C	carbon
calcd	calculated
CBMG	chlorobenzoyl mansonone g
CC	column chromatography
$CDCl_3$	deuterate chloroform
CFU	colony forming unit
CH_2Cl_2	dichloromethane
Cm	centimeter
d	doublet
DCM	dichloromethane

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dd	doublet doublet
ddd	doublet doublet doublet
DHI	5,6-dihydroxyindole
DHICA	5,6-dihydroxyindole-2-carboxylic acid
DMSO-d_6	deuterate dimethylsulfoxide
DNA	deoxyribonucleic acid
Dopa	3,4-dihydroxyphenylalanine
DPPH	2,2-Diphenyl-1-picrylhydrazyl
EtOAc	ethyl acetate
EtOH	ethanol
ERα	estrogen receptor alpha
FIC	fractional inhibitory concentration
g	gram
H_2O	water
H_2O_2	hydrogen peroxide
HBTA	5-hydroxy-1,4-benzothiazinylalanine
HR-ESI-MS	high reseonance electrospray ionization mass spectroscopy
IC_{50}	half maximal inhibition concentration
ICAQ	indole-2-carboxylic acid-5,6-quinone
IQ	indole-5,6-quinone
J/Hz	coupling constant
KCCM	Korean culture center of microorganism

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M	molar
MHz	megahertz
MeOH	methanol
MBC	minimum bactericidal concentration
MIC	minimum inhibitory concentration
mg	milligram
mL	milliliter
mm	millimeter
mM	millimolar
MRSA	methicillin resistant *Staphylococcus aureus*
Na_2CO_3	sodium carbonate
NB	nutrient broth
nM	nanomolar
NMR	nuclear magnetic resonance
NO	nitric oxide
OH	hydroxide
ORAC	Oxygen radical absorbance capacity
PPARγ	Peroxisome proliferator-activated receptor gamma
ppm	part per million
R	antioxidant radical
RNA	ribonucleic acid
ROS	reactive oxygen species
R^2	regression

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rRNA	ribosomal ribonucleic acid
S	singlet
SD	standard deviation
TCI	tokyo chemical industry
TRP	tyrosinase related protein
tRNA	transfer ribonucleic acid
TSM	traditional system of medicine
TYR	tyrosinase
U	unit
UV	ultraviolet

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CHAPTER I
INTRODUCTION

1.1 The use of natural products as medicinal source

Natural products have served as an important source of drugs since ancient times and about half of the useful drugs of today are derived from natural sources. [1]. The folk knowledge traditions which are mostly orally transmitted, are more diverse, ecosystem and ethnic community specific with household level health practices (home remedies for primary healthcare, food recipes, rituals, customs). These are generated over centuries by communities and use components of ecosystems (plants, animals and mineral/metal derivatives) that are primarily locally available, easily accessible and often cost-effective. [2] In order to recognize and to know the use of plants, people used "trial and error" method. Those experiences lead to the knowledge establishment of potentially useful medicinal plants. [3] The practice of traditional medicine is widespread in China, India, Japan, Pakistan, Sri Lanka, and Thailand. In China, about 40% of the total medicinal consumption is attributed to traditional tribal medicines. In Thailand, herbal medicines make use of legumes encountered in Caesalpiniaceae, Fabaceae, and Mimosaceae. [4]

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Information about the medicinal plants used in the drugs of traditional system of medicine (TSM) has been noteworthy, especially as a principal in new single molecule medicine discovery for modern system of medicine. Isolation by using

various separation techniques, chemical properties and spectral characteristics are necessity for determining their chemical natures of such compounds and establishing their correct structures. [5]

Certain chemical constituents from plants have previously been reported as excellent antioxidants, anti-tyrosinase, α-glucosidase inhibitor and anti-bacterial. [6-10] Therefore, the investigation of active chemical constituents from plants and lichens are noteworthy.

1.2 Reactive oxygen species (ROS) and its relations with antioxidants

ROS and free radicals are originated from human metabolic process or exogenous resources. The formation of free radicals continuously happens in the cells due to enzymatic and non-enzymatic reactions. [11] ROS are differentiated by their capacity to cause oxidative damage to proteins, DNA, and lipids. [12]

When cellular antioxidant defense system is overwhelmed by ROS through ROS level increasing or decreasing in cellular antioxidant capacity, oxidative stress occurs. [13] Several diseases are reported related to the oxidative stress formation in human body such as aging, atherosclerosis, kidney disease, neurodegenerative disorder and cancer. [14] The free radicals and ROS formation with their consequences can be seen in Figure 1.1.

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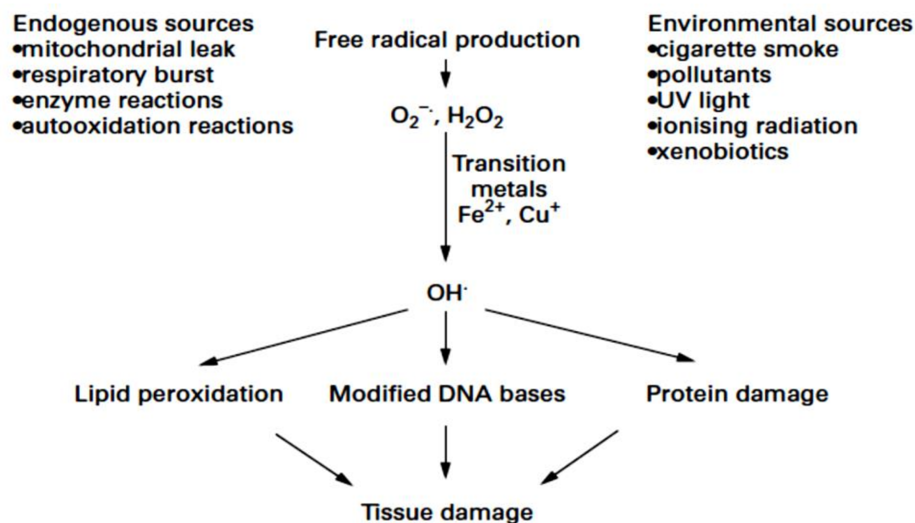

Figure 1.1 Major sources of free radicals in the body and the consequences of free radical damage. [15]

ROS could normally be scavenged by endogenous or exogenous antioxidants. Antioxidant means a substance that when its presence, even at low concentration is capable to reduce or delay the oxidation process. [16] Antioxidants may work by different mechanisms for example suppressing the formation of active species, seizing metal ions, free radical scavenging and repairing or clearing the damage cells. Recently, several antioxidants have been discussed to induce the biosynthesis of other antioxidants or defense enzymes. [17] Nowadays scientists are interested in natural antioxidants to replace the synthetic ones due to arising side effects. The natural antioxidants as human consumption can be prepared as functional foods and nutraceuticals that play important role as free radical and other reactive species defenses in human body. [18]

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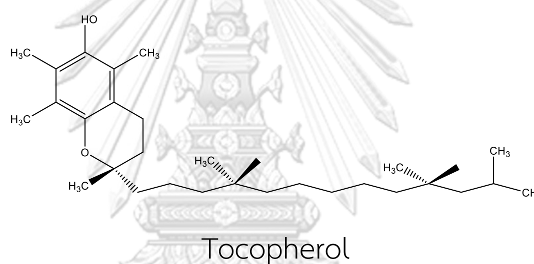
There were several reports regarding plant extracts and compounds as antioxidants. Previously, it was reported that the ethanol extracts of *K. angustifolia* **(Roxb.) Warb.** possessed high antioxidant activity with EC_{50} 13.90±1.35 μg/mL. [19]

Plant antioxidants usually contain phenolic acids, phenolic diterpenes, flavonoids, volatile oils and pigments which have potential in hydrogen donation. [17] Phenolic compounds may inhibit free radical formation and/or interrupt propagation of autoxidation. Tocopherol (**1**) is an example compound that act exquisitely in those pathways. [20]



Tocopherol

1.3 Melanin production and its relations with tyrosinase activity

Melanin is formed by the process called melanogenesis. Melanin plays significance role for the pigmentation of human skin, eye and hair. Several enzymatic catalyzed and chemical reactions are elaborated in melanogenesis process. However, enzymes such as tyrosinase and tyrosinase-related protein-1 (TRP-1) and TRP-2 have a big part in melanin synthesis. [21]

Tyrosinase (monophenol monooxygenase, E:C:1.14.18.1), also known as polyphenol oxidase, is a copper-containing enzyme widely distributed in nature. [22] Tyrosinase specifically is a key enzyme that catalyzes a rate limiting step in melanin

synthesis. Tyrosinase downregulation is the most well-known method to inhibit melanogenesis. The eumelanin and pheomelanin formation are explained in Figure 1.2. [21]

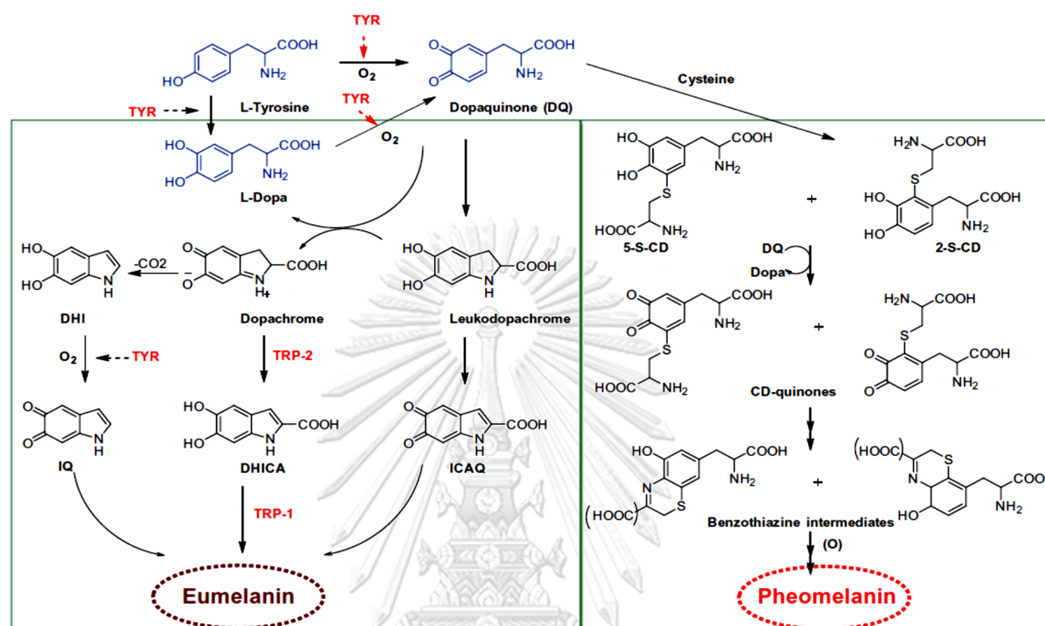


Figure 1.2 Melanogenesis pathway (production of eumelanin and pheomelanin). (Tyr: tyrosinase; DQ: dopaquinone; L-Dopa: L-3:4-dihydroxyphenylalanine; DHICA: 5,6-dihydroxyindole-2 carboxylic acid; DHI: 5,6-dihydroxyindole; ICAQ: indole-2-carboxylic acid-5,6-quinone; IQ: indole-5,6-quinone; HBTA: 5-hydroxy-1,4-benzothiazinylalanine). [21]

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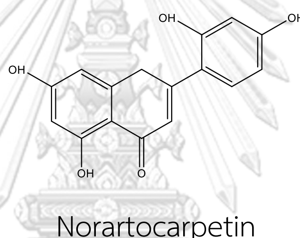
The exaggerated tyrosinase production will cause various dermatological disorders such as melasma, age spots, and discomfort to some people especially in aesthetic views. [23] Therefore, many cosmetic companies and researchers are interested in investigating the inhibition of tyrosinase enzyme.

Tyrosinase inhibitors work through several mechanisms: (1) intrusion of its transcription and/or glycosylation, (2) control its post-transcriptional, (3) by products reduction, (4) inhibition by different modalities. [24]

There are various chemical constituents from plants notably known for their ability against tyrosinase, one of the examples is a flavone norartocarpetin (**2**) isolated from *Morus ihou* (S.) Koidz**.** Noratocarpetin inhibited tyrosinase enzyme ten times higher than the positive control kojic acid with IC_{50} 1.2 μM. The kinetic study of this compound showed it processed *via* reversibly competitive pathway. [25]



Norartocarpetin

1.4 Diabetes mellitus and its relation with α-glucosidase enzyme

One of known metabolic disorders is diabetes mellitus. The characteristics of this disease is the increment of blood glucose level due to the abnormal postprandial. [26]

There are two main types of diabetes, types I and II. Diabetes type I arise when body less or stop producing insulin, whereas diabetes type II arises when body produce less insulin or having troubled with the using of insulin. [27, 28]

Type I diabetes resulted from autoimmune destruction of β-cells of pancreas. Those destruction caused permanent insulin deficiency. Therefore, patients must be

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received insulin injection regularly for treatment. This type of diabetes accounts 5-10% of diabetes population. Typically affected children and adolescents, sometimes young adults. [29]

Type II diabetes dominate 90-95% of diabetes population, this type of diabetes caused by multifactor. The markers of pathophysiological of type II diabetes are β-cells dysfunction, chronic inflammation and insulin resistance. Those factors lead to hinder the control of blood glucose levels and cause complications. This type diabetes also known as non-dependent diabetes mellitus (NDDM). Type II diabetes could be prevented by healthy diet, since one of major factors that caused diabetes is obesity that responsible for β-cells defects and peripheral tissue insulin resistance respectively. If diabetes type I mostly affected young generations, diabetes type II mostly affected people with age more than 45 years old. [30, 31] The difference between health pancreas and pancreas in type I and II diabetes mellitus is displayed in Figure 1.3.

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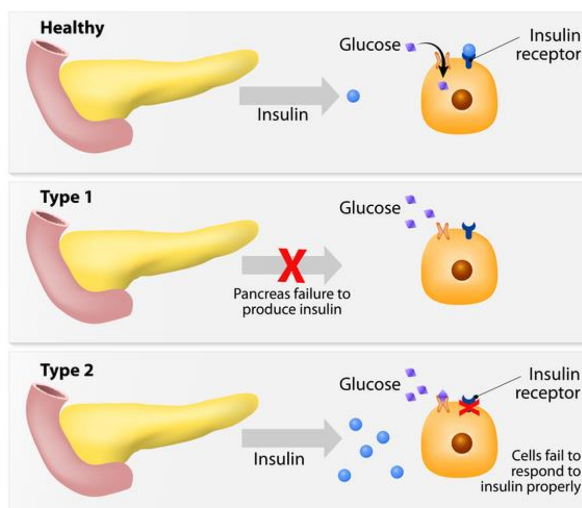

Figure 1.3 The difference between health pancreas and pancreas in type I and II diabetes mellitus.

Source: https://ghr.nlm.nih.gov/condition/type-2-diabetes

Diabetes mellitus type II can be treated by controlling the postprandial hyperglycemia. α-glucosidase enzyme which secreted from intestinal chorionic epithelium is responsible for the degradation of carbohydrates. α-glucosidase inhibitors worked by inhibiting the process of digestion and carbohydrate absorptions. Thus, the post prandial blood glucose concentration is reduced and becomes normal. [26] The mechanism of α-glucosidase inhibitors is explained at Figure 1.4.

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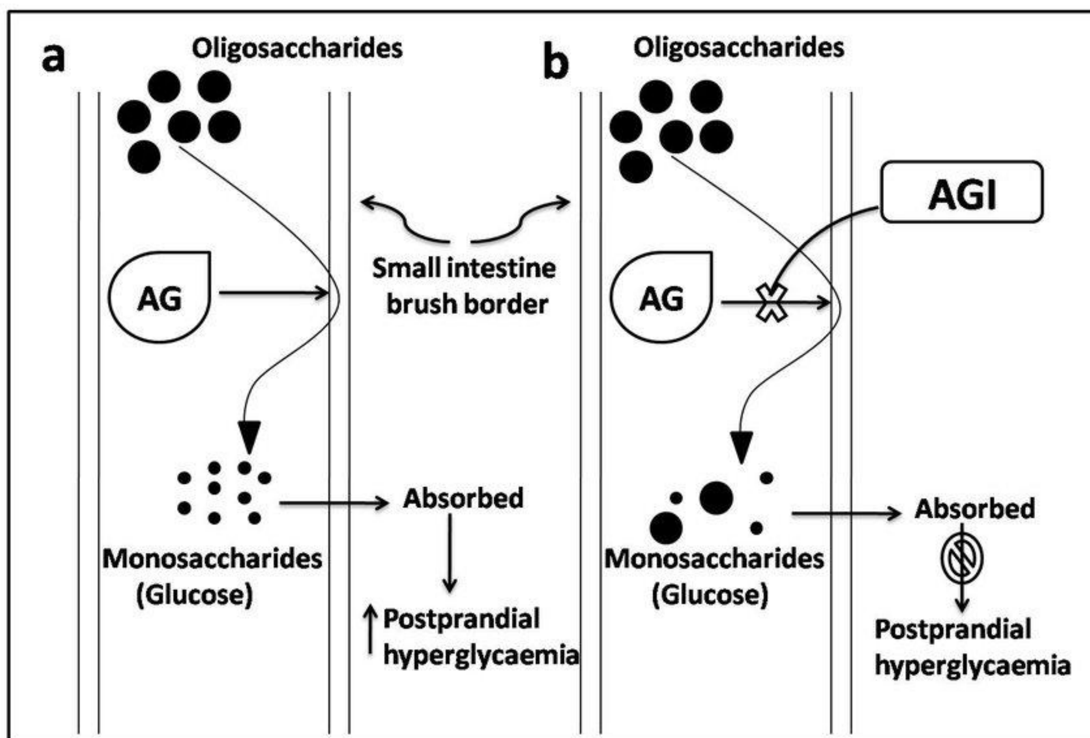

Figure 1.4 The mechanism of α-glucosidase inhibitors [32]

The well-known α-glucosidase inhibitors such as acarbose (**3**) and miglitol (**4**) possessed sugar moieties in the structure. Those moieties allowed the compounds to bind with α-glucosidase through the carbohydrate site. However, there are several reports regarding their side effects towards gastrointestinal. [33]

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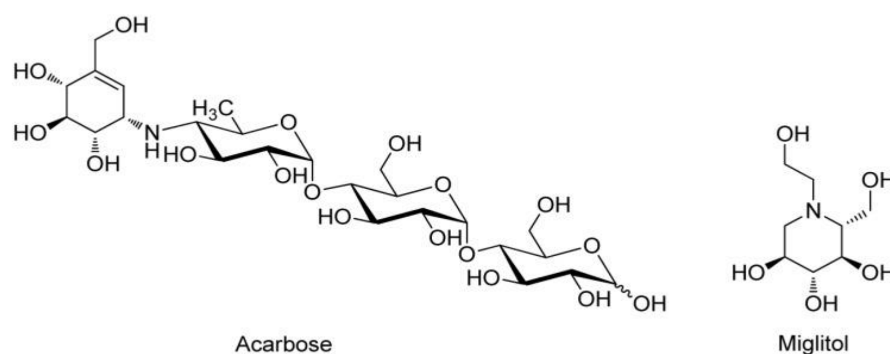

Due to several side effects from commercial α-glucosidase inhibitors, many researches focus on finding α-glucosidase inhibitors from plants. The methanol extract

from trunk bark of *Euonymus laxiflorus* Champ (ELC extract) showed potential α-glucosidase inhibitor with IC_{50} = 0.36 mg/mL against rat-derived α-glucosidase. The α-glucosidase activity towards *Saccharomyces cerevisiae* and *Bacillus stearothermophilus* with IC_{50} = 1.32 and 5.15 μg/mL, respectively. [34]

α-glucosidase inhibitors isolated from *Calea ternifolia* Kunth. plant including 6-hydroxyacetyl-5-hydroxy-2,2-dimethyl-2H-chromene (**5**), caleins C (**6**) and isorhamnetin (**7**) displayed IC_{50} 0.42, 0.28, and 0.16 mM, respectively whereas acarbose as a positive control showed IC_{50} 1.7 mM. The kinetic study of three compounds revealed that calein C and isorhamnetin act as mixed-type inhibitors, while 6-hydroxyacetyl-5-hydroxy-2,2-dimethyl-2H-chromene displayed noncompetitive inhibitor. The docking study of two compounds showed that caleins C and isorhamnetin bound to the enzyme at the catalytic site. [35]

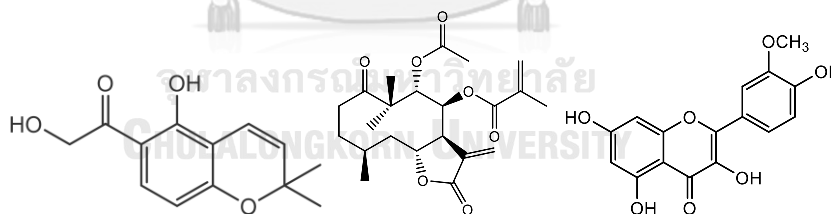

Figure 1.5 6-hydroxyacetyl-5-hydroxy-2,2-dimethyl-2H-chromene (**5**), calein C (**6**) and isorhamnetin (**7**)

1.5 Bacteria and antibacterial activities from natural products

Bacteria are single-cell microorganisms consisting of DNA and RNA. Bacteria also show essential life processes such as growth, metabolism and reproduction. [36] Bacteria cell structure displayed in Figure 1.5.

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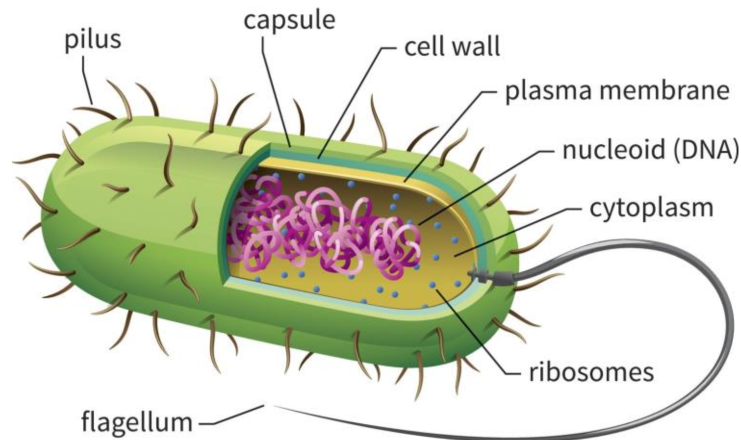

Figure 1.5 Bacteria cell structure.

Source: https://biologywise.com/organelles-their-functions

Bacteria exist in several forms such as spherical or ellipsoidal, rod like or cylindrical and spiral or helicoidal. The spherical are famously known as coccus or cocci, which occur in different types as well. Single coccus (monococcus), diplococcus, streptococcus and staphylococcus. The cylindrical cells are known as bacilli (singular bacillus). The third shape or helical bacteria known as spirillum is formed as spirally twisted cells. There is also short-half spiral cell which commonly known as comma or vibrio. The cells shape is affected the generic name of many bacteria. [37] Bacteria also differentiated by the staining of their cell wall. This staining used to understand cell wall containing peptidoglycan (murein), which show two distinct structural types. There are two main structural types including Gram-positive and Gram-negative bacteria. [36] [38]

Gram-positive cell walls are simple in structure, consist of several layers of peptidoglycan that connected to each other to form a strong, rigid scaffolding.

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Furthermore, they consisted of techoid acid (acidic polysaccharides) which contained phosphate groups that impart an overall negative charge to cell surface. Contradictory with Gram-positive, Gram-negative cells have less peptidoglycan layer, make the wall less sturdy, yet the structure is more complex due to the presence of lipoprotein layer, polysaccharide and outer membrane (phospolipid). [38] The differences of gram-positive and gram-negative bacteria cell walls can be seen in Figure 1.6.

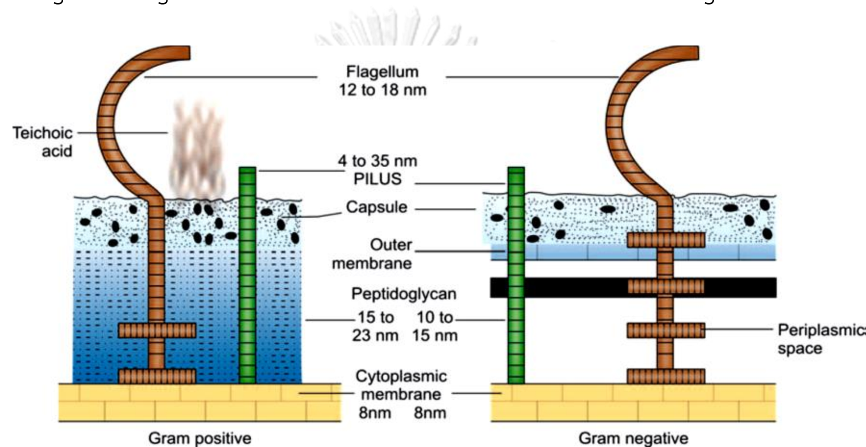

Figure 1.6 Differences of Gram-positive and Gram-negative bacteria cell walls

Bacteria cause numerous infectious diseases and the treatment is mostly based on the use of antibiotics. [39] Antibiotics were differentiated by their mechanisms. Bactericidal antibiotics were capable to kill bacteria, whereas bacteriostatic antibiotics were simply slow down the bacteria growth [40] Despite of their abilities as antimicrobial drugs, pathogenic bacteria indicated resistant effect towards some antibiotics, the screening of new antibacterial compounds should be continued and developed. [41, 42]

The use of plants and their phytochemical contents, and lichen extracts, with established antimicrobial properties, could be of great significance in preventive and

therapeutic approaches as an alternative form of health care. Natural antimicrobial agents also did not show any side effects in extended use which is different from synthetic one. [41, 43]

Herb such as *Persicaria odorata* (Lour) Sojak was also known for its biological activities. The essential oil of *P. odorata* was active as antibacterial agents towards *Salmonella* type bacteria and *Eschericia coli*. [44, 45]

Several compounds from *Parmelia subrudecta* Nyl. such as lecanoric acid (**8**) showed activity against Gram-positive bacteria such as *Staphylococcus aureus* SG 511 (MIC, 200 μg/mL), *Staphylococcus aureus* MRSA (MIC, 1000 μg/mL), and *Mycobacterium tuberculosis* (MIC, 100 μg/mL). Usnic acid (**9**) was active against several bacteria but was inactive against *Mycobacterium smegmatis* and *Candida albicans* Bayer-Ruck. These compounds showed activity against important Gram-positive pathogens like mycobacteria and multiresistant *Staphylococci*. [46]

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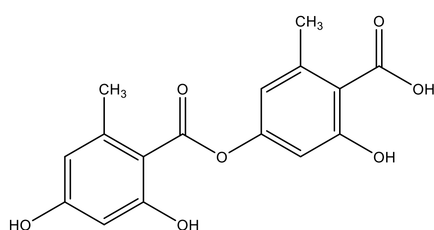
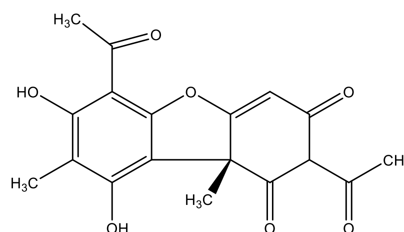


Lecanoric acid



Usnic acid

CHAPTER II

BIOACTIVE CONSTITUENTS OF

LICHEN *Parmotrema dilatatum* (Vainio) Hale

2.1 Introduction

Lichens are unique organisms that grow very slow but capable of living in extreme conditions and environment. [47] Lichen grows as a symbiotic organism between fungus and algae or cyanobacterium. [48] Lichens have been differentiated into three types of thalli including fruticose, foliose and crustose. Fruticose lichens form as strap shaped or thread like lobes with a radial thallus, which formed similar as branch tree or shrub. Foliose lichens appear to be similar as leaves on higher plants, formed by flattened lobes. Crustose lichens form like crust, attached to the substrate intimately and almost inseparable, usually growth in soil, rocks or tree barks. [49] The morphological of lichens explained at Figure 2.1.

Figure 2.1 Major morphological type of lichen thallus. (a) crustose (b) foliose (c) fruticose [50]

Lichens have been used as medicinal purpose since century. In ayurverdic medication, lichens were used to treat respiratory diseases while in unani medication which is substantially based on Ibn Sina's the Canon of Medicine, they used lichens for various stomach disorders and even for liver treatment. [51, 52]

Until recently, the exploration of bioactive constituents from lichens are noteworthy however less documented. [53] Lately, Vietnamese lichens were disclosed to possess chemical diversity. [53, 54] Those bioactive constituents sometimes comprise of an extraordinary skeleton. [55]

Several lichen metabolites of various chemical classes, including the aliphatic acids, depsides and depsidones, dibenzofurans, diterpenes, anthraquinones, naphthoquinones, xanthones have been reported with various biological activities such as cytotoxic, fungicidal, antimicrobial, antioxidant, and anti-inflammatory. [56]

Monoaromatic compounds from lichen including methyl haemmatommate (**10**), methylß-orcinolcarboxylate (**11**) and 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**12**) were found to inhibit some reactive oxygen species, the growth of bacteria, fungi and have potency as anticancer agents. [57-62]

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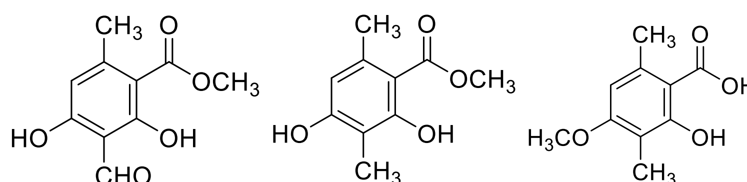


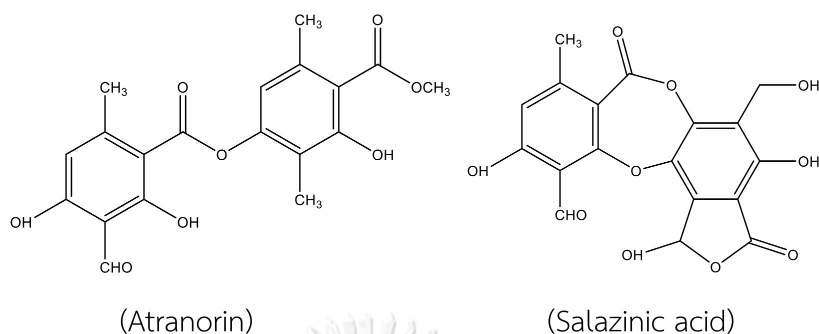
methyl haemmatommate (**10**), methylß-orcinolcarboxylate (**11**), 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**12**)

Lichen *P. dilatatum* (Vain.) Hale (Figure 2.2) is a foliose lichen which can be easily found in South-East Asia. [63] Originated from family Parmeliaceae, *Parmotrema genus* is characterized by large foliose thalli with broad lobes, commonly with a broad erhizinate marginal zone on the lower surface, pored epicortex, thick-walled hyaline ellipsoid ascospores, sub lageniform or filiform conidia and with or without marginal cilia. [64]

Figure 2.2 *Parmotrema dilatatum* (Vain.) Hale

Atranorin (**13**) and salazinic (**14**) acid are major components that can be isolated from *P. dilatatum*. [65] Several biological activities of atranorin and salazinic acid were reported. Previously, atranorin was made into cream and tested for wound healing activity towards rats. The topical application of atranorin cream towards male rats was proved to enhance the healing process. [66] In 2011, the anti-inflammatory activity and toxicity studies of atranorin were determined. Atranorin with dosage 100 and 200 mg/kg displayed noteworthy anti-inflammatory activity (paw edema and leukocyte migration).

The subchronic toxicity or cytotoxicity of atranorin towards rats were also not significance. [67]



(Atranorin) (Salazinic acid)

Salazinic acid isolated from lichen *Parmotrema cetratum* (Ach.) Hale, showed anti-tumor activity against K562 cells with concentration that inhibited cell growth by 50% (GI_{50} 64.36 μM), HT-29 (67.91 μM) and B16-F10 (78.64 μM). Salazinic acid also inhibited 88% tumor growth in B16-F10 melanoma cell. [68]

The biological activities of compounds isolated from *P. dilatatum* were scarce. In 2004, protocretaric acid isolated from *P. dilatatum* could release nitric oxide 1.5 folds greater than a positive control in the culture of mice peritoneal macrophages, by the liberation of H_2O_2 and NO. [69] The cytotoxicity of protocetraric acid (**15**) against melanoma cells was evaluated in 2013. The high selectivity index on UACC-62 cells made protocetraric acid a prospective candidate for the study of melanomas in experimental models. [70]

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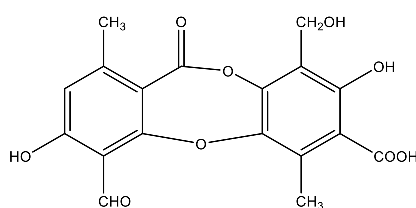
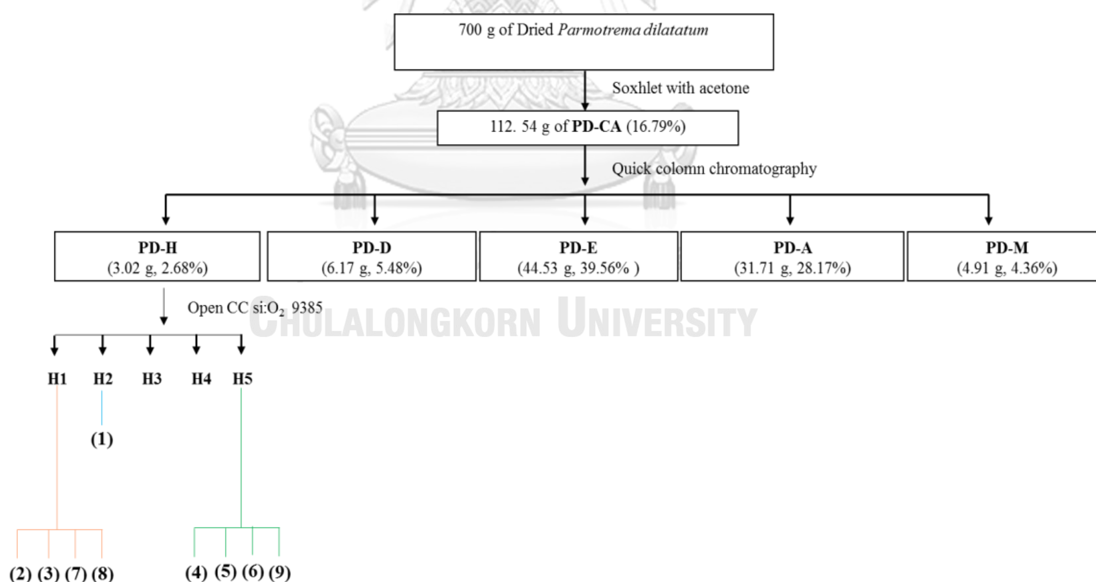


Protocretaric acid

2.2 Results and discussion

2.2.1 Extraction and isolation

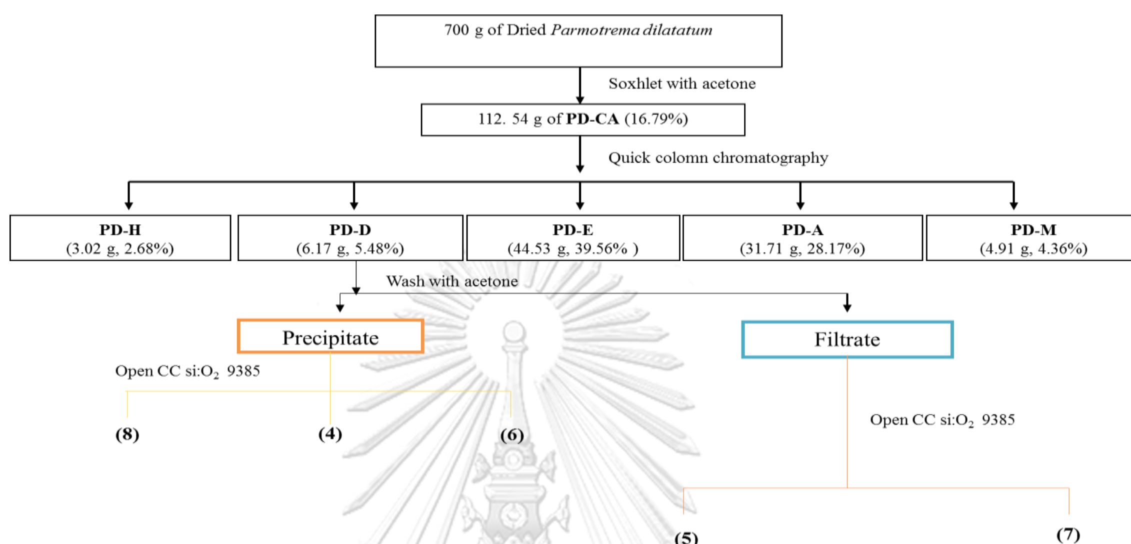
The acetone extract of the whole thallus of *P. dilatatum* was successively partitioned into *n*-hexane, dichloromethane, ethyl acetate, acetone, and methanol fractions. The isolation of *n*-hexane fraction resulted nine compounds including methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl)benzoate (**PD.1**), hopane-16ß,22-diol (**PD.2**), methyl orsellinate (**PD.3**), methyl haematomate (**PD.4**), methyl β-orcinolcarboxylate (**PD.5**), 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**PD.6**), atranol (**PD.7**), atranorin (**PD.8**) and lecanorin (**PD.9**). The description of the isolation of *n*-hexane fraction are explained in Scheme 2.1.



Scheme 2.1 Extraction, separation and isolation scheme of n-hexane fraction *from P. dilatatum*

Five compounds were obtained from the dichloromethane fraction including atranorin (**PD.8**), methyl haemmatommate (**PD.4**), 2-hydroxy-4-methoxy-3,6-

dimethylbenzoic acid (**PD.6**), methyl β-orcinolcarboxylate (**PD.5**) and atranol (**PD.7**). The description of isolation of the dichloromethane fraction are explained in Scheme 2.2.



Scheme 2.2 Extraction, separation and isolation scheme of the dichloromethane fraction from *P. dilatatum*

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2.2.2 Structure elucidation of isolated compounds

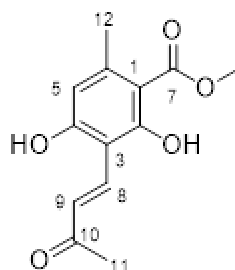
2.2.2.1 Compound PD.1

5 mg white amorphous powder was isolated from fraction **H2** and coded as compound **PD.1**. The tentative ^{1}H- and ^{13}C-NMR chemical shifts of **PD.1** are elucidated as described in Table 2.1.

Table 2.1 The tentative ^{1}H- and ^{13}C-NMR chemical shift assignment of **PD.1**

methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate (**PD.1**)		
Position	^{1}H-NMR	^{13}C-NMR
1		103.3
2		165.0
3		107.5
4		161.0
5	6.35, 1H, *s*	111.6
6		141.4
7		172.2
8	7.83, 1H, *d*, J = 16.4	133.5
9	7.11, 1H, *d*, J = 16.4	129.3
10		197.9
11	2.15, 3H, *s*	14.3
12	2.36, 3H, *s*	23.6
2-OH	12,76, 1H, *br*	
7-OMe	3.16, 3H, *s*	51.8

Based on ^{1}H- and ^{13}C-NMR spectra and MS of **PD.1**, it can be identified as methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate.



Methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate structure

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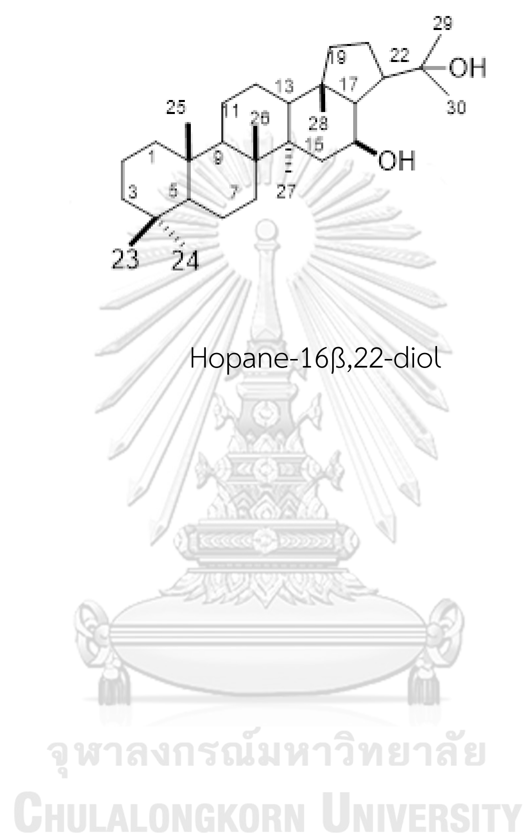
2.2.2.2 Compound PD.2

PD.2 (11 mg) was obtained from fraction **H1**The structure was elucidated by ^{1}H- and ^{13}C-NMR. **PD.2** was identified as hopane-16β,22-diol. [71] The tentative ^{1}H- and ^{13}C-NMR chemical shift assignment of **PD.2** is displayed in Table 2.2.



Hopane-16β,22-diol

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Table 2.2 The tentative ^{1}H- and ^{13}C-NMR chemical shift assignment of **PD.2**

Hopane-16ß,22-diol (2)		
Position	^{1}H-NMR	^{13}C-NMR
1	-	39.9
2	-	18.1
3	-	42.1
4		33.3
5	-	53.8
6	-	21.3
7	-	33.9
8		40.9
9		49.3
10		38.6
11	-	20.6
12	-	23.6
13	-	48.9
14		39.0
15	-	43.6
16	3.72, 1H, *ddd*, J = 10.5, 4.5, 2.5 Hz	66.6
17	1.93, 1H, *dd*, J = 10.0, 4.5 Hz	60.0
18		44.7
19	-	41.5
20	-	26.1
21	2.10, 1H, *dd*, J = 19.5, 8.5 Hz	50.4
22	3.81, 1H, *s*	71.6
23	0.81, 3H, *s*	36.6
24	0.71, 3H, *s*	21.9
25	0.92 (3H, *s*	16.9
26	0.97, 3H, *s*	16.8
27	1.03, 3H, *s*	18.0
28	0.94, 3H, *s*	15.9
29	1.07, 3H, *s*	28.9
30	1.12, 3H, *s*	30.8
16-OH	3.89, 1H, *d*, J = 8.1 Hz	

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2.2.2.3 Compound PD.3

PD.3 (21 mg) was obtained as pale-yellow amorphous powder from fraction **H1**. The structure was elucidated by ^{1}H-NMR and compared with data in the literature. [72] The literature and ^{1}H-NMR gave similar spectra as those of **PD.3**. The compound was identified as methyl orsellinate. The ^{1}H NMR spectrum of **PD.3** is displayed in Figure 2.3 and the tentative ^{1}H NMR chemical shift is presented in Table 2.3.

Table 2.3 The tentative ^{1}H-NMR chemical shift assignment of **PD.3** and methyl orsellinate

Position	**PD.3** (DMSO-d_6, 400 MHz)	methyl orsellinate (acetone-d_6, 300 MHz)[72]
3	6.16, 1H, s	6.26, 1H, s
5	6.15, 1H, s	6.22, 1H, s
6-Me	2.27, 3H, s	2.40, 3H, s
2-OH		11.60, 1H, s
4-OH		9.60, 1H, s
7-OMe	3.79, 3H, s	3.90, 3H, s

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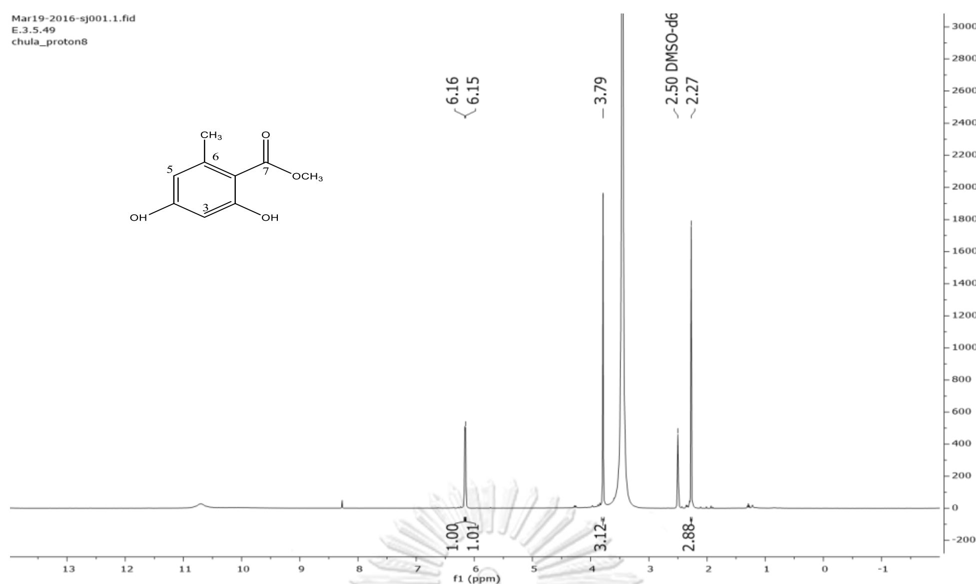

Figure 2.3 The ^{1}H-NMR spectrum of methyl orsellinate

2.2.2.4 Compound PD.4

76.5 mg of **PD.4** was obtained as pale-yellow needles from both *n*-hexane and dichloromethane fractions. Based on ^{1}H NMR spectral analysis and database from literature, **PD.4** was identified as methyl haemmatommate. [73] The ^{1}H NMR spectrum of **PD.4** is presented in Figure 2.7 and the tentative ^{1}H NMR chemical shift is collected in Table 2.4 below and the spectra showed at Figure 2.4.

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Table 2.4 The tentative ^{1}H-NMR chemical shift assignment of **PD.4** and methyl haemmatommate

Position	**PD.4** ($CDCl_3$, 400 MHz)	Methyl haemmatommate ($CDCl_3$,400 MHz) [73]
5	6.28, 1H, s	6.29, 1H, s
6-Me	2.52, 3H, s	2.53, 3H, s
2-OH	12.41, 1H, s	12.41, 1H, s
4-OH	12.88, 1H, s	12.88, 1H, s
3-CHO	10.33, 1H, s	10.34, 1H, s
7-OMe	3.95, 3H, s	3.96, 3H, s

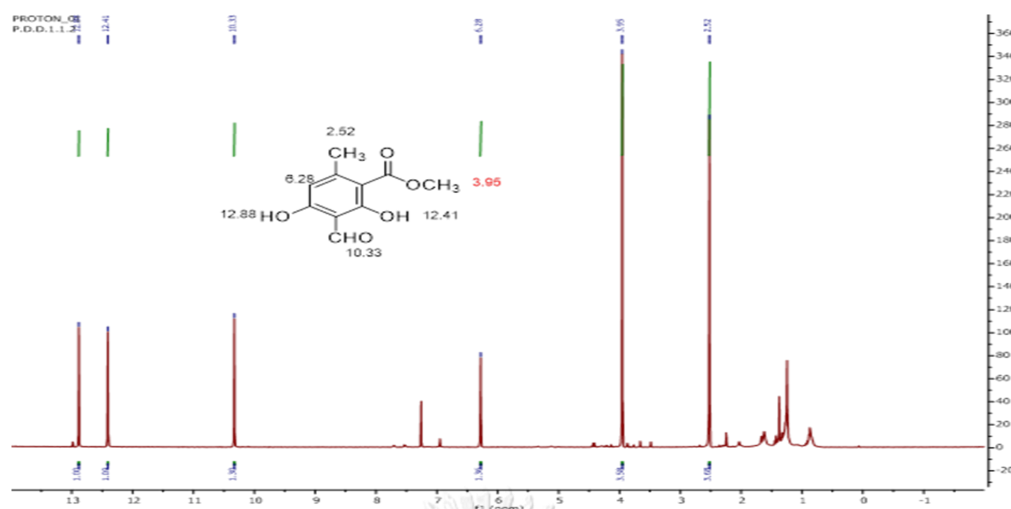

Figure 2.4 The ^{1}H-NMR spectrum of methyl haemmatommate

2.2.2.5 Compound PD.5

PD.5 was obtained from fraction **H5** in *n*-hexane and fraction **D-2.3** after purification with 30% ethyl acetate in n-hexane to obtain 1.48 g of **4** as pale yellow needle. Based on ^{1}H NMR spectrum analysis and database, **5** was identified as methyl β-orcinolcarboxylate. [73] The ^{1}H NMR spectrum of **5** showed at Figure 2.5 and the tentative ^{1}H NMR chemical shift is collected in Table 2.5.

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Table 2.5 The tentative ^{1}H-NMR chemical shift assignment of **PD.5** methyl β-orcinolcarboxylate

Position	**PD.5** (acetone-d_6,400 MHz)	methyl β-orcinolcarboxylate ($CDCl_3$, 400 MHz) [73]
5	6.32, 1H, s	6.21, 1H, s
6-Me	2.41, 3H, s	2.45, 3H, s
2-OH	12.01, 1H, s	12.04, 1H, s
4-OH	8.93, 1H, s	5.30, 1H, s
3-Me	2.05, 3H, s	2.10, 3H, s
7-OMe	3.91, 3H, s	3.92, 3H, s

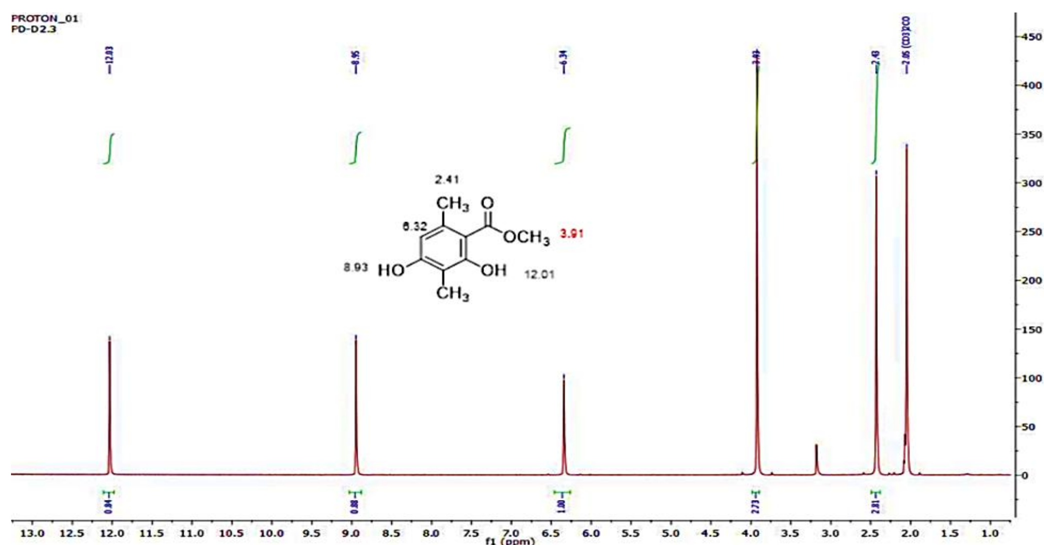

Figure 2.5 The ^{1}H-NMR spectrum of methyl β-orcinolcarboxylate

2.2.2.6 Compound PD.6

PD.6 (21 mg) was obtained mg as white needle from *n*-hexane and dichloromethane fractions. Based on ^{1}H NMR spectral analysis and database, **PD.6** was identified as 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid. [61] The ^{1}H NMR spectrum of **PD.6** is presented in Figure 2.6 and the tentative ^{1}H NMR chemical shift is collected in Table 2.6.

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Table 2.6 The tentative ^{1}H-NMR chemical shift assignment of **PD.6** 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid

Position	**PD.6** ($CDCl_3$, 400 MHz)	2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid ($CDCl_3$, 400 MHz) [61]
5	6.20, 1H, s	6.13, 1H, s
6-Me	2.49, 3H, s	2.38, 3H, s
2-OH	12.08, 1H, s	11.96, 1H, s
4-OMe	3.92, 3H, s	3.85, 3H, s
3-Me	2.10, 3H, s	2.03, 3H, s

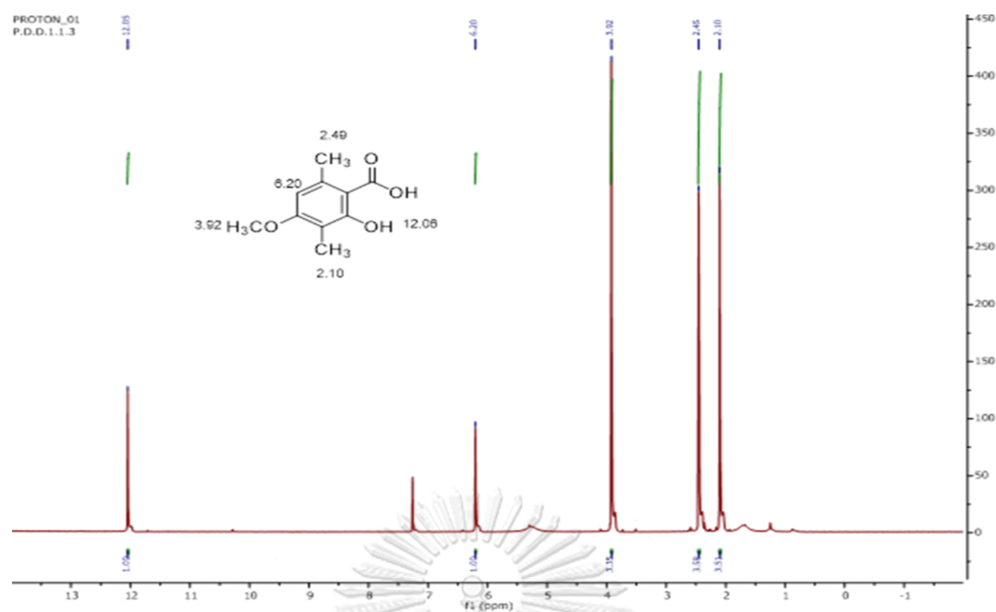

Figure2.6 The ^{1}H-NMR spectrum of 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid

2.2.2.7 Compound PD.7

PD.7 (18 mg) was obtained as yellow needle from fraction **H1** from *n*-hexane and **D-2.3** from dichloromethane. Based on ^{1}H NMR spectrum analysis and database, **PD.7** was identified as atranol. [74] The ^{1}H NMR spectrum of **PD.7** is shown in Figure 2.7 and the tentative ^{1}H NMR chemical shift is collected in Table 2.7.

Table 2.7 The tentative ^{1}H-NMR chemical shift assignment of **PD.7** and atranol

Position	**PD.7** (acetone-d_6, 400 MHz)	atranol (acetone-d_6, at 60 MHz) [74]
3	6.25, 1H, s	6.25, 1H, s
5	6.25, 1H, s	6.25, 1H, s
6-Me	2.23, 3H, s	2.23, 3H, s
3-CHO	10.26, 1H, s	10.27, 1H, s

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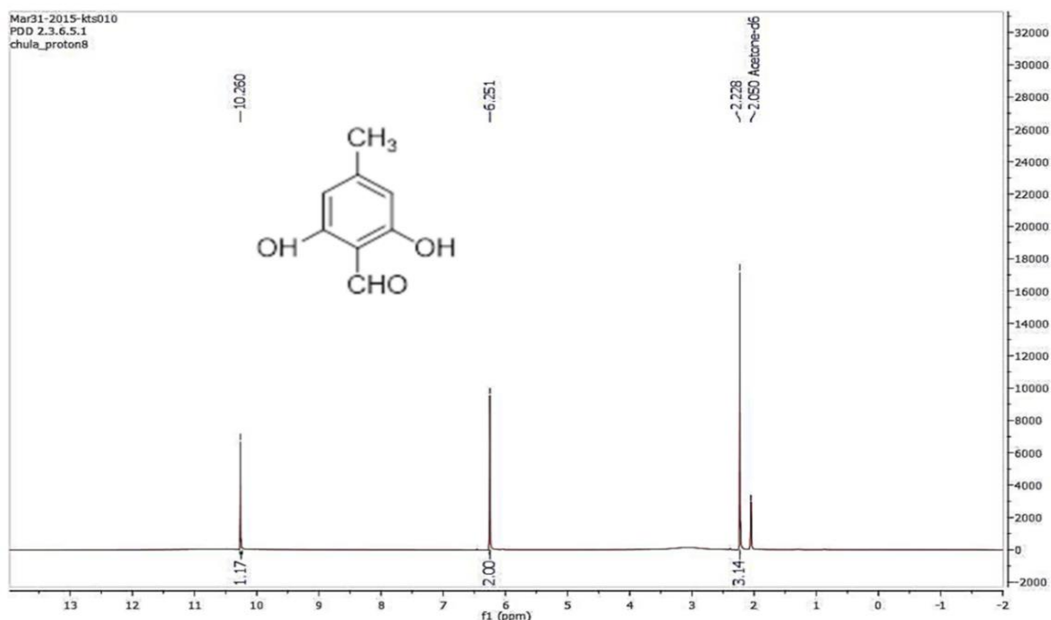

Figure 2.7 The ^{1}H-NMR spectrum of atranol

2.2.2.8 Compound PD.8

1.11 g of white needle crystal was obtained from fraction **H1** in *n*-hexane and **D1** in dichloromethane separation. **PD.8** was then elucidated by ^{1}H-NMR and database comparison and was confirmed as atranorin. [75] The ^{1}H NMR spectrum of **PD.8** is presented in Figure 2.8 and the tentative ^{1}H NMR chemical shift is collected in Table 2.8.

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Table 2.8 The tentative ^{1}H-NMR chemical shift assignment of **PD.8** and atranorin

Position	**PD.8** ($CDCl_3$, 400 MHz)	Atranorin ($CDCl_3$, 400 MHz) [75]
5	6.51, 1H, s	6.39, 1H, s
6-Me	2.68, 3H, s	2.67, 3H, s
2-OH	12.51, 1H, s	12.49, 1H, s
4-OH	12.98, 1H, s	12.54, 1H, s
3-CHO	10.35, 1H, s	10.30, 1H, s
5′	6.40, 1H, s	6.50, 1H, s
3′-Me	2.09, 3H, s	2.07, 3H, s
6′-Me	2.54, 3H, s	2.53, 3H, s
2′-OH	11.96, 1H, s	11.94, 1H, s
7′-OMe	3.98, 3H, s	3.97, 3H, s

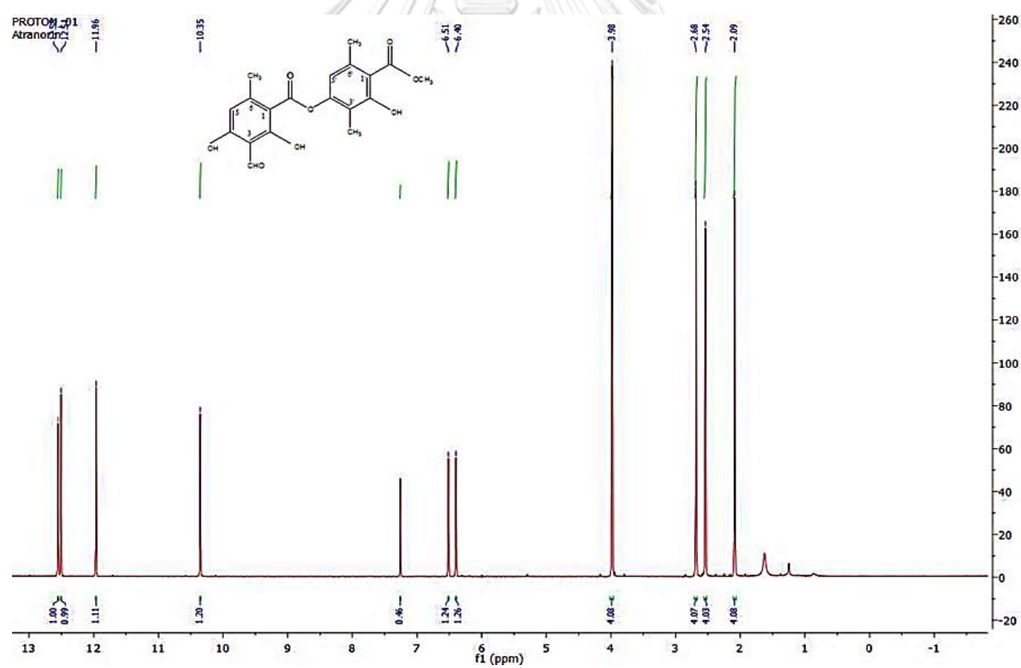

Figure 2.8 The ^{1}H-NMR spectrum of atranorin

2.2.2.9 Compound PD.9

PD.9 (7 mg) was obtained from fraction **H5** from *n*-hexane as yellow crystal and identified by ^{1}H NMR spectrum analysis and database from literature. There were some

slightly differences in data spectra, as in literature they used acetone-d_6 and measured at 300 MHz. After consideration, **PD.9** was identified as lecanorin. [76] The ^1H NMR spectrum of **PD.9** is presented in Figure 2.9 and the tentative ^1H NMR chemical shift is collected in Table 2.9.

Table 2.9 The tentative ^1H-NMR chemical shift assignment of **PD.9** and lecanorin

Position	**PD.9** ($CDCl_3$, 400 MHz)	lecanorin (acetone-d_6, 300 MHz) [76]
3	6.59, 1H, s	6.37, 1H, d, 2.3 Hz
5	6.59, 1H, s	6.28, 1H, d, 2.3 Hz
6-Me	2.62, 1H, s	2.28, 3H, s
2-OH	11.43, 1H, s	
1′	6.50, 1H, s	6.80, 1H, s
3′	6.32, 1H, s	6.60, 1H, s
5′	6.31, 1H, s	6.15, 1H, s
6′-Me	2.34, 1H, s	2.24, 3H, s

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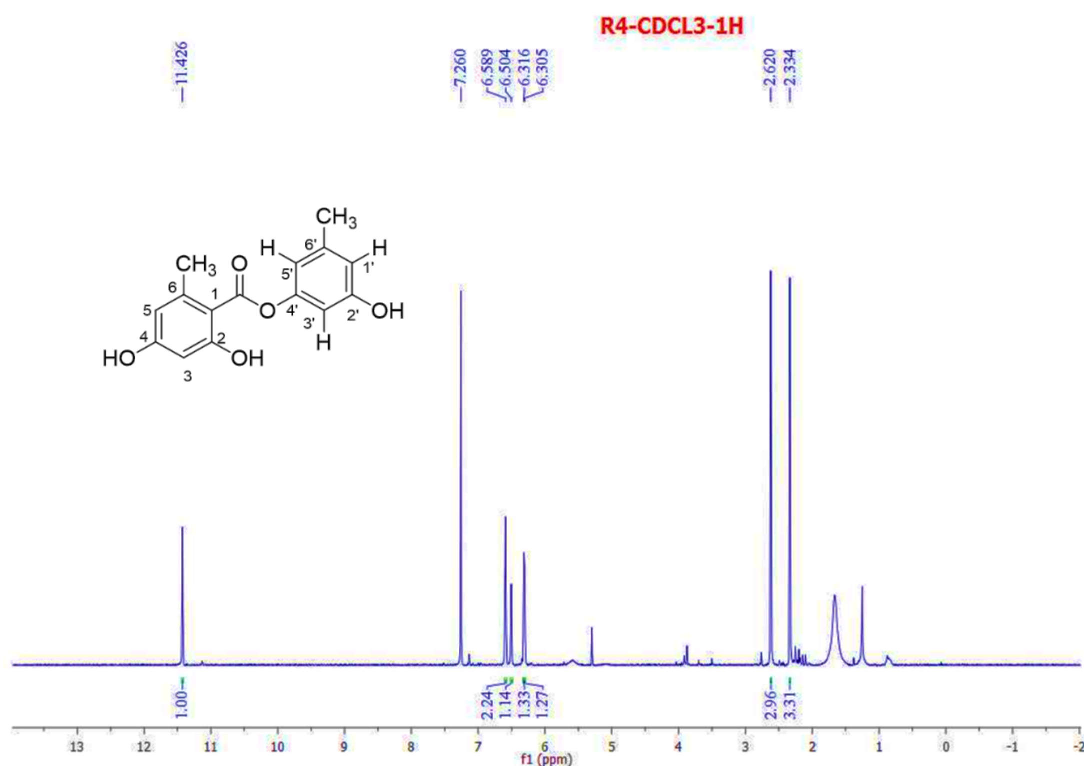

Figure 2.9 The ^{1}H-NMR spectrum of lecanorin

2.2.3 Biological activity

2.2.3.1 DPPH Inhibitory activity of lichen extract, fractions and compounds

The DPPH preliminary of lichen extract and fractions was presented by their inhibition concentration at fifty percent. Ascorbic acid was used as a positive control. The activity was indicated by the change of DPPH color from violet into pale yellow.

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The IC_{50} value of DPPH inhibition from lichen extract, fractions and compounds was evaluated by various concentrations. Then the results were placed at linear regression and the concentration was calculated by the equation. The results showed that acetone fraction exhibited the highest antioxidant activity compared to other fractions with IC_{50} value 48.9±0.59 µg/mL. The lowest activity was observed from

hexane fraction with IC_{50} more than 1,000 µg/mL. The results of DPPH assay are presented in Table 2.10.

Table 2.10 DPPH IC_{50} value of lichen extract and fractions

Sample code	IC_{50} value of lichen extract and fraction (µg/mL)
Crude *Parmotrema* extract (PD-CA)	104.4±3.40
Parmotrema hexane fraction (PD-H)	>1000
Parmotrema dichloromethane fraction (PD-D)	431.4±2.21
Parmotrema ethyl acetate fraction (PD-E)	121±0.29
Parmotrema acetone fraction (PD-A)	48.9±0.59
Parmotrema methanol fraction (PD-M)	158.2±0.21

Standard deviation was calculated by means of three replications

There were several reports on lichen extracts from *Parmotrema* genus. 70% of the methanolic extract of lichen *P. reticulatum* (Taylor) M. was reported to show high antioxidant activity towards hydroxyl and hyphochlorus radical, but gave moderate activity against DPPH, superoxide, singlet oxygen and nitrogen radical species. [77]

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Two lichens from Parmaliaceae family including *P. sulcata* Taylor and *Flavorparmelia caperata* (L.) were also reported for their DPPH inhibitory activity with IC_{50} values of 584.22 and 549.01 µg/mL, respectively. [78]

The DPPH activity of lichen extracts mostly affected by solvent used for extraction. Since most of phenolic compounds were isolated from polar extracts.

The DPPH inhibition activity of lichen compounds was presented as their IC_{50} values. The monoaromatic compounds exhibited higher inhibition activity compared to depsides. Their activity is presented in Table 2.11.

Table 2.11 IC_{50} value of DPPH assay from lichen compounds

Compounds	DPPH assay IC_{50} value (μM)
methyl orsellinate (**PD.3**)	>1000
methyl haemmatommate (**PD.4**)	587.2±0.35
methyl β-orcinolcarboxylate (**PD.5**)	>1000
2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**PD.6**)	>1000
atranol (**PD.7**)	87.5±1.74
atranorin (**PD.8**)	>1000
lecanorin (**PD.9**)	NT
ascorbic acid	1.1±0.93

NT: not tested; Standard deviation was calculated by means of three replications

Atranol showed excellent activity towards DPPH with IC_{50} value of 87.5±1.74 μM followed by methyl haemmatommate with 587.2±0.35 μM. The activity of compounds lower than the commercial standard ascorbic acid showed the outstanding IC_{50} value of 1.1±0.93 μM. Compared with other monoaromatics, the aldehyde group in atranol and methyl haemmatommate at *para* position played an important role in DPPH inhibition.

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2.2.3.2 $ABTS^{\bullet+}$ Inhibitory activity of lichen extract, fractions and compounds

Another antioxidant assay that tested with lichen is $ABTS^{\bullet+}$. The decolorization of $ABTS^{\bullet+}$ were measured as percentage inhibition. The mixture showed color change, from blue into colorless that confirmed the ability of extracts or compounds to inhibit

$ABTS^{•+}$ radical cation. The $ABTS^{•+}$ radical cation of extract, fraction and compounds are presented in Tables 2.12 and 2.13, respectively.

Table 2.12 $ABTS^{•+}$ IC_{50} value of lichen extract and fractions

Sample code	IC_{50} value of lichen extract and fraction (µg/mL)
Crude *Parmotrema* extract (PD-CA)	57.5±0.28
Parmotrema hexane fraction (PD-H)	>1000
Parmotrema dichloromethane fraction (PD-D)	9.3±0.07
Parmotrema ethyl acetate fraction (PD-E)	20.1±0.25
Parmotrema acetone fraction (PD-A)	774.8±0.29
Parmotrema methanol fraction (PD-M)	121±0.37

Standard deviation was calculated by mean of three replications

Based on Table 2.12, the dichloromethane fraction of lichen showed the highest activity towards $ABTS^{•+}$ with IC_{50} value of 9.3±0.07 µg/mL, while hexane fraction showed the lowest activity with IC_{50} value more than 1,000 µg/mL.

The $ABTS^{•+}$ activity of lichen was rarely determined. For lichen *Parmotrema austrosinese*, there was only one report. The lichen possessed great inhibitory activity at both low and high molecular weight fractions with 80 and 70% inhibition, respectively. [79] The $ABTS^{•+}$ activity of *P. dilatatum* was never documented before, but as it perceived that almost every fraction gave excellent IC_{50} value except for hexane fraction. This result may lead to the development of other antioxidant determination.

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Table 2.13 ABTS$^{\bullet+}$ activity of lichen compounds

Compounds	ABTS assay IC_{50} value (µM)
methyl orsellinate (**PD.3**)	NT
methyl haemmatommate (**PD.4**)	241.66±0.64
methyl β-orcinolcarboxylate (**PD.5**)	61.76±1.17
2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**PD.6**)	NT
atranol (**PD.7**)	25.22±0.15
atranorin (**PD.8**)	>1000
lecanorin (**PD.9**)	NT
ascorbic acid	17.43±0.04

NT: not tested; Standard deviation was calculated by mean of three replications

The highest activity was also observed from atranol with IC_{50} value of 25.2±0.15 µM followed by methyl β-orcinolcarboxylate which gave IC_{50} value of 61.76±1.17 µM and methyl haemmatommate with IC_{50} value of 25.2±0.15 µM. Apparently, the positive control ascorbic acid still showed its excellent activity with IC_{50} value of 17.4±0.04 µM.

Methyl β-orcinolcarboxylate was reported to be active against nitrogen reactive species more than radical oxygen species. This result offered that the monoaromatic compounds in lichen possessed different mechanism as antioxidant agents. [59]

2.2.3.4 Tyrosinase inhibitory activity of lichen extract, fractions and compounds

The skin whitening activity of lichen extract, fractions and compounds were determined by tyrosinase inhibition. The tyrosinase inhibition of extract, fractions and compounds are displayed in Table 2.14 and 2.15, respectively.

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Table 2.14 IC_{50} value of tyrosinase activity of lichen extract and fractions

Sample code	IC_{50} value of lichen extract and fractions (µg/mL)
Crude *Parmotrema* extract (PD-CA)	> 500
Parmotrema hexane fraction (PD-H)	>500
Parmotrema dichloromethane fraction (PD-D)	17.05±1.23
Parmotrema ethyl acetate fraction (PD-E)	334.8±1.54
Parmotrema acetone fraction (PD-A)	> 500
Parmotrema methanol fraction (PD-M)	> 500

Standard deviation was calculated by mean of three replications

Tyrosinase inhibition which provided by lichen extract and fractions showed that dichloromethane fraction presented exceptional fifty percent inhibition at very low concentration with IC_{50} value of 17.05±1.23 µg/mL. The activity then followed by ethyl acetate fraction with IC_{50} value of 334.8±1.54 µg/mL, respectively. While the crude acetone extract, hexane, acetone and methanol fractions showed the lowest activity with IC_{50} more than 500 µg/mL.

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In recent reports several lichen extracts such as *Stereocaulon ramulosum*, *Cladonia dimorphoclada*, *Stereocaulon microcarpum* and *Cladia aggregate* were active against tyrosinase enzyme. [80]

Table 2.15 IC_{50} value of tyrosinase activity of lichen compounds

Compounds	Tyrosinase assay IC_{50} value (µM)
methyl orsellinate (**PD.3**)	NT
methyl haemmatommate (**PD.4**)	>200
methyl β-orcinolcarboxylate (**PD.5**)	>200
2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**PD.6**)	NT
atranol (**PD.7**)	34.6±0.62
atranorin (**PD.8**)	9.2±1.25
lecanorin (**PD.9**)	NT
kojic acid	36.1±1.07

NT: not tested; Standard deviation was calculated by mean of three replications.

Some isolated compounds presented very good activity towards tyrosinase which were better than a positive control, kojic acid. Atranorin as a major compound in the dichloromethane fraction and a depside, have an outstanding tyosinase inhibition with IC_{50} value of 9.22±1.25 µM which was four times higher than kojic acid (IC_{50} value of 36.08±1.07 µM). Another compound that also gave admirable activity is atranol which showed antioxidant activity as well in DPPH and ABTS inhibition. Atranol presented IC_{50} value of 34.6±0.62 µM which slightly higher than kojic acid. Other compounds showed activity that could inhibit fifty percent of tyrosinase more than 200 µM. The result presented in Table 2.15 is reported for the first time from lichen *P. dilatatum.*

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The activity of methyl haemmatommate and methyl ß-orcinolcarboxylate against tyrosinase was recorded previously. At 100 μM, the activities of both compounds were not significant. [67]

It was reported previously that at 0.5 mM of methyl orsellinate could activate tyrosinase, which makes the possibility of this compound as substrate. While *n*-octyl orsellinate showed uncompetitive inhibition against tyrosinase. Thus, it was concluded that enzyme hydrophobic site accepted eight carbon of an alkyl chain in the *n*-octyl orsellinats and made compound more active. [81]

2.2.3.5 α-Glucosidase inhibitory activity of lichen extract, fractions and compounds

There were several ways to determine *α*-glucosidase activity of prospective plants, lichen or compounds. In this research, the activity was determined by using *α*-glucosidase isolated from yeast and 4-nitrophenyl *α*-D-glucopyranoside as a substrate. Lichen extracts and fractions overall gave excellent activity with IC_{50} value range from 0.7-24 μg/mL. Methanol fraction possessed high activity by inhibition fifty percent of enzyme *α*-glucosidase activity at 0.7±0.18 μg/mL. Acetone fraction presented the second highest activity with IC_{50} value of 0.8±0.24 μg/mL, followed by dichloromethane, and hexane fractions, respectively. The crude acetone extract of *P. dilatatum* also showed superb activity with IC_{50} value 6.6±0.15 μg/mL. All the data was recorded in Table 2.16.

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Table 2.16 α-Glucosidase inhibitory activity of lichen extract and fractions

Sample name	IC_{50} value of lichen extract and fraction (μg/mL)
Crude *Parmotrema* extract (PD-CA)	6.6±0.15
Parmotrema hexane fraction (PD-H)	24±0.56
Parmotrema dichloromethane fraction (PD-D)	9.5±0.51
Parmotrema ethyl acetate fraction (PD-E)	2.2±0.17
Parmotrema acetone fraction (PD-A)	0.8±0.24
Parmotrema methanol fraction (PD-M)	0.7±0.18

Standard deviation was calculated by mean of three replications

α-Glucosidase activity of compounds was determined in various concentrations to obtain IC_{50} value. The results of *α*-glucosidase activity of lichen compounds are displayed in Table 2.17.

Table 2.17 α-Glucosidase inhibitory activity of lichen compounds

Compounds	IC_{50} (μM)
methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate (**PD.1**)	77.9±0.82
hopane-16β,22-diol (**PD.2**)	>200
methyl orsellinate (**PD.3**)	NT
methyl haemmatommate (**PD.4**)	48.7±1.59
methyl β-orcinolcarboxylate (**PD.5**)	>200
2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**PD.6**)	>200
atranol (**PD.7**)	>200
atranorin (**PD.8**)	>200
lecanorin (**PD.9**)	NT
acarbose	93.6±0.49

NT: not test; Standard deviation was calculated by mean of three replications

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Methyl haemmatommate and methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate exhibited potent α-glucosidase inhibitory activity with IC_{50} values of 48.7±1.59 and 77.9±0.82 µM, respectively. Methyl haemmatommate with aldehyde group at position 3 showed better activity compared to methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate that contained α,ß-unsaturated ketone substituent. The methyl ester substituent in methyl haemmatommate also enhanced the activity greatly compared to atranol which possessed aldehyde group at position 3 but lack of methyl ester substituent at position 1 and 2-hydroxy-4-methoxy-3,6-dimethyl benzoic acid which possessed carboxylic acid constituent instead of methyl ester at position 1.

In previous researches, phenolic compounds found to be active as α-glucosidase and α-amylase enzyme inhibitory. Therefore, this result will be important used for type 2 diabetes treatment improvement. [82]

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2.2.3.6 Antibacterial activity of lichen extracts and compounds

The antibacterial activity of lichen extract, fractions and compounds towards pathogen bacteria was determined by using agar well diffusion. The antibacterial activity of extract, fractions and compounds are displayed in Table 2.18 and 2.19 respectively.

Table 2.18 Antibacterial activity of lichen extracts

Sample code	Concentration	Inhibition zone (mm)				
		Salmonella typhi ATCC 422	*Propiniobacterium acnes* KCCM 4147	*Staphylococcus aureus* ATCC 25923	*Streptococcus sobrinus* KCCM 11898	*Streptococcus mutans* ATCC 25175
(+) Chlorampenicol	0.5 mM	19.00±1.14	25.00±1.00	31.00±1.00	22.00±1.14	26.00±1.00
(-) Acetone	-	-	-	-	-	-
PD-CA	1000 ppm	13.00±0.89	11.00±1.14	11.00±1.00	14.00±1.14	13.00±1.14
PD-H	1000 ppm	11.00±0.89	8.00±1.14	6.00±1.00	10.00±1.00	13.00±0.89
PD-D	1000 ppm	12.00±1.00	12.00±1.58	10.00±1.00	13.00±0.44	10.00±2.07
PD-E	1000 ppm	11.00±1.14	11.00±1.00	11.00±0.89	13.00±0.44	13.00±0.89
PD-A	1000 ppm	8.00±0.00	9.00±0.44	9.00±1.14	11.00±0.89	10.00±0.89
PD-M	1000 ppm	6.00±0.00	11.00±0.81	10.00±0.00	8.00±0.00	11.00±1.14

Criteria of clear zone inhibition (including the diameter of well in mm) 6.0 = no activity; 6.1–8.0= weak; 8.1-10= moderate; 10.1-13= Good; 13.1-15= very good; > 15= excellent. **PD-CA**= crude *P. dilatatum* acetone extract; **PD-H**= *P. dilatatum* hexane extract; **PD-D**= *P. dilatatum* dichloromethane extract; **PD-E**= *P. dilatatum* ethyl acetate extract; **PD-A**= *P. dilatatum* acetone extract; **PD-M**= *P. dilatatum* methanol extract.

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Research regarding lichen as antibacterial are well-known; however, the antibacterial activity of *P. dilatatum* (Vainio) Hale. has never been reported. The results revealed that the bacterial inhibition ranges from 11-14 mm which was good inhibition based on the clear zone inhibition criteria. The fractions mostly showed moderate to good inhibition towards all types of bacteria. The activity of the dichloromethane fraction was dominate with clear zone inhibition range from 10-13 mm. The ethyl acetate fraction also exhibited potential inhibition towards all types of bacteria. The lowest activities were observed from the methanol fraction with clear zone inhibition 6-11 mm. The report of antibacterial of lichen towards oral bacteria was primarily documented here.

Numerous reports on lichen antibacterial activity especially belong to Parmaliaceae have been reported. The acetone, chloroform, diethyl ether, petroleum ether and methanol extracts of *Xanthoparmelia pokornyi* (Körb.) O. Blanco, A. Crespo, Elix, D. Hawksw. and Lumbsch were active towards some food borne bacteria and fungi. [83] These results were similar to those obtained from lichen *Parmelia sulcata* Nyl. Extracts, except for its petroleum ether extract. [84] The ethanol extract of *P. Kamstchandalis* Ach. also exhibited potential antibacterial activity towards Gram positive and negative bacteria with diameter of zone inhibition range from 8-20 mm. [85] The methanol and acetone extract of *P. caperata* (L.) Ach. and *P. pertusa* (Hoffm.) Mass. were active towards several pathogen bacteria and fungi compared with other lichens. [86]

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Table 2.19 Antibacterial activity of lichen compounds

Sample code	Concentration	**Inhibition zone (mm)**				
		S. typhi ATCC 422	*P. acnes* KCCM 4147	*S. aureus* ATCC 25923	*S. sobrinus* KCCM 11898	*S. mutans* ATCC 25175
(+) Chlorampenicol	0.5 mM	19.00±0.44	25.00±0.44	31.00±0.57	22.00±0.50	26.00±0.50
(-) Acetone	-	-	-	-	-	-
AT	1 mM	16.00±0.89	15.00±0.44	11.00±0.50	15.00±0.44	14.00±0.57
ATR	1 mM	8.00±0.95	11.00±0.95	8.00±0.57	10.00±0.57	8.00±0.89
MBO	1 mM	8.00±0.44	10.00±0.89	9.00±0.57	12.00±0.89	12.00±0.50
MB	1 mM	9.00±0.57	10.00±0.95	9.00±0.95	12.00±0.44	10.00±0.89
MH	1 mM	10.00±0.57	11.00±0.50	10.00±0.95	10.00±0.89	9.00±0.50
MO	1 mM	6.00±1.00	11.00±0.57	9.00±0.50	11.00±0.44	11.00±0.95
LEC	NT	NT	NT	NT	NT	NT

NT mean the compound was not tested for this activity. Criteria of clear zone inhibition (including the diameter of well in mm) 6.0 = no activity; 6.1–8.0= weak; 8.1-10= moderate; 10.1-13= Good; 13.1-15= very good; > 15= excellent. **AT**= atranorin; **ATR** = atranol; **MBO** = methyl β-orcinolcarboxylate; **MB** = 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid; **MH** = methyl haemmatommate; **MO** = methyl orsellinate; **LEC** = lecanorin

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Followed the activity of lichen extracts and fractions, the antibacterial activities of isolated compounds were determined by agar well diffusion. Based on Table 2.19, atranorin, the depside as well as the major compound of the dichloromethane fraction showed quite outstanding activity towards all types of bacteria. The interesting inhibition was towards Gram-negative bacteria *S. typhi* with diameter zone of inhibition 16.00±0.89 mm. Atranorin also gave very good activity towards *P. acne* and *S. sobrinus* with inhibition around 15 mm. Atranorin as one of known major compounds found in many lichen species possessed potential as antibacterial agents as described in several reports. The antibacterial activity of atranorin reported by Thadhani *et al.*, which inhibited *S. typhi*, *B. subtilis* and *E. coli* with diameter of inhibition 12, 12 and 13 mm, respectively at concentration of 100 μg/mL. [87]

The second major compound, methyl β-orcinolcarboxylate showed promising activity towards *S. mutans and S. sobrinus* with diameter zone inhibition around 12 mm. Quite a few of research related to this compound has been established. Methyl β-orcinolcarboxylate from *P. Kamstchandalis* showed potential activity towards Gram-positive and Gram-negative bacteria with diameter zone inhibition at 200 μg/disc range from 8-15 mm. [85]

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Based on the activity and the amount of sample, atranorin and methyl β-orcinolcarboxylate were chosen to determine MIC test. The test was focused on Gram-positive bacteria. The MIC and MBC results are presented in Table 2.20.

Table 2.20 MIC and MBC of lichen compounds

Sample code	**Concentration (µM)**									
	S. typhi		*P. acnes*		*S. aureus*		*S. sobrinus*		*S. mutans*	
	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC	MIC	MBC
AT	NT	NT	1	>1	1	>1	1	>1	1	>1
ATR	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MBO	NT	NT	1	>1	1	>1	0.5	>1	0.25	>1
MB	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MH	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
MO	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT
LEC	NT	NT	NT	NT	NT	NT	NT	NT	NT	NT

NT: not test. **AT**= atranorin; **ATR** = atranol; **MBO** = methyl β-orcinolcarboxylate; **MB** = 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid; **MH** = methyl haemmatommate; **MO** = methyl orsellinate; **LEC** = lecanorin

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Atranorin revealed MIC towards all tested bacteria at the maximum concentration 1 mM and MBC more than 1mM. The results can be seen in Figure 2.20.

Atranorin isolated from lichens collected in Chilean area was tested against three strains each of methicillin susceptible and MRSA isolated from cystic fibrosis patients. MIC of atranorin was ranged from 64-128 µg/mL and MBC value from 128->128 µg/mL gave moderate activity compared to usnic acid that exhibited MIC value from 2-8 µg/mL and MBC value 64->64 µg/mL. [88]

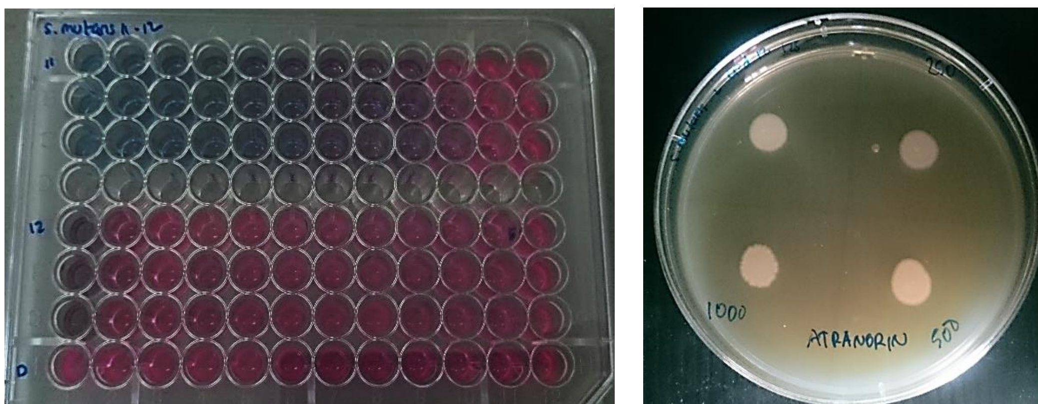

Figure 2.13 Left side the MIC of atranorin (code 12); right side the MBC of atranorin towards *S. mutans*

2.3 Experimental Section

2.3.1 General Experimental Procedures

The NMR spectra of lichen compounds were recorded on a Bruker AM-400 MHz. All chemical shifts were quoted on the δ scale in ppm using residual solvent signals as the internal standard ($CDCL_3$ 7.26 ppm for ^1H NMR and 77.2 ppm for ^{13}C NMR; acetone-d_6: 2.04 ppm for ^1H NMR, 29.8 and 206.0 ppm for ^{13}C NMR; DMSO-d_6: 2.50 ppm for ^1H NMR, 39.5 ppm for ^{13}C NMR). Open-air column chromatographies were performed on silica gel 40-63 µm, Himedia Laboratories, Mumbai, India. 70-230 mesh (Merck Grade 7734), pore size 60 Å, 230-400 (Merck Grade 9385). TLC analyses were carried out on precoated silica gel 60F254 plates (Merck KGaA, Germany). The plates were monitored under UV-light exposure and/or treated with a solution of 5% vanillin in acidic ethanolic solution prior to heating. Tyrosinase and α-glucosidase enzymes, *L*-tyrosine, *p*-nitrophenyl-α-D-glucopyranoside, ABTS, DPPH all were purchased from Sigma-Aldrich.

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2.3.2 Lichen Material

The thalli of the lichen were separated from rocks in Lam Dong Province, Vietnam, in August-September 2013. This species was determined as *Parmotrema dilatatum* (Vain.) Hale by Dr. Wetchasart Polyiam (Lichen Research Unit, Department of Biology, Faculty of Science, Ramkhamhaeng University, Bangkok, Thailand). A voucher specimen (No. US-B033) was deposited at the Herbarium of the Department of Organic Chemistry, University of Science, National University, Ho Chi Minh City, Vietnam.

2.3.3 Extraction and Isolation of Compounds

The dried thalli of *P. dilatatum* (700 g) were air dried, grounded and extracted with acetone. The filtered solution was evaporated to dryness, to afford a crude acetone extract (112.5 g). The crude acetone extract was applied to silica gel solid-phase extraction and successively eluted with *n*-hexane, DCM, EtOAc, acetone, and MeOH to afford the corresponding fractions: **PH** (3.02 g), **PD** (6.2 g), **PEA** (44.5 g), **PA** (31.7 g), and **PM** (5.0 g).

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PH (3.02 g) was applied to normal phase silica gel column chromatography (CC) and eluted with solvent system *n*-hexane:EtOAC (8.0:2.0) to obtain five fractions: **H1** (1.57 g), **H2** (0.37 g), **H3** (0.32 g), **H4** (0.19 mg), and **H5** (0.55 g).

H1 was subjected further into normal phase CC and isocratically eluted with *n*-hexane:EtOAc: acetone: acetic acid (8.0:2.0:0.4:0.1, v/v/v/v) to obtain four compounds: **PD.2** (11 mg), **PD.3** (21 mg), **PD.7** (13 mg), and **PD.8** (210 mg). Furthermore, **H2** was

chromatographed, using similar solvent system as previously described above to obtain **PD.1** (5.0 mg). **PD.H5** was then purified by CC to obtain four compounds including **PD.4** (67 mg), **PD.5** (25 mg), **PD.6** (3 mg), and **PD.9** (7 mg).

Methyl (*E*)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl)benzoate (PD.1), white amorphous powder. ^{1}H NMR (500 MHz, acetone-d_6, δ, ppm, J/Hz): 12,76 (1H, *br*, 2-OH), 7.83 (1H, *d*, J = 16.4, H-8), 7.11 (1H, *d*, J = 16.4), 6.35 (1H, *s*, H-5), 3.16 (3H, *s*, 7-OCH_3), 2.36 (3H, *s*, H-12), 2.15 (3H, *s*, H-11). ^{13}C-NMR (125 MHz, DMSO-d_6, δ, ppm): 197.9 (C-10), 172.2 (C-7), 165.0 (C-2), 161.0 (C-4), 141.4 (C-6), 133.5 (C-8), 129.3 (C-9), 111.6 (C-5), 107.5 (C-3), 103.3 (C-1), 51.8 (7-OCH_3), 23.6 (C-12), 14.3 (C-11). HR-ESI-MS m/z 249.0754 $[M-H]^-$ (calcd. for C_{13} $H_{13}O_5$-H, 249.0763).

Hopane-16β,22-diol (PD.2), ^{1}H NMR (500 MHz, DMSO-d_6, δ, ppm, J/Hz): 3.89 (1H, *d*, J = 8.1 Hz, 16-OH), 3.81 (1H, *s*, 22-OH), 3.72 (1H, *ddd*, J = 10.5, 4.5, 2.5 Hz, H-16), 2.10 (1H, *dd*, J = 19.5, 8.5 Hz, H-21), 1.93 (1H, *dd*, J = 10.0, 4.5 Hz, H-17), 1.12 (3H, *s*, H-30), 1.07 (3H, *s*, H-29), 1.03 (3H, *s*, H-27), 0.97 (3H, *s*, H-26), 0.94 (3H, *s*, H-28), 0.92 (3H, *s*, H-25), 0.81 (3H, *s*, H-23), 0.71 (3H, *s*, H-24). ^{13}C-NMR (125 MHz, DMSO- d_6, δ, ppm): 71.6 (C-22), 66.6 (C-16), 60.0 (C-17), 53.8 (C-5), 50.4 (C-21), 49.3 (C-9), 48.9 (C-13), 44.7 (C-18), 43.6 (C-15), 42.1 (C-3), 41.5 (C-19), 40.9 (C-8), 38.6 (C-10), 39.9 (C-1), 39.0 (C-14), 36.6 (C-23), 33.9 (C-7), 33.3 (C-4), 30.8 (C-30), 28.9 (C-29), 26.1 (C-20), 23.6 (C-12), 21.9 (C-24), 21.3 (C-6), 20.6 (C-11), 18.1 (C-2), 18.0 (C-27), 16.9 (C-25), 16.8 (C-26), 15.9 (C-28).

D1 from the dichloromethane fraction was obtained by washed the crystal form in the dichloromethane fraction with hexane and continued with acetone. A white

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brownish crystal then subjected to silica and column chromatography repeatedly with solvent system *n*-hexane:DCM:MeOH 3:2:0.05. From the purification of atranorin, there was a fraction containing a mixture of atranorin (**PD.8**) and other two compounds. Fraction was then purified by using solvent system *n*-hexane:DCM:MeOH = 3:2:0.1 to obtain **PD.4** and **PD.6**.

4.46 g of **D2** was coated on silica gel high-purity grade (Merck Grade 7734), pore size 60 Å, 70-230 mesh. The coating sample was then subjected on silica gel high-purity grade (Merck Grade 9385), pore size 60 Å, 230-400 mesh particle size as stationary phase. A mixture of n-hexane and EtOAc was used as an eluent by increasing the polarity. Fractions were collected in erlenmeyer about 200 mL. Each fraction was then confirmed by TLC. The fractions with the similar spots or bands appearance were collected as major fraction. Sub-fractions from **D2** column chromatography is displayed in Table 2.21.

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Table 2.21 Sub-fractions from **D2** column chromatography

Fraction	Solvent composition and ratio	Volume
D-2.1	100 % of *n*-hexane-5 % Ethyl acetate in *n*-hexane	7200 mL
D-2.2	5 % Ethyl acetate in *n*-hexane	2800 mL
D-2.3	5 % -10% Ethyl acetate in *n*-hexane	4000 mL
D-2.4	10% Ethyl acetate in *n*-hexane	1600 mL
D-2.5	15-20 % Ethyl acetate in *n*-hexane	4500 mL
D-2.6	40-60 % Ethyl acetate in *n*-hexane	4000 mL
D-2.7	80-100% Ethyl acetate in *n*-hexane	4000 mL

D-2.3 was further purified with 30% EtOAc in *n*-hexane to obtain 7 fractions with one fraction appeared almost pure. This fraction was recrystallized to gain 1.23 g of **PD.5** as yellow needle shape crystal. Based on ^{1}H NMR spectrum analysis and database from literature **PD.5** was identified as methyl β-orcinol carboxylate.

Furthermore, one more compound was obtained from **D-2.3** by using isocratic solvent system *n*-hexane:acetone 4:2. **PD.7** was obtained about 15 mg as yellow needle shape. Based on ^{1}H NMR spectrum analysis and database from literature, **PD.7** was identified as atranol.

2.3.4 DPPH inhibition activity of lichen extracts and compounds

The antioxidant activity was followed the method described by Rankovic *et al.*, 2012) with modification. [89] Samples were prepared in 10% DMSO and two-fold dilution was performed on 96 well plate by using multichannel micropipette, while ascorbic acid was prepared in MeOH. 0.05 mg/mL of DPPH solution was prepared in MeOH. 50 µL of samples were placed on 96 well plate and then 100 µL of DPPH solution was added. The mixtures were homogenized and incubated in dark place at room temperature for 30 minutes. The DPPH absorbance was measured by using microplate reader (BIOTEK power waver xs2) at 517 nm. Ascorbic acid was used as the positive control. The percentage inhibition was calculated using the following equation:

$$\text{DPPH scavenging effect (\%)} = [(A_0 - A_1)/A_0] \times 100$$

where A_0 is the absorbance of the negative control and A_1 is the absorbance of reaction mixture of extract or compounds or standard.

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The inhibition concentration at 50% inhibition (IC_{50}) was the parameter used to compare the radical scavenging activity.

2.3.5 $ABTS^{\bullet+}$ inhibition activity of lichen extracts and compounds

The assay was performed by using modified method from Fu, Zhang, Guo, and Chen. [90] $ABTS^{\bullet+}$ and potassium persulfate were diluted in distilled water and placed in dark at room temperature for 12-16 h. The stock solution was then diluted with distilled water to obtain an absorbance of 0.7 ± 0.02 at 734 nm (BIOTEK power waver xs2). All samples were prepared in 10% DMSO. 50 µL of various concentration of samples were placed on 96 well plate and then 150 µL of $ABTS^{\bullet+}$ solution was added. The absorbance of reaction was measured immediately or up to 3 min. Percentage inhibition and IC_{50} value calculation was calculated alike DPPH inhibition assay calculation.

2.3.6 α-glucosidase Inhibitory Assay

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α-Glucosidase inhibitory activity was determined according to [91] 0.1 U/mL of α-glucosidase and 1 mM *p*-nitrophenyl-α-D-glucopyranoside as a substrate were dissolved in 0.1 mM pH 6.9 phosphate buffer. 10 µL of inhibitor and 40 µL of enzyme was preincubated at 37 °C for 10 min, afterwards 50 µL of substrate was then added to the reaction mixture. The enzymatic reaction was carried out at 37 °C for 20 min and stopped by adding 1 M Na_2CO_3 (100 µL). Enzymatic activity was quantified by measuring absorbance at 405 nm (ALLSHENG micro plate reader AMR-100). All samples

were analyzed in triplicate at different concentrations to obtain the IC_{50} value of each extract and compound, and the mean values were retained.

The inhibition percentage (%) was calculated by the following equation:

$$\text{Inhibition (\%)} = [(A_{sample}/A_{control})/ A_{control}] \times 100$$

where $A_{control}$ is the absorbance of the negative control and A_{sample} is the absorbance of reaction mixture with compounds or extracts or standard.

2.3.7 Tyrosinase Inhibitory Assay

The activity of extracts and compounds towards tyrosinase enzyme was determined by using 96-well micro plate with modification. [92] Extracts and compounds were dissolved in 10% DMSO in buffer. *L*-tyrosine was used as substrate. Two-fold dilution of sample was done to obtain various desire concentrations. The mixture of 50 µL of sample solution and 50 µL tyrosinase from mushroom (250 U/mL) was placed into 96 well plate and incubated for 5 minutes. Subsequently, 50 µL solution of 5 mM *L*-tyrosine as a substrate was added into the mixtures and then incubated further for 30 minutes. The activity was measured at 490 nm by using micro plate reader (ALLSHENG micro plate reader AMR-100). The tyrosinase inhibition was calculated from the following formula (1) and IC_{50} was determined for each sample.

$$\%\, Tyrosinase\ inhibition = \frac{\Delta Acontrol - \Delta A\ sample}{\Delta A\ control}\ x\ 100 \qquad (1)$$

Where "$\Delta Acontrol$" was the absorbance value at 490 nm without the test sample and "$\Delta Asample$" was the absorbance value of mixture contained the sample.

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2.3.8 Determination of antibacterial activities by agar well diffusion test

The antibacterial activity towards bacteria were determined with modified agar well diffusion method described by Karuppiah and Mustaffa [93] against tooth decay bacteria *Streptococcus mutans* ATCC 25175 and *Streptococcus sobrinus* KCCM 11898. Bacteria causing skin disease such as *Staphylococcus aureus* ATCC 25923 and *Propinibacterium acnes* KCCM 4147. Extracts, fractions and compounds were also tested with Gram-negative bacteria caused typhi fever *Salmonella typhi* ATCC 422.

Microorganisms were inoculated in nutrient broth and left overnight. The density of each microbial suspension was equal to that of 1-1.5x10^8 cells/mL (standardized by 0.5 McFarland). About 25 mL of nutrient agar was transferred into 9 cm sterilized petri dish. The plates were left at room temperature to let medium solidify. 100 µL of bacteria sub culture then swabbed over the agar media and allow to dry. Wells were made by using a sterile corked borer (6 mm). 30 µL of compounds and antibiotics were placed into the wells. Negative control used solvent for dissolving the sample. Clear zone inhibition was measured after incubation overnight at 37 ^{o}C. All experiments were done in triplicates and the results were expressed as means values.

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Diameter of inhibition zone including diameter of well (6 mm) was observed with range of activity as described here where 6.0 mm means no activity, 6.1 – 8.0 mm means compound show weak activity, 8.1 – 10.0 mm means moderate activity, 10.1 – 13.0 mm means good, 13.1 – 15.0 mm means very good activity and more than 15 mm means compounds show excellent activity.

2.3.9 Minimum inhibitory concentration (MIC) and minimum bactericidal concentration of lichen compounds (MBC)

MIC was conducted by micro dilution method with some modifications using 96-well micro-titer plates. [94, 95] It was determined by selecting the lowest concentration of sample which show inhibition with colorimetric change. Bacterial culture was inoculated in the nutrient broth and incubated at 37 ^{o}C for 18-24 h. The bacterial suspensions were adjusted to the 0.5 McFarland standard (approximately 1 to 1.5x10^{8} CFU/mL). Various concentrations were used for each compound and tetracycline. 50 μL of nutrient broth (NB) were added to all wells and the serial dilution were performed using the 8 multichannel pipettes.

The excess amount of compound was discarded in the end of wells from serially descending concentrations. After that, 40 μL NB will be added in each well and 10 μL of bacteria suspension. The plates will be prepared in triplicate and incubated at 37 ^{o}C for 18-24 h. After the incubation, 10 μL of 0.01% resazurin as oxidation-reduction indicator were added into each well of the 96-well micro-plate and the plates were incubated for 10 minutes. Any color change observed from purple to pink or colorless was taken as positive with cell viability. The lowest concentration of compound at with no color change from blue to purple or pink was recorded as the MIC value. The test was performed as triplicates and concurrently with commercial antibiotic (tetracycline) as a positive control.

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MBC was conducted by taking 10 μL of three various concentrations which were two-fold higher than MIC and placed in the nutrient agar media. The plates were incubated for 18-24 h and the concentration which showed no growth of bacteria colony was noted as MBC.

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CHAPTER III

BIOLOGICAL ACTIVITIES OF *Knema angustifolia* (Roxb.) Warb., *Persicaria odorata* (Lour.) Sojak. AND *Mansonia gagei* Drumm. MAJOR CONSTITUENTS

3.1 Introduction

3.1.1 *Knema angustifolia* (Roxb.) Warb.

Knema genus belongs to Myristicaceae, widely distributed around Africa, Australia and Asia. In Thailand, there are 12 species of *Knema* genus. [96] Several *Knema* species have been used for medicinal purpose especially for their barks and seeds. [97] Previously, it was reported that *K. furfuracea* Warb. and *K. globularia* (Lam.) Warb. from Thailand were used to treat sores and acne. [98] The figure of *K. angustifolia* plant and stem cross section is displayed in Figure 3.1.

Figure 3.1 *K. angustifolia (Roxb.) Warb.* plant (left), plant stem cross section (right)

Source: Google; Personal documentation

Knema angustifolia (Roxb.) Warb. is known as “Kamlang leuat ma” in Thai or “horse blood”. [99] While in Indonesia, Malaysia and Singapore this plant known as “Mendarahan”. The name is presumably due to red resin color produced by plant.

The stems of *K. angustifolia* are well known as whole body tonic. [99] Several species of *Knema* from Thailand, for instance *K. elegans*, *K. furfuraceae*, *K. laurina* and *K. tunuinervia* were reported to contain anacardic acid, cardanols, acetophenone derivatives, isocoumarins, and neolignans. [100] The woods of *K. austrosiamensis* was reported to consist of stilbenes while new sesquiterpenes were reposted from the stem barks of *K. patentinervia*. [101, 102] The chemical constituents of *Knema* genus is displayed in Figure 3.2.

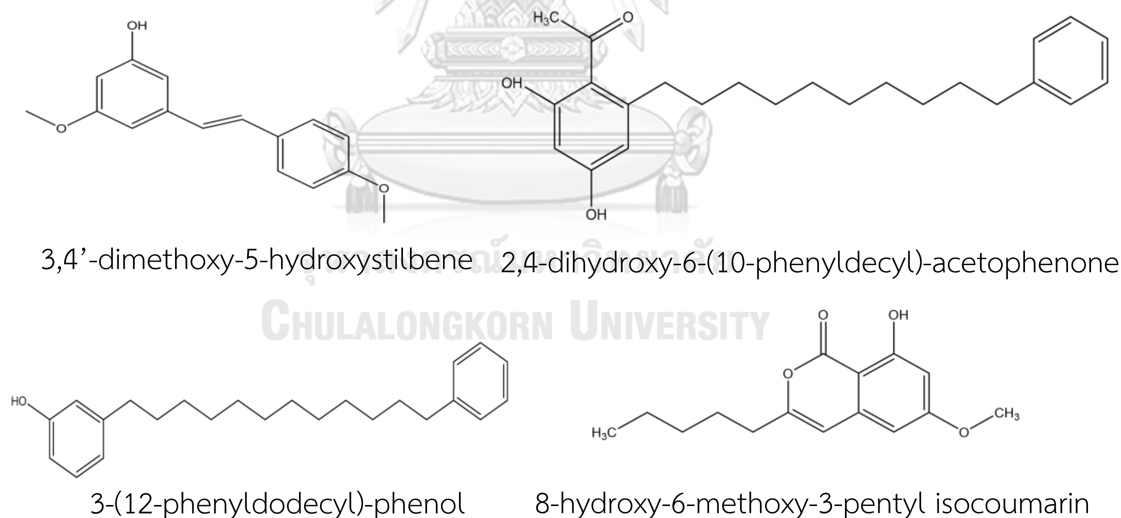

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Figure 3.2 *Knema* genus chemical constituents

3.1.2 *Persicaria odorata* (Lour.) Sojak.

Persicaria odorata (Lour.) Sojak. (synonym: *Polygonum odoratum* Lour) (Figure 3.3). was famous as herb in various South East Asian cuisines. [103] Famously known

as Vietnamese coriander in English. In Vietnam, it is called as “rau răm”, while in Singapore, Malaysia and Indonesia this herb is called “daun kesum” or “daun laksa” due to the use of the leaves in laksa dish. In Thai, *P. odorata* is known as “praew”. [104]

Figure 3.3 *P. odorata* Lour. (Sojak.)

Source: www.123rf.com; https://essentialoils.com.my/mint-vietnamese-mint-essential-oil.htm

The plant was believed to cure fever, restrain thirst, for injuries, including antimicrobial property, anti-inflammatory activity, antitumor-promoting activity, antioxidative property and insect antifeedant activity. [105] The aerial part of *P. odorata* mostly contained essential oil such as (*Z*)-3-hexenal, (*Z*)-3-hexenol, decanal, undecanal, and dodecanal (Figure 3.4). [103]

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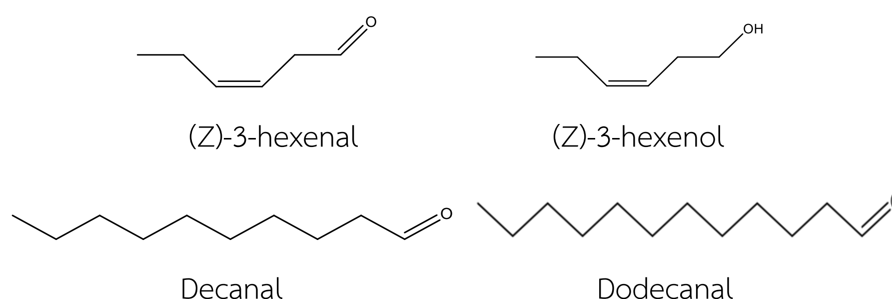

Figure 3.4 Major constituents from *P. odorata*

3.1.3 *Mansonia gagei* Drumm.

Another local plant that utilized for medicinal purpose is *Mansonia gagei* Drumm. (Figure 3.5) which was native to Thailand. This plant belongs to Sterculaceae family, locally known as "chan-hom, chan-pha-ma, chan-khao, or chan-cha-mod". People in Thai used the heartwoods of this plant as cardiac stimulant, refreshment agent, anti-depressant, antiemetic and onilivertigo. [106, 107]

Figure 3.5 *M. gagei* Drumm. tree (left) and its heartwood (right)

The extracts of *M. gagei* Drumm. were reported for their significant biological activities as well as their major constituents. Mansonone G as a major constituent and mansonone E (Figure 3.6), both were identified as 1,2-naphthoquinone. Both compounds possessed interesting biological activities such as antibacterial, anticancer and antifungal. [106]

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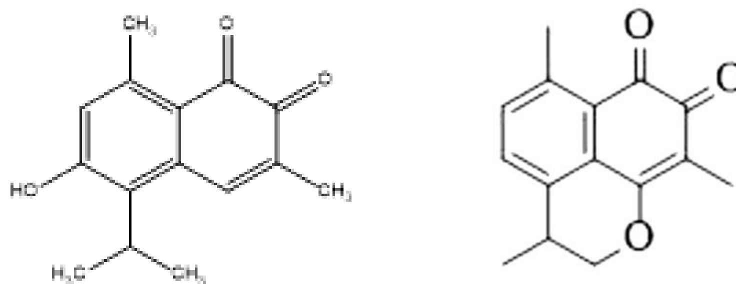

Figure 3.6 Mansonone G (left), mansonone E (right)

3.2 Results and discussion

3.2.1 Extraction of the stems of *K. angustifolia* (Roxb.) Warb.

The extraction of *K. angustifolia* stem was done subsequently in four organic solvents, including *n*-hexane, dichloromethane, ethyl acetate and methanol. 600 g of dried ground *K. angustifolia* were extracted using soxhlet with 5 L of each solvent for 3 times. The results of extraction are shown in Table 3.1.

Table 3.1 Weight and percent yield of *K. angustifolia* extraction

Extract	Weight (g)	Percent yield (%)
n-hexane	7.02	1.17
dichloromethane	1.38	0.23
ethyl acetate	12	2
methanol	65.68	11

The stems of *K. angustifolia* were extracted with different solvents to obtain various extracts based on polarity. The dichloromethane extract yielded the lowest amount of extract 1.38 g while 65.68 g of extract could be obtained from methanol extraction. Various solvent with different polarities may tranquil the isolation process

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and resulted different biological activities. Therefore, to extract different compounds from plants with a high precision, solvents with different polarities must be used. [108]

3.2.2 Extraction of the leaves of *P. odorata*

Dried ground leaves (700 g) of *P. odorata* Lour. Sojak extracted by soxhlet subsequently using *n*-hexane, dichloromethane and methanol. The results of extraction are shown in Table 3.2.

Table 3.2 Weight and percent yield of *P. odorata* extraction

Extract	Weight (g)	Percent yield (%)
n-hexane	14.55	2.01
dichloromethane	6.15	0.8
methanol	89.35	12.76

Different from *K. angustifolia*, the leaves of *P. odorata* were extracted with three different solvents. The consideration for choosing suitable solvents to extract sample is the target isolated compound. In *P. odorata* the major compounds were usually found in *n*-hexane and methanol extracts.

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3.2.3 Extraction of the heartwoods of *M. gagei*

From 10 kg of the heartwood extraction with ethyl acetate, 575 g of crude extract with 5.75% of percent yield could be obtained. The *M. gagei* heartwood was not subsequently extracted, due to the major compounds of this plant such as mansonones G and E could be isolated in dichloromethane and ethyl acetate.

3.2.4 Biological activities of *K. angustifolia*

3.2.4.1 DPPH and $ABTS^{•+}$ inhibition activity of *K. angustifolia* extracts and compounds

The antioxidant activities of extracts and compounds were determined by the ability to decolorize DPPH and $ABTS^{•+}$ reagent that mostly known for antioxidant testing model. The antioxidant activities of extracts are shown in Table 3.3.

Table 3.3 DPPH and $ABTS^{•+}$ inhibition activity of *K. angustifolia* extracts

Sample code	$IC_{50\ of}$ DPPH (µg/mL)	IC_{50} of ABTS (µg/mL)
n-hexane extract	13.6±0.45	775.3±0.25
Dichloromethane extract	39.7±0.12	120.5±0.39
Ethyl acetate extract	3.5±0.24	46.6±0.43
Methanol extract	1.8±0.20	188.4±0.30

Standard deviation was calculated by mean of three replications.

The antioxidant activities of *K. angustifolia* extracts revealed that all extracts had antioxidant potential. The difference was the concentration of 50 percent inhibition towards reagents that caused radical occurrence. Among them, the ethyl acetate extract showed excellent activities in most of antioxidant activities by inhibiting DPPH and $ABTS^{•+}$ with IC_{50} values of 3.5±0.24 and 46.6±0.43 µg/mL. However, the methanol extract gave the highest activities by inhibiting 50% of DPPH at 1.8±0.20 µg/mL. The polarity of solvent used in extraction would give high probability to obtain excellent antioxidants. [109]

Four major compounds from the ethyl acetate extract of *K. angustifolia* were tested for antioxidant assay. Those compounds included 11-*O*-acetyl bergenin, bergenin, quercetin and catechin. Their structures are depicted in Figure 3.7 and the antioxidant activities are presented in Table 3.4.

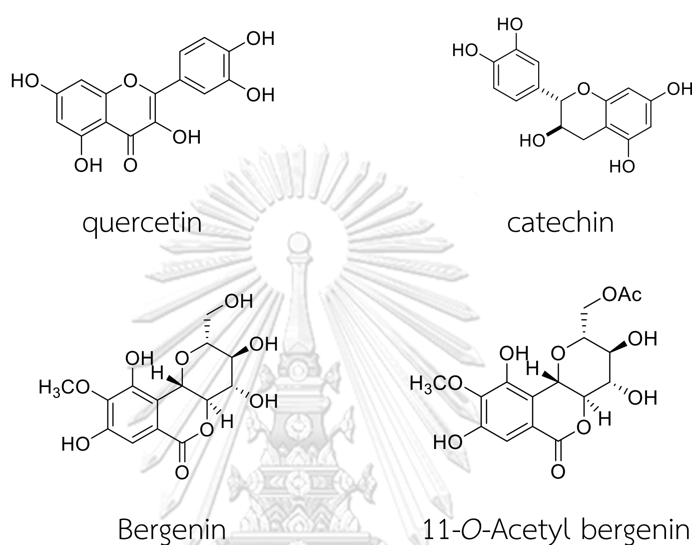

Figure 3.7 *K. angustifolia* chemical constituents

Table 3.4 DPPH and ABTS$^{\bullet+}$ inhibition activity of compounds isolated from *K. angustifolia*

Compounds	IC_{50} of DPPH (μM)	IC_{50} of ABTS$^{\bullet+}$ (μM)
11-*O*-acetyl bergenin	> 1 mM	14.3±0.79
Bergenin	> 1 mM	13.4±0.94
Quercetin	0.9±1.04	11.1±0.62
Catechin	2.1±0.24	13.3±0.25
Ascorbic acid	1.1±0.93	17.4±0.04

Standard deviation was calculated by mean of three replications

For the isolated compounds from *K. angustifolia*, quercetin showed the highest activity against DPPH and ABTS$^{\bullet+}$ with IC_{50} values of 0.9±1.04 and 11.1±0.62 μM, respectively. Quercetin also exhibited antioxidant activity higher than ascorbic acid as

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a positive control. Meanwhile, bergenin and 11-*O*-acetyl bergenin showed DPPH inhibition with IC_{50} values more than 1 mM. However, they showed significant inhibition towards $ABTS^{•+}$ with IC_{50} values of 13.4±0.94 and 14.3±0.79 μM respectively. All compounds showed higher activity against $ABTS^{•+}$ compared to a positive control, ascorbic acid.

Many researches regarding flavonoids as antioxidants. They might prevent the radical damage in several ways. The known mechanism usually called as direct scavenging of free radicals. Radicals will be oxidized by flavonoids, which causing radical less reactive and become more stable. Due to the high reactivity of flavonoid hydroxyl groups, make the radical inactive. [110]

3.2.4.2 α-Glucosidase inhibitory assay of *K. angustifolia* extracts and compounds

The α-glucosidase activities of *K. angustifolia* extracts and compounds were determined. The activities of plant extracts and compounds are displayed in Tables 3.5 and 3.6, respectively.

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Table 3.5 α-Glucosidase inhibitory assay of *K. angustifolia* extracts

Sample code	IC_{50} (μg/mL)
Hexane extract	20.9±0.40
Dichloromethane extract	10.1±0.32
Ethyl acetate extract	0.8±0.96
Methanol extract	0.2±0.33

Standard deviation was calculated by mean of three replications

The inhibitory activity of the extracts from *K. angustifolia* against α-glucosidase was tremendous. The range of IC_{50} values started from 0.2 up to 20.9 μg/mL. The activity was affected by the polarity of solvent. The more polar solvent, the better activity result. This might be due to the capability of each solvent to extract specific constituents possessed anti α-glucosidase activity.

Table 3.6 α-Glucosidase inhibitory assay of *K. angustifolia* compounds

Compounds	IC_{50} (μM)
11-*O*-acetyl bergenin	87.7±1.01
Bergenin	NA
Quercetin	71.2±1.04
Catechin	151.6±1.88
Acarbose	93.6±0.49

NA: not active; Standard deviation was calculated by mean of three replications

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The isolated flavonoids from *K. angustifolia* including quercetin and catechin showed potent α-glucosidase inhibitory with IC_{50} values of 71.2±1.04, and 151.6±1.88 μM. Previous researches reported that the activity of commercial diabetic drug, acarbose sometimes was lower than some flavonoids. The kinetic mechanism of flavonoids towards α-glucosidase was also determined. [26, 111, 112] 11-*O*-Acetyl bergenin displayed excellent α-glucosidase activity with IC_{50} value of 87.7±1.01 μM, while bergenin itself displayed no activity. Substitution of acetyl group in 11-*O*-acetyl bergenin led to the increase in anti α-glucosidase activity compared to the hydroxyl group at bergenin. Previously, it was reported that the derivatives of bergenin gave

promising α-glucosidase inhibition than bergenin. [113] To the best of our knowledge this is the first report of 11-*O*-acetyl bergenin as α-glucosidase inhibitor.

3.2.4.3 Tyrosinase inhibitory assay of *K. angustifolia* extracts and compounds

The stem extracts and compounds of *K. angustifolia* were further tested for their potential as skin whitening agents through tyrosinase inhibition. The tyrosinase inhibition of extracts is displayed by their IC_{50} value in Table 3.7.

Table 3.7 Tyrosinase inhibitory assay of *K. angustifolia* extracts

Sample code	IC_{50} (µg/mL)
Hexane extract	50.2±0.69
Dichloromethane extract	118.9±1.83
Ethyl acetate extract	51.6±1.27
Methanol extract	115.9±0.76

Standard deviation was calculated by mean of three replications.

The *n*-hexane and ethyl acetate extracts showed potent anti-tyrosinase activity with IC_{50} values of 50.2±0.69 and 51.6±1.27 µg/mL, respectively. The *n*-hexane extract showed the highest activity compared to the other extracts. The methanol and dichloromethane extracts showed the lowest among other extracts with IC_{50} values of 115.9±0.76 and 118.9±1.83 µg/mL, respectively.

The compounds were also tested for their anti-tyrosinase activity. The tyrosinase inhibition is presented in Table 3.8.

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Table 3.8 Tyrosinase inhibitory assay of *K. angustifolia* compounds

Compounds	IC_{50} (µM)
11-*O*-acetyl bergenin	NA
Bergenin	NA
Quercetin	43.3±0.26
Catechin	>200
Kojic acid	36.1±1.07

NA: not active; Standard deviation was calculated by mean of three replications

Quercetin isolated from the ethyl acetate extract of *K. angustifolia* gave the highest anti-tyrosinase activity compared to the other compounds with IC_{50} value of 43.3±0.26 µM. Quercetin is known for its tyrosinase activity, quercetin inhibited enzyme by inhibiting the diphenolase activity through chelating copper. [114] Moreover, the activity of quercetin is comparable to kojic acid with IC_{50} value of 36.1±1.07 µM. Catechin, in the opposite site gave the activity of IC_{50} > 200 µM. In the previous paper, it was reported that catechin did not show any inhibition towards tyrosinase, compared to its derivatives gallocatechin and epi-gallocatechin gallate that inhibited tyrosinase higher than arbutin as a positive control. [115]

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3.2.4.4 Determination of antibacterial activity from *K. angustifolia* extracts by agar well diffusion

The antibacterial activity of *K. angustifolia* extracts is shown in Table 3.9.

Table 3.9 Antibacterial activity of *K. angustifolia* extracts by agar well diffusion

Sample code	Concentration	Inhibition (mm)				
		Salmonella typhi ATCC 422	*Staphylococcus aureus* ATCC 25923	*Streptococcus mutans* ATCC 25175	*Propinibacterium acne* KCCM 4147	*Streptococcus sobrinus* KCCM 11898
(+) Chloramphenicol	0.5 mM	19.00±0.00	31.00±0.00	26.00±0.00	25.00±0.00	22.00±0.00
(-) Acetone	-	-	-	-	-	-
Hexane	1000 ppm	6.00±0.00	12.00±0.58	11.00±0.25	14.00±0.00	14.00±0.00
Dichloromethane	1000 ppm	6.00±0.25	9.00±1.00	11.00±0.66	9.00±0.00	11.00±0.90
Ethyl acetate	1000 ppm	7.00±0.66	10.00±0.25	12.00±0.00	9.00±1.00	9.00±0.66
Methanol	1000 ppm	8.00±0.45	11.00±0.45	10.00±0.58	8.00±0.00	9.00±0.25

Criteria of clear zone inhibition (including the diameter of well in mm) 6.0 = no activity; 6.1–8.0= weak; 8.1-10= moderate; 10.1-13= Good; 13.1-15= very good; > 15= excellent

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The antibacterial activity of *K. angustifolia* extracts was determined by agar well diffusion towards several types of pathogen bacteria. The n-hexane extract was mostly active towards Gram-positive bacteria with diameter inhibition zone range from 11-14 mm. However, the extract showed no activity towards Gram-negative bacteria that cause typhi fever *Salmonella typhi*. This might be due to the lack ability of extracts to enter the cell walls of *S. typhi* which is more complex than Gram positive bacteria. [36]

3.2.5 Biological activities of *P. odorata*

3.2.5.1 DPPH and $ABTS^{\bullet+}$ inhibition activity of *P. odorata* extracts and compounds

The extracts and major compounds of *P. odorata* leaves were tested for their antioxidant activities towards DPPH and $ABTS^{\bullet+}$. The activities of extracts and compounds are presented as IC_{50} value in Tables 3.10 and 3.11, respectively.

Table 3.10 DPPH and $ABTS^{\bullet+}$ inhibition activity of *P. odorata* extracts

Sample code	IC_{50} of DPPH (µg/mL)	IC_{50} of ABTS (µg/mL)
n-hexane extract	> 1000	> 1000
Dichloromethane extract	> 1000	> 1000
Methanol extract	22.3±0.24	154.4±0.49

Standard deviation was calculated by mean of three replications.

From data presented in the table above, the methanol extract from *P. odorata* was found to be active towards DPPH and $ABTS^{\bullet+}$ compared to the other two extracts. The IC_{50} values for DPPH and $ABTS^{\bullet+}$ were 22.3±0.24 and 154.4±0.49 µg/mL. In 2015, it was reported that the methanol extract of *P. odorata* consisted of various flavonoids

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including rutin as a major constituent, catechin, quercetin, kaempferol and isorhamnetin. [116] Thus, flavonoids in *P. odorata* methanol extract should be responsible for its antioxidant activities.

Table 3.11 DPPH and $ABTS^{\bullet+}$ inhibition activity of *P. odorata* compounds

Compounds	IC_{50} of DPPH (μM)	IC_{50} of $ABTS^{\bullet+}$ (μM)
Decanal	> 1 mM	> 1 mM
Dodecanal	> 1 mM	> 1 mM
Ascorbic acid	1.12±0.93	17.4±0.04

Standard deviation was calculated by mean of three replications.

Compared to the extracts, the major compounds in *P. odorata* which were decanal and dodecanal showed low antioxidant activities with IC_{50} more than 1 mM. The hydrophobicity of compounds might become as main factors why both compounds had low activity towards two antioxidants testing models used in this research.

3.2.5.2 α-Glucosidase inhibitory assay of *P. odorata* extracts and compounds

The anti-diabetic activities of the leave extracts and compounds from *P. odorata* were determined by their capabilities to inhibit enzyme α-glucosidase, which widely known as one of anti-diabetic assays. The α-glucosidase inhibition activity of extracts and compounds are displayed in Tables 3.12 and 3.13, respectively.

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Table 3.12 α-Glucosidase inhibitory assay of *P. odorata* extracts

Sample code	IC_{50} (µg/mL)
Hexane extract	>500
Dichloromethane extract	>500
Methanol extract	41.3±1.46

Standard deviation was calculated by mean of three replications.

As enlightened above that methanol extract of *P. odorata* contained some important flavonoids for antioxidants. Flavonoid such as rutin, as its major constituents was also reported for α-glucosidase inhibition activity. Rutin was a potent α-glucosidase with IC_{50} value of 0.037 µM. [117] Therefore, the α-glucosidase activity of the methanol extract was due to the presence of rutin in the methanol extract.

Table 3.13 α-Glucosidase inhibitory assay of *P. odorata* compounds

Compounds	IC_{50} (µM)
Decanal	>200
Dodecanal	>200
Acarbose	93.63±0.49

Standard deviation was calculated by mean of three replications.

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The activity of two major constituents of *P. odorata* including decanal and dodecanal towards α-glucosidase were also reported. It was found that both compounds had IC_{50} more than 200 µM. The essential oil of the leaves of *P. odorata* and dodecanal which is the major compound, were known mostly for their antimicrobial activity. [44, 118]

3.2.5.3 Tyrosinase inhibitory assay of *P. odorata* extracts and compounds

The leave extracts and compounds of *P. odorata* were tested for its potential as skin whitening agents through enzyme tyrosinase inhibition. The activities of extracts and compounds are displayed by their IC_{50} value in Tables 3.14 and 3.15, respectively.

Table 3.14 Tyrosinase inhibitory assay of *P. odorata* extracts

Sample code	IC_{50} (µg/mL)
PO Hexane extract	NA
PO Dichloromethane extract	NA
PO methanol extract	226.9±1.77

NA: not active; Standard deviation was calculated by mean of three replications.

The methanol extracts of *P. odorata* gave the highest inhibitory activity towards tyrosinase. This is probably caused by the presence of some flavonoids such as quercetin, which has been reported as tyrosinase inhibitors. [116]

Table 3.15 Tyrosinase inhibitory assay of *P. odorata* compounds

Compounds	IC_{50} (µM)
Decanal	NA
Dodecanal	NA
Kojic acid	36.1±1.07

NA: not active; Standard deviation was calculated by mean of three replications

Similar to other activities, both major compounds from *P. odorata* did not inhibit tyrosinase, which might be due to the lack of binding ability of these compounds to the active site of the enzyme.

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3.2.5.4 Determination of antibacterial activity from *P. odorata* extracts by agar well diffusion

The antibacterial activity of *P. odorata* extracts by agar well diffusion

Table 3.16 Antibacterial activities *P. odorata* extracts by agar well diffusion test

No.	Sample code	Concentration	Inhibition (mm)				
			Salmonella typhi ATCC 422	*Staphylococcus aureus* ATCC 25923	*Streptococcus mutans* ATCC 25175	*Propinibacterium acne* KCCM 4147	*Streptococcus sobrinus* KCCM 11898
1.	(+) Chlorampenicol	0.5 mM	19.00±0.00	31.00±0.00	26.00±0.00	25.00±0.00	22.00±0.00
2.	(-) Acetone	-	-	-	-	-	-
3.	Hexane	1000 ppm	7.00±0.25	9.00±0.66	10.00±0.58	8.00±1.00	11.00±0.25
4..	Dichloromethane	1000 ppm	6.00±0.45	10.00±0.00	8.00±0.25	6.00±0.58	10.00±0.00
5.	Methanol	1000 ppm	6.00±0.58	10.00±0.90	10.00±0.90	8.00±0.25	10.00±0.66

Criteria of clear zone inhibition (including the diameter of well in mm) 6.0 = no activity; 6.1–8.0= weak; 8.1-10= moderate; 10.1-13= Good; 13.1-15= very good; > 15= excellent

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The antimicrobial activity of *P. odorata* showed weak to good activities towards Gram-positive bacteria with inhibition range from 6-11 mm with *n*-hexane overall give better activities compared to other extracts. Whereas, the activitiy of *P. odorata* leaves extracts towards Gram-negative showed weak activities with inhibition range from 6-7 mm.

3.2.6 Biological activities of *M. gagei*

3.2.6.1 DPPH and $ABTS^{•+}$ inhibition activity of *M. gagei* extracts and compounds

The extract and major compounds of *M. gagei* heartwood were tested for its antioxidant activities towards DPPH and $ABTS^{•+}$. The activities of extracts and compounds are presented as IC_{50} value in Table 3.17.

Table 3.17 DPPH and $ABTS^{•+}$ inhibition activity of *M. gagei* extracts and compounds

Sample code and compounds	IC_{50} of DPPH	IC_{50} of ABTS
Mansonia ethyl acetate extract	10.1±0.24 μg/mL	29.4±0.64 μg/mL
Mansonone G	> 1 mM	> 1 mM
Mansonone E	> 1 mM	> 1 mM
Ascorbic acid	1.12±0.93 μM	17.4±0.04 μM

Standard deviation was calculated by mean of three replications.

The ethyl acetate extract of *M. gagei* showed potent antioxidant activity towards DPPH and $ABTS^{•+}$ with IC_{50} values of 10.1±0.24 and 29.4±0.64 μg/mL. The methanolic extracts previously reported to inhibit DPPH radical scavenging with IC_{50} <0.02 mg/mL. [119] Those results were comparable with those have been obtained in this research. Thus, *M. gagei* might showing potential to another antioxidant activity as

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well. However, the major compounds of *M. gagei* including mansonones G and E gave low activities in both antioxidant tests. These results were akin with previous results using TLC autobiography for DPPH assay, which explained that mansonones G and E were inactive as antioxidant at concentration 10 μg/mL, while mansonone N showed antioxidant activity at concentration 2.5 μg/mL. [106]

3.2.6.2 α-Glucosidase inhibitory assay of *M. gagei* extracts and compounds

The anti-diabetic activity of heartwood extracts and compounds from *M. gagei* was determined by α-glucosidase inhibitory assay. The activity is shown in Table 3.18.

Table 3.18 α-Glucosidase inhibitory assay of *M. gagei* extracts and compounds

Sample code and compounds	IC_{50}
Mansonia ethyl acetate extract	5.9±1.49 μg/mL
Mansonone G	NA
Mansonone E	>200 μM
Acarbose	93.63±0.49 μM

NA: not active; Standard deviation was calculated by mean of three replications

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The ethyl acetate extract of *M. gagei* presented high α-glucosidase activity with IC_{50} value of 5.9±1.49 μg/mL. However, mansonone G was inactive towards α-glucosidase and mansonone E showed IC_{50} >200 μM. Thus, the α-glucosidase activity of the ethyl acetate extract might come from other compounds or the work of synergism activity of compounds in the ethyl acetate extract.

3.2.6.3 Tyrosinase inhibitory assay of *M. gagei* extracts and compounds

The heartwood ethyl acetate extract and compounds of *M. gagei* were tested for their potential as skin whitening agents through enzyme tyrosinase inhibition. The activities of extracts and compounds are displayed by their IC_{50} value in Table 3.19.

Table 3.19 Tyrosinase inhibitory assay of *M. gagei* extracts and compounds

Sample code and compounds	IC_{50}
Mansonia ethyl acetate extract	452.2±0.62 μg/mL
Mansonone G	>200
Mansonone E	NA
Kojic acid	36.1±1.07

NA: not active; Standard deviation was calculated by mean of three replications

The ethyl acetate extract of *M. gagei* presented low α-glucosidase activity with IC_{50} value 452.2±0.62 μg/mL. The major compound mansonone E was inactive and mansonone G showed IC_{50} >200 μM. Thus, the tyrosinase activity of the ethyl acetate extract might come from other compounds contained in the ethyl acetate extract. The synergism effect between compounds in the ethyl acetate extract probably played important role as well in tyrosinase inhibitory activity.

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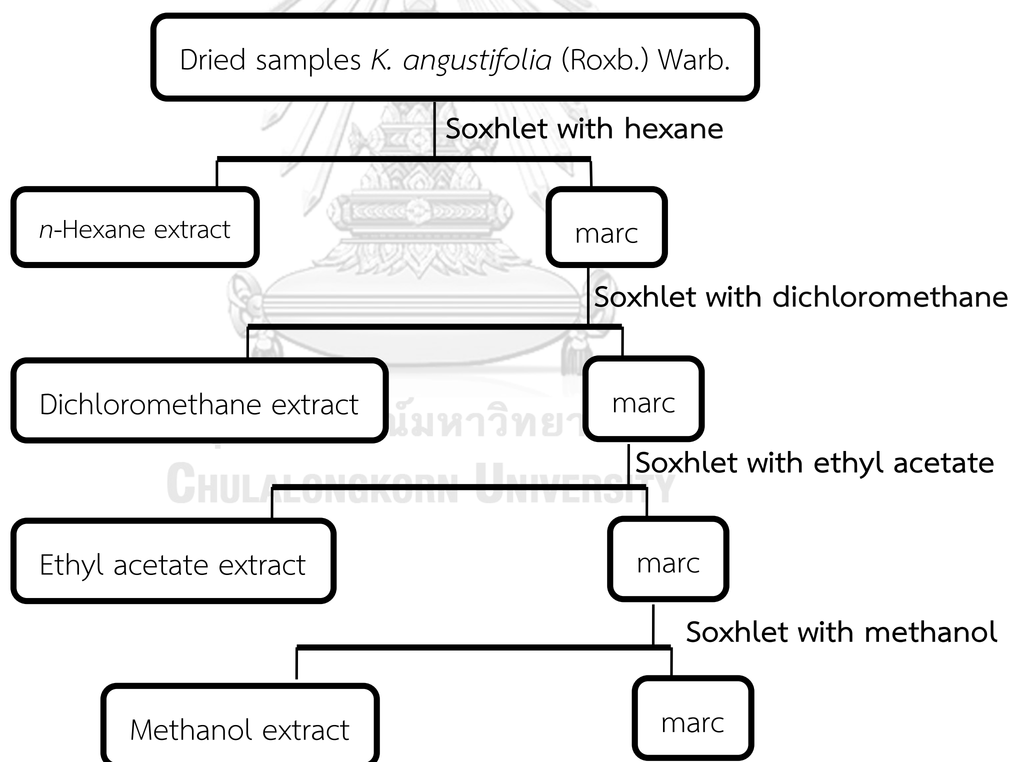
3.3 Experimental Section

3.3.1 *K. angustifolia* (Roxb.) Warb. extraction

Dried stems of *K. angustifolia* were purchased from herb store in Mukdahan province in December 2015. 600 g of dried stems were powdered and refluxed subsequently from non-polar solvent into the polar one. 5 L of solvents were used in each extraction. The temperature used was adjusted based on the boiling point of each solvent. The liquid extracts were then evaporated under pressured to obtain various crude extracts. The extraction is described in Scheme 3.1.



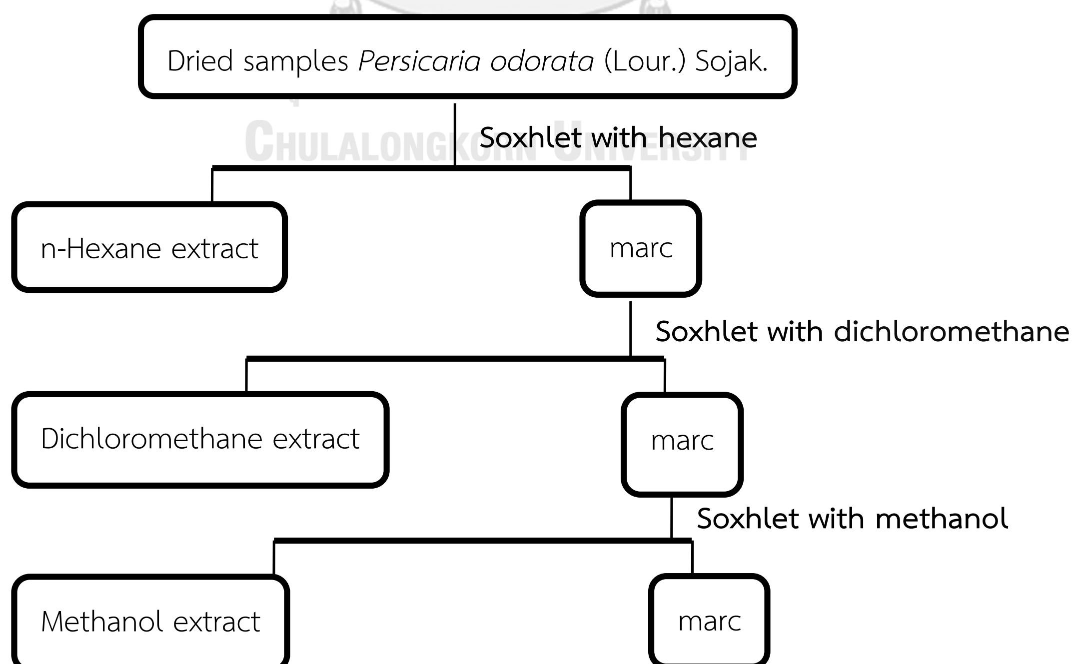
Scheme 3.1 Extraction of *K. angustifolia* (Roxb.) Warb. stems bark

3.3.2 Major constituents of *K. angustifolia* (Roxb.) Warb.

The major constituents were obtained from the separation of the ethyl acetate extract by Ms. Thi Kim Dung Le. The constituents including bergenin, 11-*O*-acetyl bergenin, quercetin and catechin were identified.

3.3.3 *Persicaria odorata* (Lour.) Sojak. extraction

Fresh leaves of *P. odorata* were purchased from Pak Klong market in Bangkok in December 2015. The sample was originated from Ayutthaya province. The fresh leaves were air dried and powdered. The powder sample (700 g) was refluxed subsequently starting from non-polar solvent into the polar one. 5 L of solvents were used in each extraction. The temperature used was adjusted based on the boiling point of each solvent. The liquid extracts then evaporated under pressured to obtained various crude extracts. The extraction is described in Scheme 3.2.



Scheme 3.2 *P. odorata* (Lour.) Sojak. Extraction

3.3.4 Major constituents of *P. odorata* (Lour.) Sojak.

The major constituents of *P. odorata* (Lour.) Sojak. including decanal and dodecanal were purchased from TCI chemicals company.

3.3.5 *Mansonia gagei* Drumm. extraction

The dried heartwoods of *Mansonia gagei* Drumm. was bought from Tai Hua Chan, the herbal drug store in Bangkok, Thailand in December 2015. Totally 10 kg of *M. gagei* heartwood powder was soaked in ethyl acetate at room temperature. After 48 h the liquids were collected and evaporated under pressured. The cycle was repeated for ten times. The crude extract was collected at one bottle.

3.3.6 Major constituents of *M. gagei* Drumm.

Mansonone G and mansonone E were isolated by Ms. Hong Truc Phan and Ms. Rita Hairani.

3.3.7 Biological activities of plants extracts and major constituents

3.3.7.1 DPPH inhibition activity of plants extracts and compounds

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The method used in this chapter was described in chapter 2

3.3.7.2 $ABTS^{\bullet+}$ inhibition activity of plants extracts and compounds

The method used in this chapter was described in chapter 2

3.3.7.3 Alpha glucosidase inhibitory assay

The method used in this chapter was described in chapter 2

3.3.7.4 Tyrosinase inhibitory assay

The method used in this chapter was described in chapter 2

3.3.7.5 Determination of antibacterial activities by agar well diffusion

The method used in this chapter was described in chapter 2

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CHAPTER IV

SYNERGISTIC EFFECT OF MANSONONE G AND ITS DERIVATIVES WITH TETRACYCLINE AND STREPTOMYCINE

4.1 Introduction

4.1.1 Mansonone G and its derivatives

One of known secondary metabolites widespread in nature is naphthoquinone. [120] The chemical structure of monomeric naphthoquinones is based on the naphthalene skeleton with carbonyl groups at positions C1 and C4 (1,4-naphthoquinones) or at C1 and C2 (1,2-naphthoquinones) as shown in Figure 4.1. [121]

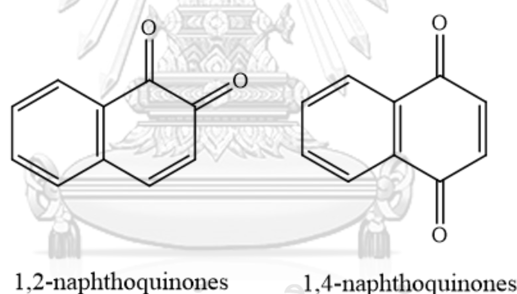

Figure 4.1 Chemical structures of 1,2-and 1,4-naphthoquinones

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Biological activities of naphthoquinones including anti-inflammatory, antioxidant, antibacterial, anticancer, anti-estrogenic, anti-larvicidal and trypanocidal activities have been addressed. [106] [122-125] One of notable naphthoquinones for further investigation is mansonone G. Mansonone G, a 1,2-naphthoquinone was a major constituent found in *Mansonia gagei* Drumm. and *Thespesia populnea* (L.) Soland. Ex Coor. [106, 126] Although mansonone G exhibited fewer biological activities than other

mansonones, this compound still provided many sites for potential improvement of its biological activities. [106, 122, 127] Therefore, researchers had attempts to modify its structure by derivatization in hoping that their biological activities would be improved. [122, 128, 129]

Recent studies have disclosed that through derivatization of 1,2-naphthoquinones by alkylation, acetylation introduction of epichlorohydrin, indole derivatives formation and many other schemes gave improvement to their biological activities. Mansonone G and certain derivatives are shown in Figure 4.2. [122, 128-130]

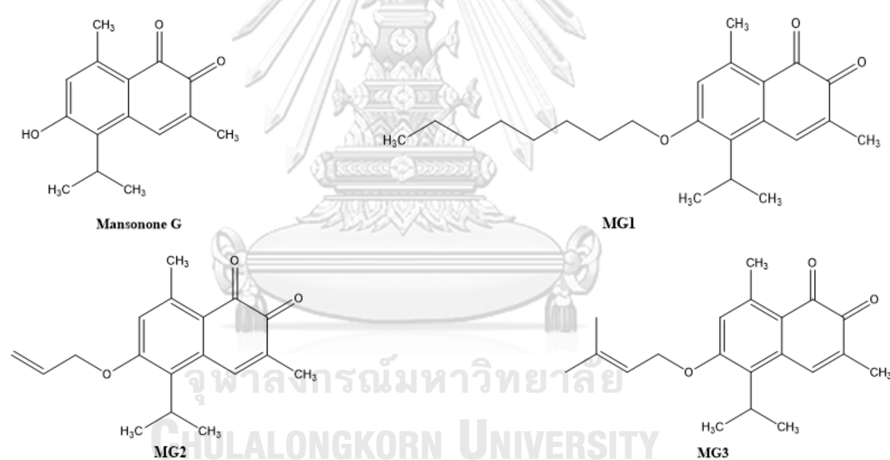

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Figure 4.2 Chemical structures of mansonone G and its derivatives

As reported in previous study, the binding of acetyl mansonone G to ERα increased by ten folds compared to the parent mansonone G with IC_{50} value 630 μM. [122]

The derivatives of mansonone G also presented excellent activities in bacterial inhibition towards *Staphylococcus aureus*. The addition of alkyl group with 8 carbon

atom, allyl, and prenyl ethers in mansonone G gave excellent clear zone inhibition of 20.0, 23.0 and 23.7 mm, respectively. The allyl and prenyl ether analogues exhibited the lowest MIC values at 0.975 µM whereas the alkyl one showed MIC value of 15.6 µM. This result also proved that the derivatives gave better activity compared to mansonone G itself. [128]

Another mansonone G derivative containing 2-chlorobenzoyl moiety (CBMG) presented excellent anti-adipogenic activity. CBMG worked with adipocyte differentiation suppression and lipid accumulation *via* suppression of PPAR**γ**-mediated adipogenic gene expression. [129]

These recent studies of biological activities from mansonone G and its derivatives are very appealing for further development.

4.1.2 Antimicrobial resistance and the synergistic effect of compounds combined with antibiotics

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The antimicrobial resistance problems have occurred since the beginning of antibiotic development. These problems potentially extend to the entire group of available therapeutic agents. Bacteria expressing multi drug resistant (MDR), extensively drug resistant (XDR) and pan drug resistant (PDR) phenotype are amongst the most important cause of infections in nosocomial and community settings and new drugs are urgently needed. [131]

MDR is defined as nonsusceptibility to at least one agent in three or more antimicrobial classes, XDR is defined as nonsusceptibility to at least one antimicrobial

agent in all but two or less antimicrobial classes and PDR is defined as nonsusceptibility to all agents in all antimicrobial classes. [132] Antibacterial resistance mechanism is displayed in Figure 4.3.

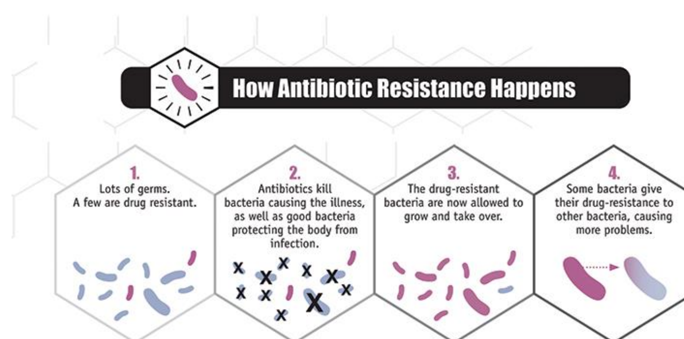

Figure 4.3 Antibacterial resistance scheme
Source: http://www.cdc.gov/drugresistance/about.html

There are several ways to reduce the development of drug resistance. One of them is to decrease the dose of antibiotics used in treatment. This can be achieved using antibiotic combinations. [133]

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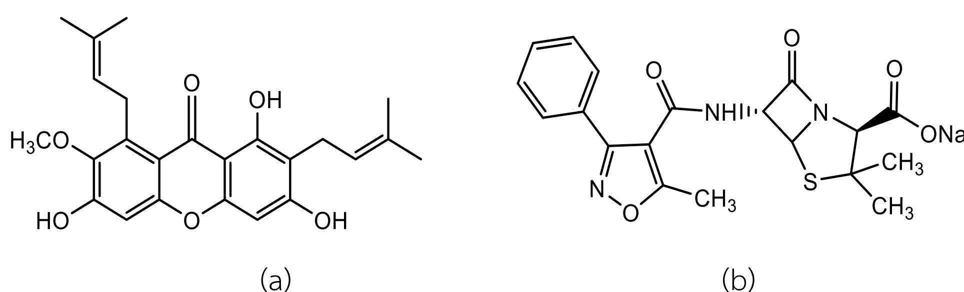
Plant-derived antibacterial are always a source of novel therapeutics. In spite of the fact that plant-derived antibacterial are less potent, plants fight infections successfully. Hence, it becomes apparent that plants adopt a different paradigm– ‘‘synergy’’– to combat infections. There are several mechanisms how plant metabolites work as modifiers on multidrug resistance. These include receptor or active side inhibitor, as inhibitor towards enzyme which degrades or modifies antibiotics, increase the permeability of outer membranes and inhibit the efflux pumps. [134]

The potential combination of chalcones derivatives with non-beta lactams antibiotics showed synergistic effect against methicillin resistant *Staphylococcus aureus* (MRSA). Ciproflaxin combined with 4-hydroxy-chalcone showed synergistic effect, which also presented by the combination with 4′-bromo-2-hydroxychalcone. Doxycycline with 4′-bromo-2-hydroxychalcone or 2′,2-dihydroxychalcone also gave synergistic effect against MRSA. The combination of gentamicin with 2′,2-dihydroxychalcone exhibited excellent result in synergistic test. These studies concluded that the synergistic mechanism of these analogues with non-beta lactam antibiotics possibly caused by the efflux pump inhibitors. [135]

The combination of the major compound α-mangostin isolated from *Garcinia mangostana* L. with oxacillin against oxacillin-resistant *Staphylococcus saprophyticus* (ORSS) was studied using checkerboard method. The MIC of the mixture between α-mangostin (2 μg/mL) and oxacillin (16 μg/mL) at a fractional inhibitory concentration index (FICI) of 0.37 showed the synergistic activity. The combination of α-mangostin with oxacillin triggered significantly peptidoglycan damage, DNA leakage and increasing the permeability of ORSS-2705 cells. [136]



(a) (b)

(a) α-mangostin and (b) oxacillin chemical structure

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The synergistic effect of artocarpin isolated from *Artocarpus heterophyllus* towards MRSA (DMST 20654), *Pseudomonas aeruginosa* (DMST 15442), and *Escherichia coli* (ATCC 25922) was investigated. Artocarpin exhibited synergistic effects when combined with ampicillin, tetracycline, norfloxacin towards MRSA. While against *P. aeruginosa* the combination was only working with tetracycline and norfloxacin. The synergistic effects were also observed by the combination of artocarpin with norfoxacin towards Gram-negative bacteria *E. coli*. The mechanism of action from this combination has not been studied. [137] These studies proved that plant secondary metabolites combination with antibiotics gave enlightenment to the antimicrobial resistance problems as one possible solution.

Artocarpin

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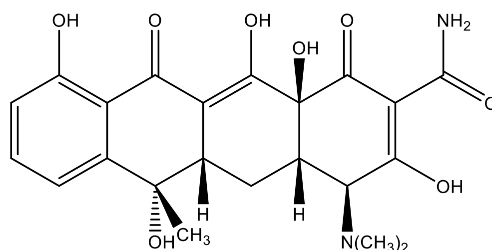
4.1.3 Tetracycline

Tetracycline is a broad-spectrum antibiotic which shows inhibitory activity against a wide range of Gram-positive and Gram-negative bacteria including certain microorganisms such as chlamydiae, mycoplasmas, rickettsiae, and protozoan parasites. [138]



Tetracycline

The core structure of tetracycline consists of four fused six-membered rings. They are usually produced by strains of *Streptomyces aureofaciens* and *S. rimosus* and, more recently, by *Micromonospora* and *Actinomadura brunea*.[139]

There are some principal members of tetracycline class such as chlortetracycline, oxytetracycline, tetracycline, demethylchlortetracycline, rolitetracycline, limecycline, clomocycline, methacycline, doxycycline, minocycline and *tert*-butylglycylamidominocycline. [138-140]

Tetracycline acts as bacteriostatic by reversibly inhibiting the bacterial peptide synthesis. Tetracycline binds to the 70S ribosome and inhibits the binding of the amino acid carrying *t*-RNA molecule to the ribosome. A site with a high affinity for tetracycline has been identified on the 30S subunit of 70S ribosome. Tetracycline also binds to and inhibits the function of eucaryotic 80S ribosomes, but to a much more limited extent, which explains the selectivity. [140, 141]

There are two mechanisms that caused bacteria show resistance towards tetracycline. Exogenous genes are acquired that encode efflux pumps, which prevent intracellular accumulation of these drugs. Alternatively, genes may be acquired that

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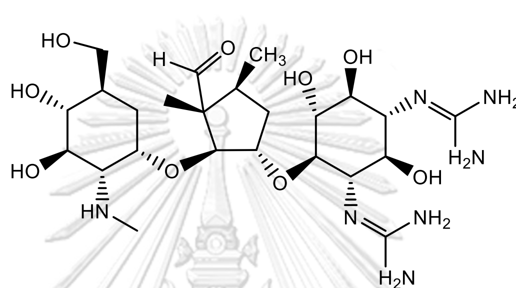
encode ribosomal protection proteins. These factors alter the conformation of the bacterial ribosome such that tetracycline no longer binds the ribosome, but protein translation remains unaffected. [40, 140]

4.1.4 Streptomycin

Aminoglycoside type of antibiotics such as streptomycin works by inhibiting bacterial *m*RNA translation. [142]



Streptomycin

Streptomycin typically used to treat tuberculosis, urinary tract, meningitis, tularemia, pneumonias, abscesses, peritonitis. Streptomycin mostly used to treat diseases that caused by Gram-negative bacteria, but it was also effective against Gram-positive bacteria. [143]

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Streptomycin acts as bactericidal antibiotic which caused the bacteria to death. The mechanism of streptomycin in Gram-negative bacteria is by cross the outer membrane. This action is due to their polar nature that make streptomycin unable to diffuse through membranes. Streptomycin has complex mechanism involving the transfer of peptidyl *t*RNA from the A-site to the P-site and damage the proofreading process that controls the accuracy of translation. The abnormal protein may affect the

physiology of bacteria, thus explained the highly bactericidal by concentration-dependent activity of aminoglycosides type of antibiotics. [144]

The bacterial resistance to aminoglycoside was defined by three mechanisms. The first mechanism is by the intracellular aminoglycoside accumulation reduction as a result from bacterial membrane alteration that reduces the uptake and/or active efflux system. For the second mechanism, the bacteria decreased aminoglycoside binding by mutation or methylation of the 16s ribosomal RNA (rRNA) binding site. The third one involved the deactivation of aminoglycoside by enzyme through *N*-acetylation, *O*-nucleotidation, or *O*-phosphorylation. [145]

4.1.5 *Staphylococcus aureus*

Staphylococci are Gram-positive bacteria, characterized as non-motile, non-spore-forming, spherical cells of 0.5 to 1.5 μm in diameter, occurring as single cocci, in pairs, as tetrads, or as short chains, which characteristically divide in more than one plane, thereby forming irregular clusters like a bunch of grapes. [146]

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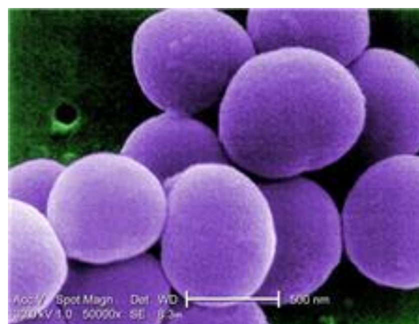

S. aureus. Source: http://www.cdc.gov/drugresistance/about.html

Staphylococcus aureus known as the most pathogen bacteria in human infection. *S aureus* is a major cause for a wide range of clinical infections such as

bacteremia and infective endocarditis along with osteoarticular, skin and soft tissue, pleuropulmonary, and device-related infections. [147]

S. aureus is famously known for its ability to become resistance to antibiotics. The antibiotic-resistance strains occurred as epidemic waves that caused by one or a few successful clones. The most common strain in these types is MRSA which now emerged as a well-known cause of community infections. Therefore, researchers focus on the developing medicine that can inhibit the resistance type of bacteria. [148]

4.1.6 *Streptococcus mutans*

Tooth decay is an infectious yet a non-threaten disease characterized by local destruction of the tooth. Even though is less threatening, but if tooth decay left untreated it will develop the infection area. The infection area can spread out to the periodontal tissue at the root apex, leading to periapical abscesses and in rare case a serious systemic infection can occur. [149, 150]

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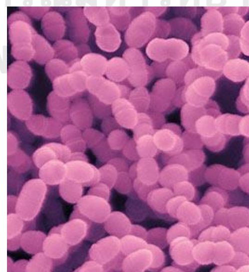

S. mutans. Source:https://microbewiki.kenyon.edu/index.php/Streptococcus_mutans-_Tooth_Decay

The cause of tooth decay mostly occurs based on the appearance of oral bacteria called *S. mutans*. *S. mutans* belongs to the *Streptococci* group of bacteria.

These bacteria are particularly effective at forming biofilms on the hard tissues of the human oral cavity. Adherence of *S. mutans* to dental surfaces is the first step in the formation of biofilms by this organism and is mediated by sucrose-dependent and sucrose-independent mechanisms**.** [151] The mechanism of biofilm resistance is shown in Figure 4.4.

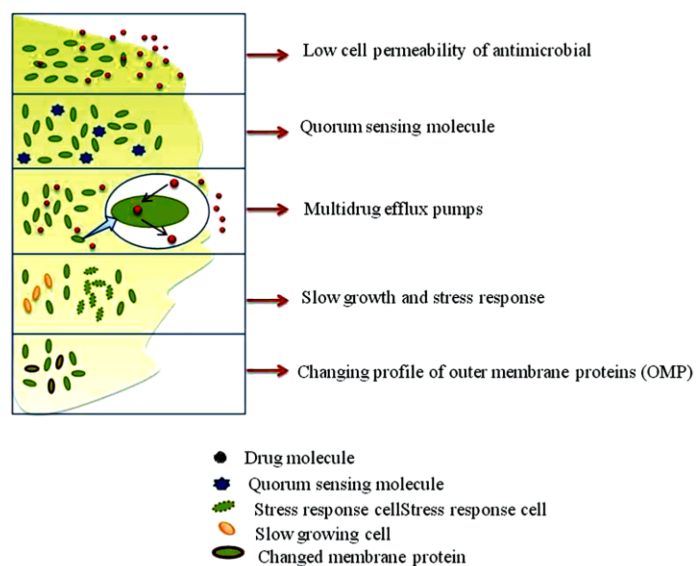

Figure 4.4 Mechanism of biofilm resistance [152]

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Even though *S. mutans* is less threatening Gram-positive bacteria, the biofilm formed by this bacteria cause resistance towards some antimicrobial agents such as amoxicillin, doxycycline, metronidazole including chlorhexidine which is used in dentifrices and mouth rinses. [153]

4.2 Results and discussion

4.2.1 Clear zone inhibition, MIC and MBC of mansonone G and its derivatives

Mansonone G and its derivatives clear zone inhibition, MIC and MBC are presented in Table 4.1.

The result in Table 4.1 showed that the agar well diffusion from mansonone G and its analogue at 1 mM displayed excellent activities, it was linear to the result reported by Hairani *et al.*, (2016) that derivatives of mansonone G gave better activity compared to the parent mansonone G. [128]

The alkyl derivative **MG1** showed the highest inhibition at 1 mM towards *S. mutans* on agar well diffusion with 23.67±0.88 mm clear zone inhibition compared to mansonone G with 17.92±1.03 mm. **MG1** and 3,3-dimethylallyl ether mansonone G (**MG3**) displayed the inhibition towards *S. aureus* with 17.75±2.00 and 18.43±1.28 mm which were better compared to the clear zone inhibition caused by mansonone G (16.33±0.78 mm). The MIC's of **MG2** and mansonone G displayed identical values with 125 μM towards *S. aureus* and *S. mutans*. The MBC value of **MG2** towards *S. mutans* gave the exact result as shown by mansonone G, while in *S. aureus* **MG2** showed better result with MBC 500 μM.

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Table 4.1 Clear zone inhibition, MIC and MBC of mansonone G and its derivatives

Sample	Clear zone inhibition at 1 mM for samples and 0.5 mM for antibiotics against *S. aureus*(mm)	MIC value towards *S aureus* (µM)	MBC value towards *S. aureus* (µM)	Clear zone inhibition at 1 mM for samples and 0.5 mM for antibiotics *S. mutans* (mm)	MIC value towards *S mutans* (µM)	MBC value towards *S. mutans* (µM)
Mansonone G	16.33 ± 0.78	125	>1000	17.92 ± 1.03	15.62	125
MG1	17.75 ± 2.00	250	>1000	23.67 ± 0.88	250	>1000
MG2	15.17 ± 0.19	125	500	19.67 ± 0.63	15.62	125
MG3	18.43 ± 1.28	250	>1000	18.25 ± 2.41	62.5	500
Streptomycin	22.67 ± 3.21	1.95		23.00 ± 0.00	0.97	
Tetracycline	25.67 ± 0.57	1.95		26.00 ± 0.00	1.95	

Criteria of clear zone inhibition (including the diameter of well in mm) (three bacteria have not been tested) 6.0 = no activity; 6.1–8.0= weak; 8.1-10= moderate; 10.1-13= Good; 13.1-15= very good; > 15= excellent.

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Mansonone G and its derivatives showed bacteriostatic activity towards *S. aureus* except for allyl ether derivative (**MG2**) which showed bactericidal activity. The minimum bactericidal activity of mansonone G and its derivative against *S. mutans* mostly displayed bactericidal activity except for **MG1** which showed bacteriostatic activity towards *S. mutans*.

4.2.2 The combination effect of mansonone G and its derivatives with agar well diffusion

Based on the result above, the combination effect between mansonone G and its derivatives with antibiotics was determined by agar well diffusion. Instead of compound alone, in one well will be consisted of sample and antibiotics. Based on the compounds and antibiotics concentration used in Table 4.1, the concentration of each agent used in combination was reduced up to 100 folds and their combination effect then observed as clear zone inhibition. The results are presented in Table 4.2.

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The combination of antibiotics with mansonone G and its derivatives on *S. aureus* showed that the clear zone inhibition were less or similar compared to the inhibition showed by antibiotics alone. This type of results also displayed by the combination of antibiotics with mansonone G and its derivatives against *S. mutans*. The necessity of using different methods to establish their synergist effect is important. Therefore, the synergistic effect of antibiotics with compounds should be validated by the common checkerboard micro dilution and their FICI (fractional Inhibition concentration index) should be calculated.

Certain synthesized compounds were able to enhance antibacterial activity. For example, diethyl 2-[(3-hydroxy-1,4-dioxo-1,4-dihydro-napthalene-2-yl)-hydrazono]malonate, the lapachol derivative showed activity two folds greater than lapachol itself towards *S. aureus*. The MIC of the derivatives gave even better result compared to the commercial antibiotics oxacillin and vancomycin towards the MRSA. The optimum density of this compound exhibited the similar result to vancomycin even at lower concentration than vancomycin itself. [154]

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Table 4.2 The antibacterial activities of antibiotics, compounds alone and combined with antibiotics towards *S. aureus* and *S. mutans*

Sample	*S. aureus*			*S. mutans*		
	Clear zone inhibition at 0.10 mM for samples and 0.05 mM for antibiotics (mm)	Clear zone combination with Streptomycin and samples (0.05 and 0.1 mM) (mm)	Clear zone combination with Tetracycline and samples (0.05 and 0.1 mM) (mm)	Clear zone inhibition at 0.10 mM for samples and 0.05 mM for antibiotics (mm)	Clear zone combination with Streptomycin and samples (0.05 and 0.1 mM) (mm)	Clear zone combination with Tetracycline and samples (0.05 and 0.1 mM) (mm)
Mansonone G	12.00 ± 0.00	18.00 ± 0.00	20.00 ± 0.00	10.91 ± 0.80	15.83 ± 0.28	18.00 ± 0.00
MG1	15.67 ± 0.57	18.00 ± 0.00	20.00 ± 0.00	13.91 ± 0.52	18.08 ± 0.14	21.50 ± 1.32
MG2	14.00 ± 1.00	19.41 ± 0.52	17.67 ± 1.15	13.67 ± 1.41	16.83 ± 0.14	20.33 ± 0.57
MG3	12.33 ± 1.15	19.33 ± 0.57	18.33 ± 1.52	11.5 ± 0.86	15.58 ± 1.50	22.00 ± 1.00
Streptomycin	21.00 ± 0.00			18.33 ± 0.57		
Tetracycline	23.33 ± 0.57			22.33 ± 0.57		

Criteria of clear zone inhibition (including the diameter of well in mm) (three bacteria have not been tested) 6.0 = no activity; 6.1–8.0= weak; 8.1-10= moderate; 10.1-13= Good; 13.1-15= very good; > 15= excellent.

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4.2.3 The combination effect of mansonone G and its derivatives by checkerboard method

The checkerboard microdilution method is a common method to study the relationship of compound combined with antibiotics. The principal of this method is using the typical of microdilution assay but with various sample concentrations. The concentration was obtained based on the MIC of compounds and antibiotics alone, furthermore various concentrations were diluted in combination. The results of synergistic effect of mansonone G and its derivatives towards *S. aureus* are presented in Table 4.3.

The combination of compounds and antibiotics displayed the synergistic effects of mansonone G and its derivatives when combined with tetracycline. The concentration of the compound was decreased 20-22 folds lower than MIC, whereas the concentration of tetracycline could decrease for 4-8 folds. The additive activity was observed by mansonone G and **MG2** with FICI value 0.99 and 0.52, respectively, while **MG1** and **MG3** showed synergistic effect when combined with streptomycin. The FICI values of **MG1** and **MG3** were 0.30 and 0.36, respectively. The combination of **MG1** and **MG3** with streptomycin decreased the concentration of each compound 8 and 6 folds, correspondingly while streptomycin was 4 folds lower than the original MIC alone.

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Table 4.3 Synergistic effects of mansonone G and its derivatives combined with antibiotics towards *S. aureus* by using checkerboard method

Sample	MIC Alone (μM)	MIC Mix (μM)	FIC Index	Interpretation	Increasing rate (fold) Mansonones/ Antibiotics
MG	125	62.5	0.99	Additive	2/2
Streptomycin	1.95	0.97			
MG1	250	15.62	0.30	Synergist	8/4
Streptomycin	1.95	0.48			
MG2	125	3.90	0.52	Additive	10/2
Streptomycin	1.95	0.97			
MG3	250	31.25	0.36	Synergist	6/4
Streptomycin	1.95	0.48			
MG	125	0.06	0.06	Synergist	22/8
Tetracycline	1.95	0.12			
MG1	250	0.12	0.24	Synergist	22/4
Tetracycline	1.95	0.48			
MG2	125	0.12	0.06	Synergist	20/8
Tetracycline	1.95	0.12			
MG3	250	0.24	0.24	Synergist	20/4
Tetracycline	1.95	0.48			

Synergism is defined as a FIC index of <0.5; additive as a FIC index of 0.5-1.0; and antagonism as a FIC index of >1.0. The lowest FIC index from each checkerboard was recorded.

S. aureus is one of important clinically Gram-positive bacteria that cause global concern due to its ability to produce resistant type of bacteria and caused numerous

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antibiotics resistance such as β-lactam antibiotics.[148, 155] Therefore, the results of combination will give benefit in future application towards resistance type of *S. aureus*.

The synergistic activity of mansonone G and its derivatives towards oral bacteria *S. mutans* were also evaluated, the data are presented in Table 4.4.

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Table 4.4 Synergistic effects of mansonone G and its derivatives combined with antibiotics towards *S. mutans* by using checkerboard method

Sample	MIC Alone (μM)	MIC Mix (μM)	FIC Index	Interpretation	Increasing rate (fold) Mansonones/ Antibiotics
MG	15.62	Na	Na	Na	Na
Streptomycin	0.97	Na			
MG1	250	31.25	0.61	Additive	6/2
Streptomycin	0.97	0.48			
MG2	15.62	7.81	0.99	Additive	2/2
Streptomycin	0.97	0.48			
MG3	62.5	7.81	0.61	Additive	6/2
Streptomycin	0.97	0.48			
MG	15.62	0.007	0.12	Synergist	22/6
Tetracycline	1.95	0.24			
MG1	250	0.12	0.12	Synergist	22/6
Tetracycline	1.95	0.24			
MG2	15.62	0.015	0.12	Synergist	20/6
Tetracycline	1.95	0.24			
MG3	62.5	0.03	0.12	Synergist	22/6
Tetracycline	1.95	0.24			

Synergism is defined as a FIC index of <0.5; additive as a FIC index of 0.5-1.0; and antagonism as a FIC index of >1.0. The lowest FIC index from each checkerboard was recorded. Na means the mic and synergistic effect are not available

The combination between compounds and streptomycin showed that the analogues of mansonone G gave additive activity with FICI value ranged between 0.61-0.99. Mansonone G itself displayed no activity when combined with streptomycin.

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Tetracycline when combined with mansonone G and its derivatives, aaall of them showed synergistic effect towards *S. mutans*. The MIC combination could decrease 22 folds for compounds and 6 folds for tetracycline.

S. mutans has been implicated as a primary causative agent of dental caries in humans. An important virulence property of the bacterium is its ability to form biofilm known as dental plaque on tooth surfaces. In addition, this organism also produces glucosyl-transferases, multiple glucan-binding proteins, protein antigenic, and collagen-binding protein, surface proteins that coordinate to produce dental plaque, thus inducing dental caries. [151] Albeit *S. mutans* is less threatening the biofilm formed by these bacteria can cause resistance against several antimicrobial agents [156]

The synergistic activity of **MG** and its derivatives was affected by the presence of 1,2-naphthoquinone skeleton. Previously, biflorin, a 1,2-naphthoquinone isolated from *Capraria biflora* L. presented tremendous result as antibacterial in Gram-positive, Gram-negative and as anti-fungi. [157, 158] Nevertheless, when the naphthalene skeleton was modified their antibacterial activities were decreased up to 10 folds except for their oxime derivatives which displayed better inhibition compared to biflorin against *S. aureus* and *E. coli* and *Enterococcus faecalis*. [159]

Amongst **MG** derivatives, the octyl ether mansonone G (**MG1**) and prenyl ether **MG3** derivatives exhibited promising results when combined with antibiotics. The combination of **MG1** or **MG2** with streptomycin showed synergistic effect against *S. aureus*, whereas the combination showed additive activities against S. *mutans*. The

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derivatives however, showed synergistic effect when combined with tetracycline against both bacteria.

Many researches explained that the presence of hydrophilic long chain in compound affected its antimicrobial activity. That was because the hydrophilic long chain would ease compound permeability into lipophilic cell wall bacteria. [124] [160-162] Compounds containing prenyl group also showed profound synergistic activities as displayed in several previous researches. [137, 162]

These results give high optimism to disclose new promising antimicrobial combination agents against clinically important Gram-positive bacteria and resistance type of bacteria strain. It can be concluded that the structural improvement in mansonone G derivatives displayed great synergistic activities compared to mansonone G itself. In the future, the synergistic activities towards resistance type bacteria and the mechanism of 1,2-naphthoquinone derivatives should be further studied.

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4.3 Experimental section

4.3.1 General experiment

Mansonone G including its derivatives such as **MG1** (octyl ether mansonone G), **MG2** (allyl ether mansonone G), **MG3** (3,3-dimethylallyl ether mansonone G) were obtained from *Mansonia gagei* Drumm. and from the synthesis of mansonone G conducted by Ms. Rita Hairani. Resazurin, tetracycline and streptomycin used in this experiment were obtained from Sigma Aldrich. The nutrient broth was obtained from Difco.

4.3.2 Agar well diffusion test

Determined by the same method as explained in Chapter 2.

4.3.3 Minimum inhibitory concentration (MIC)

Determined by the same method as explained in Chapter 2.

4.3.4 Combination effects of mansonone G and its derivatives with tetracycline and streptomycin

The combination effects of mansonone G and its derivatives was used the exact method as agar well diffusion, but instead of using 30 µL of single compound, 15 µL of compound and 15 µL of tetracycline or streptomycin were combined in one well. Several concentrations were used in this method.

4.3.5 Checkerboard method micro-dilution assay

The *in vitro* interactions between tetracycline and streptomycin with mansonone G and its derivatives were investigated by a two-dimensional checkerboard micro-dilution assay, using a 96-well micro-titration plates with some modification. [163, 164] The range of concentrations tested for each drug was 2- to 128-fold lower than the MIC calculated alone. A stock solution of tetracycline, mansonone G and its derivatives was prepared in 10% DMSO with nutrient broth medium. In each well of the microplate 25 µL of microbial growth medium were added. Compound dilutions were started from half of MIC concentration and then the serial dilution was performed using the 8 multichannel pipettes from A2-A12 and then another dilution was performed from A2-A12 to G2-G12. The excess amount of compound was discarded in

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the end of wells from serially descending concentrations. The antibiotics were initially prepared to obtain two folds until 64 folds of final concentration. An aliquot of 25 µL of concentrated antibiotic was added to columns B1 to B12 until G1-G12. Columns B1 to G1 contained only the antibiotic while columns A1-A12 only the compound. Wells H1-H4 was the drug free well used as growth control. Finally, 50 µL of 1-1.5 × 10^8 CFU/ml bacteria suspension were added to each well of the micro-plate. The micro-titer plates were incubated at 37 °C for 18 h.

After the incubation, 10 µL of 0.01% resazurin as oxidation-reduction indicator will be added into each well of the 96-well micro-plates and the plates will be incubated for 10 minutes. Any color change observed from purple to pink or colorless was taken as positive with cell viability. The lowest concentration of compound at with no color change from blue to purple or pink was recorded as the MIC of combination.

The nonparametric approach is based on the fractional inhibitory concentration index (FICI) model expressed as:

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$$\Sigma FIC = FIC_A + FIC_B = MIC_{AB}/MIC_A + MIC_{BA}/MIC_B$$

where MIC_A and MIC_B are the MICs of drugs A and B when acting alone and MIC_{AB} and MIC_{BA} are the MICs of drugs A and B when acting in combination.

Among all ΣFICs calculated for each micro-plate, the FICI was determined as the lowest ΣFIC (ΣFICmin) when the highest ΣFIC (ΣFICmax) was smaller than 4. In this experiment all FICIs where lower than 4, thus all FIC index where calculated as

ΣFICmin.In this method, synergism is defined as a FIC index of <0.5; additive as a FIC index of 0.5-1.0; and antagonism as a FIC index of >1.0.

CHAPTER V
CONCLUSIONS

Nine compounds were isolated from *n*-hexane and dichloromethane fraction of *P. dilatatum* including Methyl (E)-2,4-dihydroxy-6-methyl-3-(3-oxobut-1-en-1-yl) benzoate (**PD.1**), Hopane-16ß,22-diol (**PD.2**), methyl orsellinate (**PD.3**), methyl haematommate (**PD.4**), methyl ß-orcinolcarboxylate (**PD.5**), 2-hydroxy-4-methoxy-3,6-dimethylbenzoic acid (**PD.6**), atranol (**PD.7**), Atranorin (**PD.8**) and lecanorin (**PD.9**).

Acetone fraction showed the highest activity as DPPH radical scavenging, while dichloromethane fraction showed the highest activity towards $ABTS^{+\bullet}$. Meanwhile, Atranol (**PD.7**) presented the highest activity towards DPPH and $ABTS^{+\bullet}$.

The tyrosinase activity of lichen extracts and fractions showed that dichloromethane fraction has potential as anti-tyrosinase activity, while atranorin (**PD.8**) play important role as anti-tyrosinase in dichloromethane fractions due to its highest activity amongst tested compounds.

Anti-diabetic activity of lichen extracts fractions and compounds were evaluated against enzyme α-glucosidase. It was shown that methanol fraction has excellent activity as alpha glucosidase inhibitors. Contrary with tyrosinase assay methyl haemmatommate (**PD.4**) showed strongest alpha glucosidase inhibitory compared to other compounds.

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The lichen extract and fraction give various antibacterial activity with crude acetone extract exhibited good to very good inhibition based on the clear zone inhibition criteria. The atranorin (**PD.8**) showed good to excellent activity compared to other compounds based on the clear zone inhibition criteria. However, atranorin (**PD.8**) and methyl ß-orcinolcarboxylate (**PD.5**) showed bacteriostatic activity.

Plants extracts including *K. angustifolia*, *P. odorata* and *M. gagei* were extracted and tested for its biological activities. Amongst them *K. angustifolia* was displayed potential activity in DPPH, ABTS, antibacterial, tyrosinase assay and alpha glucosidase assay.

The major constituents from those three plants were also tested for its biological activity. Quercetin exhibited prospective antioxidants, anti-tyrosinase and anti-diabetic activities amongst other compounds.

Compound **MG1** and **MG3** derivatives of mansonone G showed synergistic effect against *S. aureus* when combined with streptomycin. While when combined with tetracycline mansonone G and all derivatives showed synergistic effect against *S. aureus* and *S. mutans*.

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APPENDIX A

NMR spectrum of compound 1 and 2

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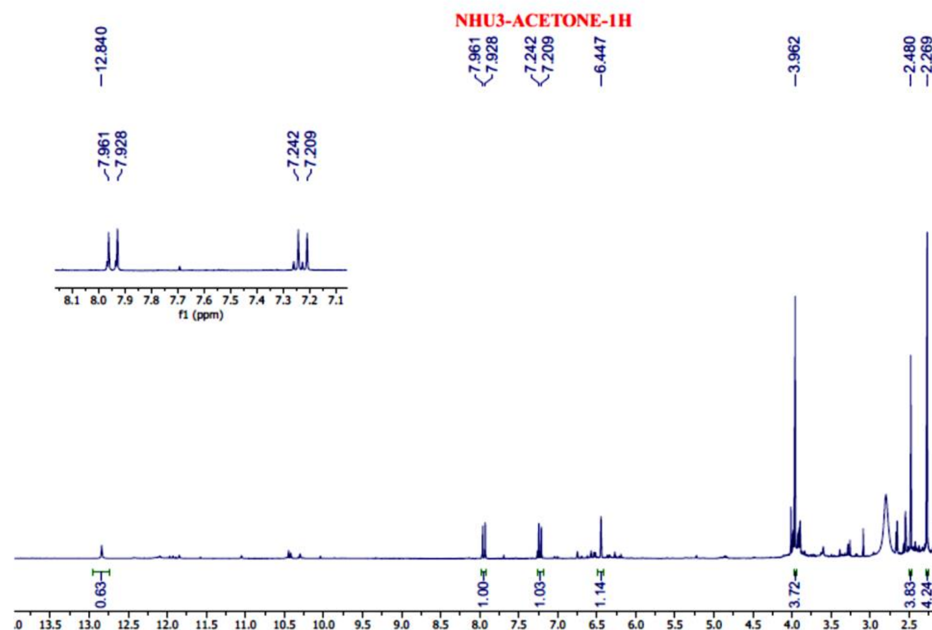

Figure A.1 The ^{1}H-NMR of compound **PD.1**

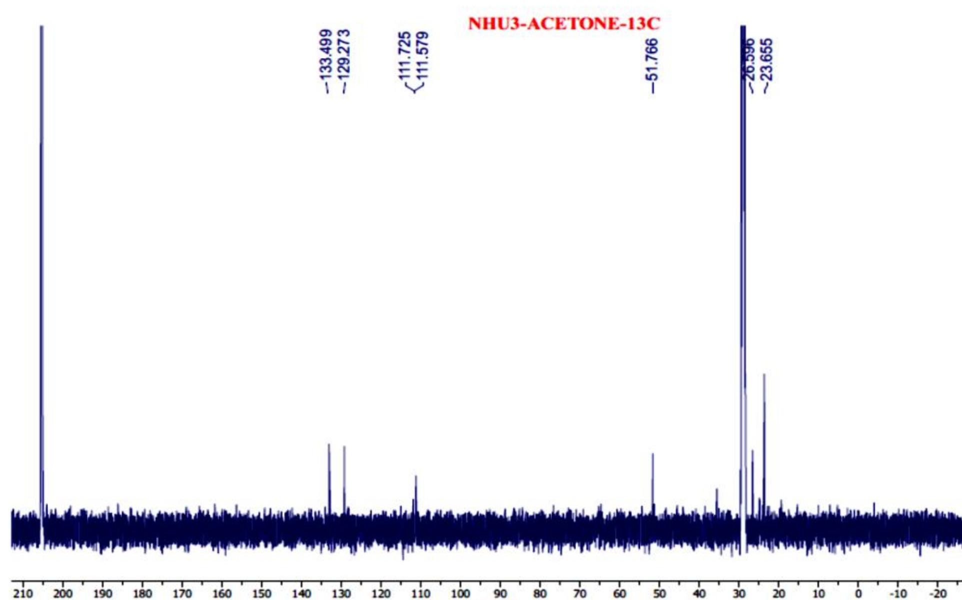

Figure A.2 The ^{13}C-NMR of compound **PD.1**

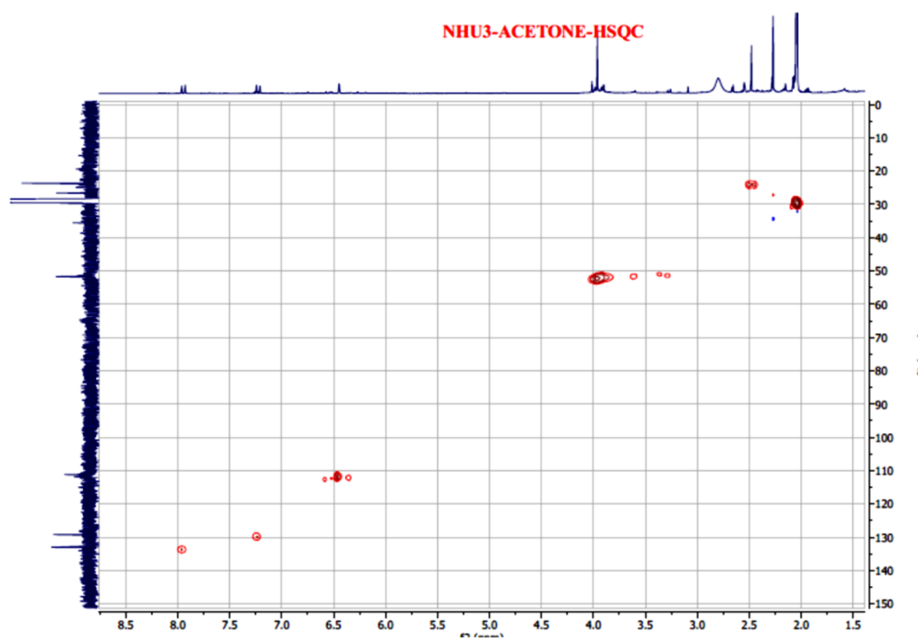

Figure A.3 HSQC of compound **PD.1**

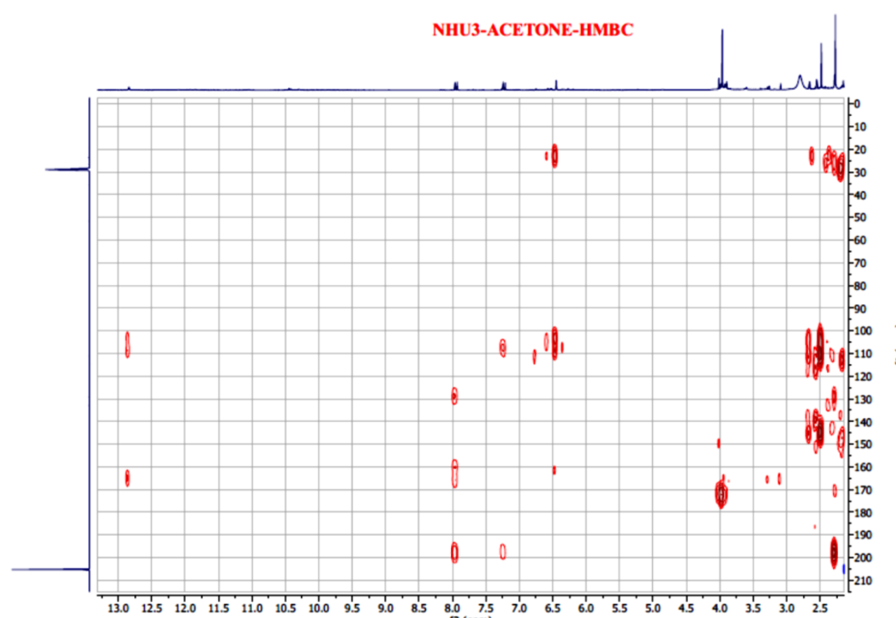

Figure A.4 HMBC of compound **PD.1**

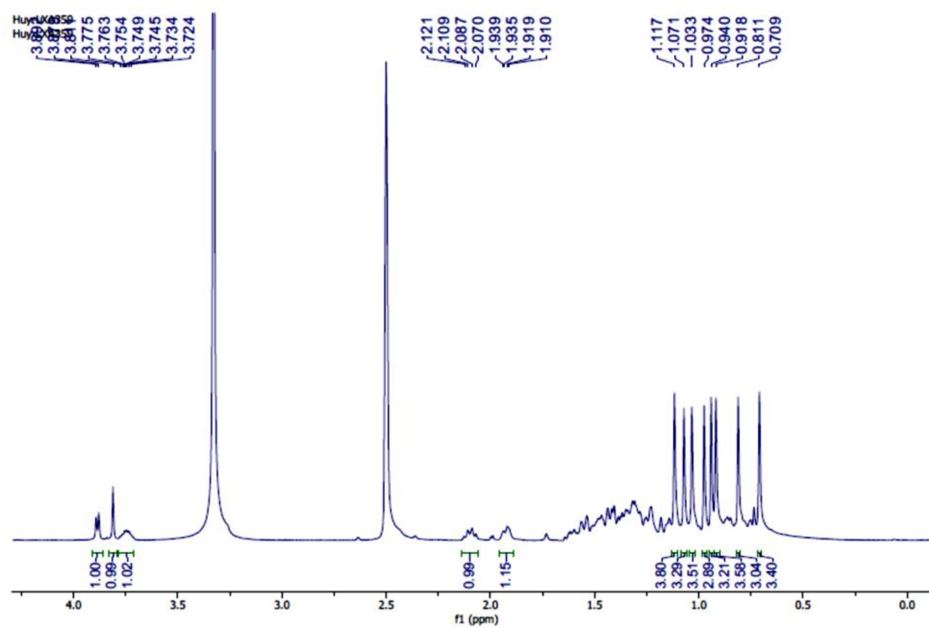

Figure A.5 The ^{1}H-NMR of compound **PD.2**

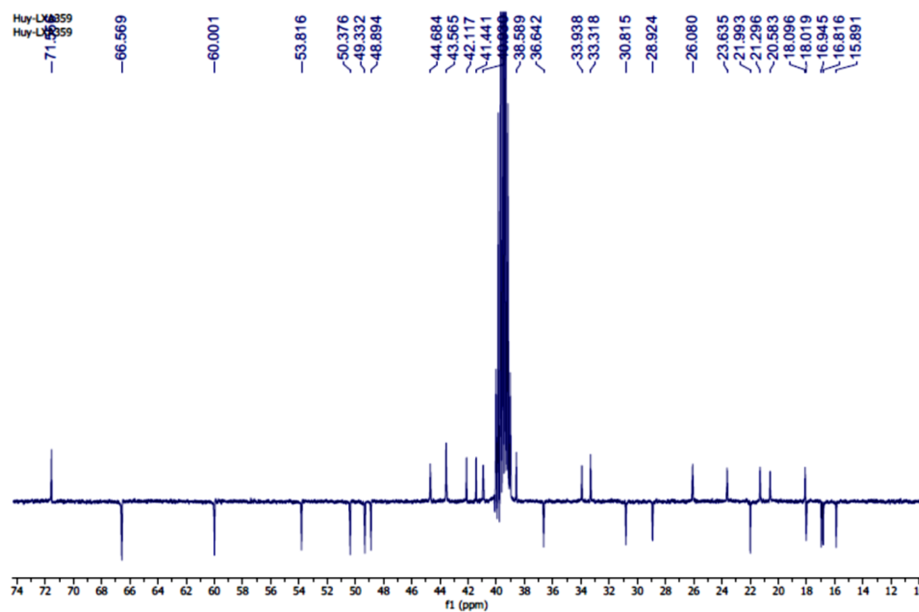

Figure A.6 The ^{13}C-NMR of compound **PD.2**

APPENDIX B

Preparation of solutions

B.1. Chemicals solution preparation

B.1.1. Preparation of 0.05 mg/mL 1, 1-diphenyl-2-picrylhydrazyl (DPPH) in MeOH

the formula of DPPH = $C_{18}H_{12}N_5O_6$ (MW = 394.32)

1) The amount of DPPH radical solution used in one plate

   (96 x 100 µL per well = 9600 µL= 9.6 mL)

2) The concentration used was 0.05 mg DPPH in 1 mL MeOH. One plate required around 10 mL of DPPH. (0.05 mg x 10 mL = 0.5 mg of DPPH)

   ∴ The amount of DPPH used in one plate is 0.5 mg. Therefore 0.5 mg of DPPH weighed and dissolve in 10 ml MeOH to provide 0.05 mg/mL DPPH radical solution

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B.1.2 Preparation of (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS and (Potassium persulfate) $K_2S_2O_8$

1) Weighed 16 mg of (2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) ABTS
2) Weighed 2.64 mg of $K_2S_2O_8$
3) Dissolved the mixture of 1 and 2 in 4 mL of distilled water

4) Incubated for 12-16 h and measure the $ABTS^{+\cdot}$ solution before use (the solution can be adjusted with distilled water with absorbance around 0.68 – 0.72) at 734nm.

B.3. Preparation of 2.5 mM L-Tyrosine

the formula of L-Tyrosine = $C_9H_{11}NO_3$ (MW= 181.19)

1) Weighed 27.18 mg L-Tyrosine and dissolve in 60 mL of 50 mM sodium phosphate buffer pH 6.8 to provide 2.5 mM L-Tyrosine

B.4. Preparation of 1 mM PNPG (4-Nitrophenyl β-D-glucopyranoside)

1) Weighed 27.11 mg of 1mM PNPG (4-Nitrophenyl β-D-glucopyranoside) and dissolve in 90 mL of 0.1 mM pH 6.9 phosphate buffer.

B.5 Preparation of 1 M Na_2CO_3

1) Weighed 10.59 g of Na_2CO_3 in 100 mL of DI water to provide 1 M Na_2CO_3

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B.2. Preparation of buffer

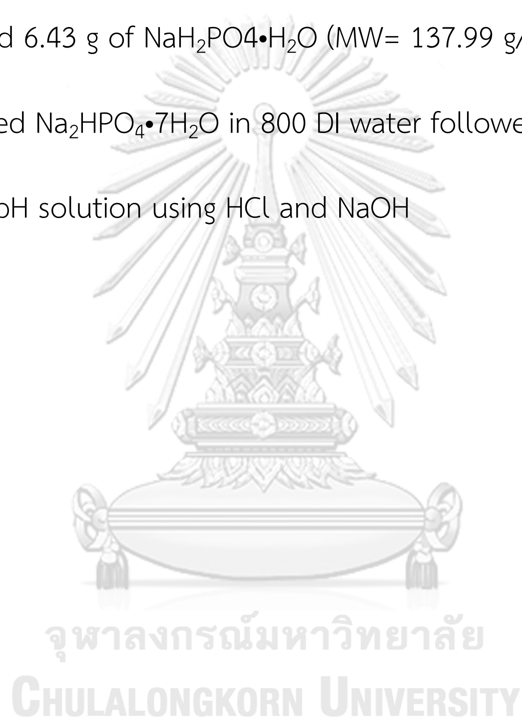
B.2.1. Preparation of 50 mM of sodium phosphate buffer (pH 6.8)

1) A: Weighed Na_2HPO_4 (MW= **141.96** g/mole) 1.42 g was dissolved in 200 mL of DI water to provide 50 mM of Na_2HPO_4.
2) B: Weigh NaH_2PO_4 (MW= 119.98 g/mol) 1.2 g was dissolved in 200 mL of DI water

3) 60 mL of solution A was combined with 140 mL of solution B. Added 300 mL of DI water and adjust the pH solution to provide sodium phosphate buffer pH 6.8.

B.2.2 Preparation of 0.1 mM of Phosphate buffer (pH 6.9)

1) Weighed 14.30 g of Na_2HPO_4•$7H_2O$ (MW= 268.03 g/mole)
2) Weighed 6.43 g of NaH_2PO4•H_2O (MW= 137.99 g/mole)
3) Dissolved Na_2HPO_4•$7H_2O$ in 800 DI water followed by NaH_2PO4•H_2O
4) Adjust pH solution using HCl and NaOH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