ความสัมพันธ์ระหว่างโครงสร้างและฤทธิ์ทางชีวภาพของฟลาโวนอยด์จากกระชายเหลือง *Boesenbergia rotunda* (L.) Mansf. และกระชายดำ *Kaempferia parviflora* Wall. ex Baker.

นางสาวกรองกาญจน์ กิ่งแก้ว

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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ปีการศึกษา 2558

STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIP OF FLAVONOIDS FROM *Boesenbergia rotunda* (L.) Mansf. AND *Kaempferia parviflora* Wall. ex Baker.

Miss Krongkan Kingkaew

A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Chemistry
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Thesis Title	STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIP OF FLAVONOIDS FROM *Boesenbergia rotunda* (L.) Mansf. AND *Kaempferia parviflora* Wall. ex Baker.
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กรองกาญจน์ กิ่งแก้ว : ความสัมพันธ์ระหว่างโครงสร้างและฤทธิ์ทางชีวภาพของฟลาโวนอยด์จากกระชายเหลือง *Boesenbergia rotunda* (L.) Mansf. และกระชายดำ *Kaempferia parviflora* Wall. ex Baker. (STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIP OF FLAVONOIDS FROM *Boesenbergia rotunda* (L.) Mansf. AND *Kaempferia parviflora* Wall. ex Baker.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.วรินทร ชวศิริ, 100 หน้า.

ฟลาวาโนนสองชนิด (20 และ 43) และฟลาโวนห้าชนิด (44-48) แยกได้จากสิ่งสกัดไดคลอโรมีเทนของกระชายเหลืองและกระชายดำตามลำดับ ได้สังเคราะห์อนุพันธ์ฟลาโวนอยด์ยี่สิบแปดชนิด (28-29, 33-34 และ 49-72) พบว่าเป็นสารใหม่เจ็ดชนิด ได้นำสารทั้งหมดมาศึกษาฤทธิ์ต้านแบคทีเรีย ฤทธิ์ต้านไทโรซิเนส และฤทธิ์การสังเคราะห์เมลานิน พบว่า 6,8-dibromo-5,7-dihydroxyflavone (33) และ 6,8-diiodo-5,7-dihydroxyflavone (34) มีฤทธิ์ต้านแบคทีเรียทั้งห้าชนิดได้ดีที่สุด และแสดงค่า MIC ที่ 31.25-62.5 μM โดย 33 และ 34 มีสมบัติเป็นสารต้านจุลชีพมีฤทธิ์ยับยั้งการเจริญเติบโตของจุลชีพสำหรับ *Propionibacterium acnes* และ *Staphylococcus aureus* และมีสมบัติเป็นสารต้านจุลชีพมีฤทธิ์ฆ่าหรือทำลายเชื้อจุลชีพสำหรับ *Streptococcus sobrinus*, *Streptococcus mutans* และ *Salmonella typhi* การศึกษาฤทธิ์การต้านแบคทีเรียของการรวมกันของ 33 และ 34 กับยาปฏิชีวนะสี่ชนิดคือ chloramphenicol, tetracycline, streptomycin และ ampicillin พบว่ามีผลเสริมฤทธิ์กัน นอกจากนี้การศึกษาฤทธิ์ต้านไทโรซิเนสของฟลาโวน สิบเจ็ดชนิด พบว่าสารทั้งหมดไม่ให้ฤทธิ์ในการต้านไทโรซิเนส โดยแสดงค่า IC_{50} มากกว่า 100 μM สำหรับฤทธิ์ในการสังเคราะห์เมลานินพบว่า หมู่เมทอกซีที่วง A ของฟลาโวนมีความสำคัญต่อฤทธิ์การกระตุ้นการเกิดเมลานิน โดยมี 5,7-dimethoxyflavone (49), 5-methoxy-7-ethoxyflavone (53), 5-methoxy-7-butoxyflavone (54) และ 5,7,2′,3′,4′-pentamethoxyflavone (65) แสดงฤทธิ์ดีกว่า theophylline สองเท่า แม้ใช้ความเข้มข้นน้อยกว่าสิบเท่า นอกจากนี้ 5,7,3′,4′,5′-pentamethoxyflavone (67) แสดงฤทธิ์ในการกระตุ้นการเกิดเมลานินได้ดีที่ความเข้มข้นต่ำ แม้ที่ความเข้มข้นเท่ากับ 3.125 μM สาร 67 ยังคงแสดงฤทธิ์ได้ดีกว่าสารควบคุมเชิงบวกถึงสองเท่า

ภาควิชา เคมี ลายมือชื่อนิสิต .....

สาขาวิชา เคมี ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

ปีการศึกษา 2558

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KEYWORDS: BOESENBERGIA ROTUNDA / KAEMPFERIA PARVIFLORA / ANTI-BACTERIAL / ANTI-TYROSINASE / MELANOGENESIS ACTIVITY

KRONGKAN KINGKAEW: STRUCTURE-BIOLOGICAL ACTIVITY RELATIONSHIP OF FLAVONOIDS FROM *Boesenbergia rotunda* (L.) Mansf. AND *Kaempferia parviflora* Wall. ex Baker.. ADVISOR: ASST. PROF. WARINTHORN CHAVASIRI, Ph.D., 100 pp.

Two flavanones (20 and 43) and five flavones (44-48) were isolated from the CH_2Cl_2 extracts of *Boesenbergia rotunda* and *Kaempferia parviflora.* Twenty-eight flavonoid derivatives (28-29, 33-34 and 49-72) were synthesized, among them seven were identified as new compounds. All collected flavonoids were examined on anti-bacterial, anti-tyrosinase and melanogenesis activities. Among twenty-two tested compounds, 6,8-dibromo-5,7-dihydroxyflavone (33) and 6,8-diiodo-5,7-dihydroxyflavone (34) exhibited the highest activity against all bacteria with MIC of 31.25-62.5 μM. They were bacteriostatic agent for *Propionibacterium acnes* and *Staphylococcus aureus,* and bactericidal agent for *Streptococcus sobrinus, Streptococcus mutans* and *Salmonella typhi.* The combination of 33 and 34 with commonly used antibiotic including chloramphenicol, tetracycline, streptomycin and ampicillin exhibited synergistic effect. Seventeen tested compounds did not show anti-tyrosinase activity (IC_{50} >100 μM). For melanogenesis activity, the methoxy group on A-ring played an important role in melanogenesis-stimulating activities. 5,7-dimethoxyflavone (49), 5-methoxy-7-ethoxyflavone (53), 5-methoxy-7-butoxyflavone (54) and 5,7,2′,3′,4′-pentamethoxyflavone (65) showed more than two-fold higher activity than theophylline although the concentration of compounds were ten-folds less. In addition, 5,7,3′,4′,5′-pentamethoxyflavone (67) showed strong activity at low concentration. At 3.125 μM, 67 still exhibited activity more than two-fold that of positive control.

Department: Chemistry Student's Signature .....
Field of Study: Chemistry Advisor's Signature .....
Academic Year: 2015

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CONTENTS

Page

Page

Page

LIST OF TABLES

Table **Page**

LIST OF FIGURES

Figure **Page**

LIST OF ABBREVIATIONS

bs	broad singlet (NMR)
calcd	calculated
$CDCl_3$	Deuterated chloroform
CH_2Cl_2	dichloromethane
d	doublet (NMR)
dd	doublet of doublets (NMR)
DMF	*N*,*N*-dimethylformamide
DMSO	dimethylsulfoxide
EtOAc	ethyl acetate
EtOH	ethanol
g	gram (s)
hr	hour (s)
HR-MS	high resolution mass spectrometry
IC_{50}	Inhibition concentration 50 %
J	coupling constant (NMR)
K_2CO_3	potassium carbonate
m	multiplet (NMR)
M	molar (s)
MBC	minimum bactericidal concentration
MeOH	methanol
mg	milligram (s)
MIC	minimal inhibitory concentration
min	minute (s)
mL	milliliter (s)
mm	millimeter (s)
mmol	millimole (s)
Na_2CO_3	sodium carbonate

nm	nanometer (s)
NMR	nuclear magnetic resonance
q	quartet (NMR)
s	singlet (NMR)
t	triplet (NMR)
TLC	thin layer chromatograph
UV	ultraviolet
µM	micromolar (s)
µL	microliter (s)
%	percent
℃	degree of Celsius
α	alpha
δ	chemical shift
δ_{H}	chemical shift of proton
δ_{C}	chemical shift of carbon
$[M+Na]^{+}$	pseudomolecular ion

CHAPTER I
INTRODUCTION

Flavonoids are a class of natural products. They are known to be present in plants including fruits, vegetables and herb. As the largest class of polyphenol, they can be classified into six major subgroups, based on their molecular structure including chalcone, flavone, flavanone, flavonol, anthocyanin, and isoflavonoid. Flavonoids are associated with a broad spectrum of health promoting effects such as antioxidant [1], antibacterial [2] and anticancer [3]. Moreover, flavonoids can be applied to cosmetic which has been suggested to protect the skin against UV-induced damage and skin aging [4].

Traditional medicine is used widely throughout Thailand. There are many Thai plants that are used for medicinal purposes such as *Boesenbergia rotunda* (fingerroot, Krachai). The crushed roots and rhizomes are applied to painful parts of the body to ease rheumatic pains, and used internally to dispel flatulence, improve the appetite and digestion, as a remedy for dry mouths, coughs and ulcers. *Kaempferia parviflora* (Thai ginseng, Krachai dum) has traditionally been used as a health promoting, stimulating and vitalizing agent. This plant is very popular for stimulating sexual performance mostly in males. It contains substantial amounts of PDE5 inhibitors, which act similarly as Viagra, with the ability to enhance sexual performance by increasing blood-flow to the testis [5] and stimulating dopaminergic functions in the hypothalamus. Furthermore, *K. parviflora* can increase sperm density and promote health, reducing triglycerides and preventing diabetes.

1.1 General characteristics of *Boesenbergia rotunda* (L.) Mansf.

B. rotunda belongs to the ginger family (Zingiberacea). The rhizomes of *B. rotunda* are often shaped like a bunch of fingers. It is a small perennial plant of about 15–40 cm in height. Its leaves are broad and light green while the leaf sheath is red. The underground portion of the plant consists of a small globular shaped central subterraneous rhizome (1.5–2.0 cm in diameter) from which several slender and long tubers sprout all in the same direction like the fingers of a hand, thus the common name fingerroot [6].

Figure 1.1 The rhizomes and plants of *B.* rotunda

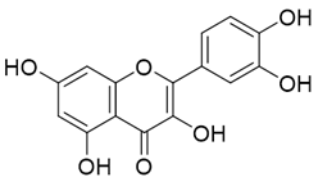
1.2 General characteristics of *Kaempferia Parviflora* Wall. Ex Baker

K. parviflora belonging to the the ginger family (Zingiberacea) is a herbaceous plant of about 50-70 cm in height. Its leaves are simple leaf with deep violet and oval shape. It is native to Thailand and has some historical and medicinal usage for treating metabolic ailments and improving vitality in Thailand and limited to surrounding regions.

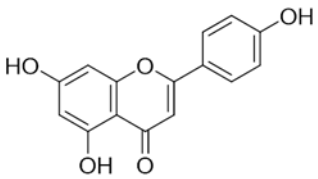
Figure 1.2 The rhizomes and plants of *K. Parviflora*

1.3 Anti-bacterial activity of flavonoids

Although some bacteria are beneficial or harmless, several are pathogenic. Pathogenic bacteria contribute to other globally important diseases, such as pneumonia, which can be caused by bacteria such as *Streptococcus* and *Pseudomonas*. Nowadays, many research groups are interested in anti-bacterial activity of flavonoids. Several isolated or synthesized flavonoids possessed anti-bacterial activity. This property of flavonoids enables them to be used extensively in the area of nutrition, food safety, and health. Quercetin (**1**) and apigenin (**2**) are among the most studied flavonoids which have been known to exhibit antibacterial activities [7].



1



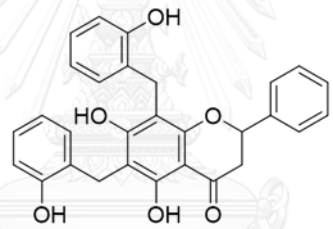
2

In 2006, Hussain and co-workers investigated 4-thioflavones and 4-iminoflavones and their anti-bacterial activity against *Escherichia coli*, *Bacillus subtilis*, *Shigella flexnari*, *Salmonella aureus*, *Salmonella typhi* and *Pseudomonas aeruginosa*. These compounds exhibited better activity than their corresponding

flavone analogues. Investigation of the compounds having substituents as F, OMe and NO_2 at 4′-position in ring-B of flavones exhibited the activity enhancement and the presence of electronegative groups in the studied compounds showed a direct relationship to the antibacterial activity [8].

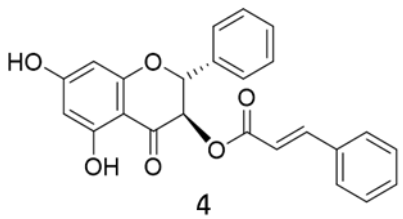
In 2008, Li and Xu reported that quercetin (**1**) extracted from lotus leaves was a promising antibacterial agent for periodontitis [9].

In 2015, Ferreira and co-workers addressed that a flavanone, dichamanetin (**3**), isolated from *Cleistochlamys kirkii*, was very active against all Gram-positive strains tested (*Staphylococcus aureus*, *Enterococcus faecalis* and *Bacillus subtillis*), displaying MIC values in the range of 1–7.5 μg/mL [10].



3

In 2016, Biva and co-workers isolated four compounds from the leaf-extract of *Eremophila alternifolia*. Pinobanksin-3-cinnamate (**4**) was the most promising antibacterial compound with significant activity (10-20 μM) against Gram-positive bacterium *Staphylococcus aureus* including methicillin resistant and biofilm forming strains[11].



4

1.4 Anti-tyrosinase activities of flavonoids

Tyrosinase is an oxidase which is the rate-limiting enzyme for controlling the production of melanin. Melanin biosynthesis starts with two-step conversion catalyzed by tyrosinase, hydroxylation of L-tyrosine to 3,4-dihydroxyphenylalanine (L-DOPA) and the oxidation of L-DOPA to dopaquinone, finally oxidative polymerization *via* several dopaquinone derivatives to yield pheomelanin (red-orange-color) and eumelanin (black-brown-color). The melanin biosynthesis pathway is shown in **Figure 1.3** [12, 13].

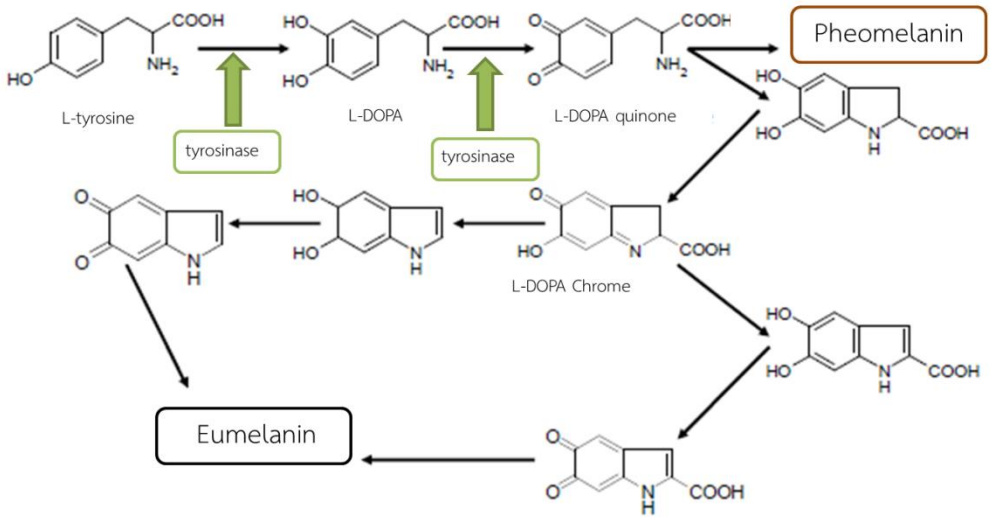
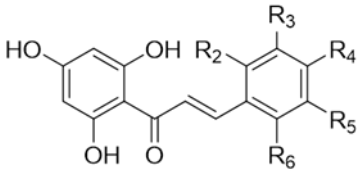


Figure 1.3 Melanin biosynthesis pathway [13]

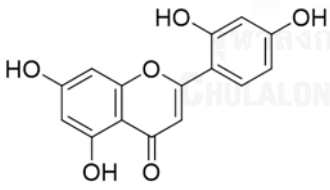
Both natural and synthetic flavonoids had been tested for anti-tyrosinase activity in order to search for whitening agents. For example, in 2007, Jun and co-workers synthesized a series of hydroxychalcones and studied their tyrosinase inhibitory activity. 2′,4′,6′-Trihydroxychalcone (**5**), 2,2′,3,4′,6′-pentahydroxychalcone (**6**), 2′,3,4,4′,5,6′-hexahydroxychalcone (**7**), 2′,4′,6′-trihydroxy-3,4-dimethoxychalcone (**8**) and 2,2′,4,4′,6′-pentahydroxychalcone (**9**) exhibited high inhibitory effects on

tyrosinase with respect to L-tyrosine as a substrate. By structure–activity relationship study, it was suggested that 2′,4′,6′-trihydroxy substructure in the chalcone skeleton be efficacious for the inhibition of tyrosinase activity. The catechol structure on B-ring of chalcones was not advantageous for the inhibitory potency [14].



5: $R_2 = R_3 = R_4 = R_5 = R_6 = H$
6: $R_2 = R_3 = OH$, $R_4 = R_5 = R_6 = H$
7: $R_2 = R_6 = H$, $R_3 = R_4 = R_5 = OH$
8: $R_2 = R_5 = R_6 = H$, $R_3 = R_4 = OCH_3$
9: $R_2 = R_4 = OH$, $R_3 = R_5 = R_6 = H$

In 2013, Ko and co-workers addressed that norartocarpetin (**10**), artogomezianone (**11**), cudraflavone A (**12**) and artonin M (**13**) from *Artocarpus altilis* inhibited melanin production by strongly suppressing tyrosinase activity [15].



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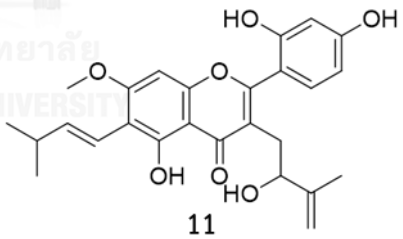

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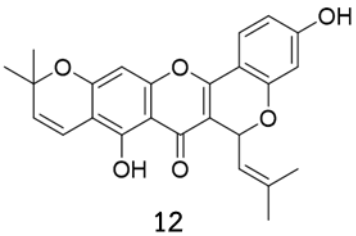

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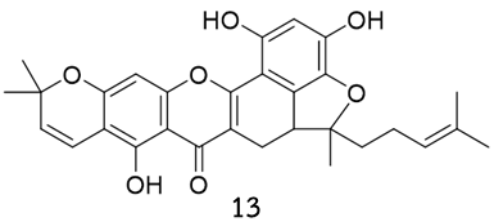
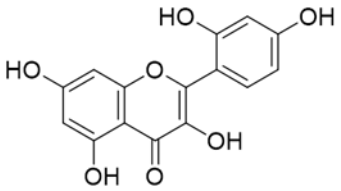


13

In 2014, Zhang and co-workers investigated morin (**14**), a flavonol that widely distributed in plants and foods of plant origin, exhibited potent tyrosinase inhibitory activity [16].



14

In 2016, Zheng and co-workers synthesized flavonoids from the reaction between 2,4-dihydroxybenzaldehyde and hydroxyacetophenones *via* Aldol, Michael, and Friedel–Crafts additions using boric acid as catalyst. These synthetic compounds were demonstrated significant tyrosinase inhibitory activities much stronger than kojic acid [17].

1.5 Melanogenesis activities of flavonoids

Melanin pigment is distributed in several tissues in human. The important role of melanin is considered to protect skin from UV damage by absorbing UV light. Melanin is produced in melanosomes by melanocytes on a complex process called melanogenesis. Keratinocytes, existing on the skin surface, get stimulated by UV irradiation to produce messengers such as α-melanocyte-stimulating hormone (α-MSH), prostaglandin, and histamine to melanocytes. Then melanin is biosynthesized by melanocyte in melanosome and transports to keratinocytes. The skin pigmentation is induced by the cornification. Similarly, hair pigmentation occurs due to melanin released on the outside of the melanocyte. The excess accumulation of melanin in the skin often causes skin problems, such as freckles chloasma and melisma so the controlling of melanogenesis is important. The mechanism of skin pigmentation is shown in **Figure 1.4** [18, 19].

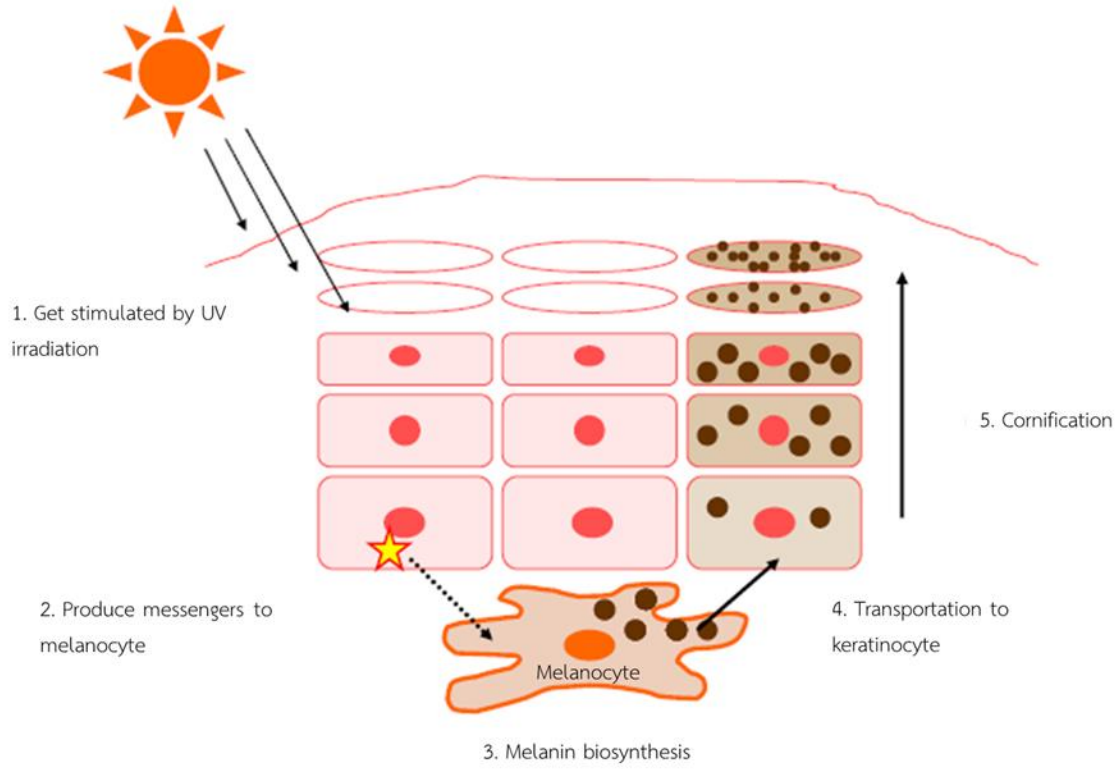
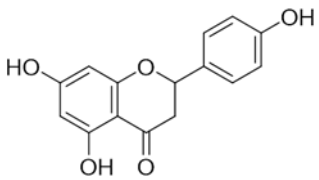


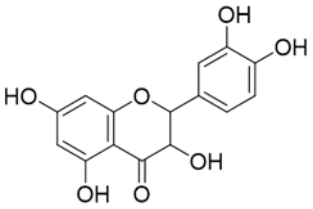
Figure 1.4 Mechanism of skin pigmentation [19]

Many research groups explored the compounds which could control melanogenesis activity. For example, in 2006, Ohguchi and co-workers found that naringenin (**15**) induced melanogenesis in mouse B16 melanoma cells, and that the major melanogenic signaling factors, such as tyrosinase, Tyrp1, Dct, and Mitf, were upregulated by naringenin [20].



15

In 2008, Boo and co-workers reported that taxifolin (**16**) and luteolin (**17**) inhibited the cellular melanogenesis as effectively as arbutin, one of the most widely used hypopigmenting agents in cosmetics [21].



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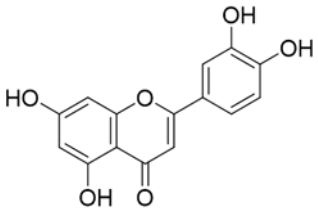


17

In 2009, Fujii and co-workers isolated flavonoids from the methanolic extract of rose hips and investigated their anti-melanogenesis activity. Among isolated compounds, quercetin (**1**) was a potent melanogenesis inhibitor and decreased the intracellular tyrosinase activity as well as the tyrosinase activity in a cell culture-free system [22].

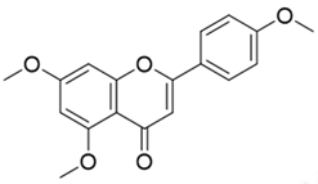
In 2013, Horibe and co-workers found that 4′-*O*-methylated flavonoids increased the melanin contents of the cells 3- to 7-fold higher than the control cells. On the other hand, the corresponding 4′-OH-type flavonoids had a significantly smaller effect [23].

In 2014, Takekoshi and co-workers reported the relationships between the chemical structures of flavonoids and their melanogenesis-promoting actions, it was inferred that a hydroxyl group bound to the phenyl group plays an important role in stimulating melanogenesis [24].

1.6 Literature review

There are many reports concerning the flavonoids isolated from *B. rotunda* and *K. parviflora* and their analogues possessing the biological activities such as

antibacterial, anticancer, anti-inflammatory, antitumor and melanogenesis activity. For example, in 2003 Yenjai and co-workers isolated nine flavonoids from *K. parviflora*. Among these 5,7,4′-trimethoxyflavone (**18**) and 5,7,3′,4′-tetramethoxyflavone (**19**) exhibited antiplasmodial activity against *Plasmodium falciparum*, with IC_{50} of 3.70 and 4.06 μg/mL, respectively [25].



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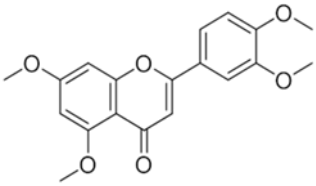
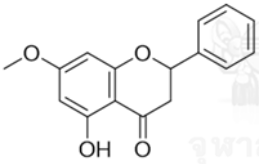


19

In 2005, Bhamarapravati and co-workers isolated pinostrobin (**20**) and red oil from *B. rotunda*, and dihydroguaiaretic acid (**21**) from *Myristica fragrans* and investigated their antibacterial activity. These compounds showed good potential to inhibit the growth of *Helicobacter pylori* [26].



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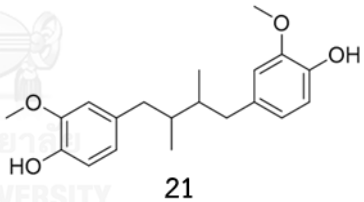
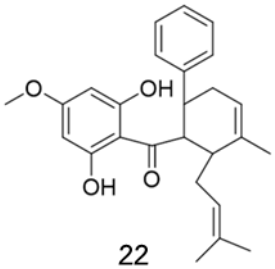


21

In 2009, Tewtrakul and co-workers isolated active compounds from the rhizomes of *B. rotunda* (panduratin A (**22**)) and *K. parviflora* (5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**23**)). Their inhibitory activities against nitric oxide (NO) production showed IC_{50} of 5.3 μM for panduratin A and 16.1 μM for 5-hydroxy-3,7,3′,4′-tetramethoxyflavone [27].



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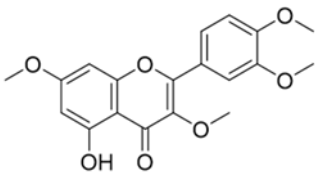
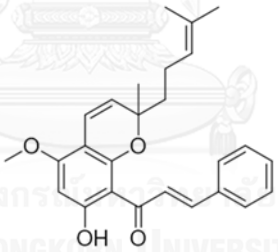


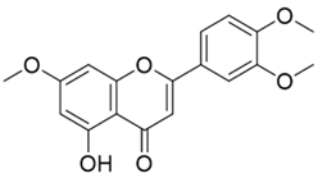
23

In 2012, Isa and co-workers investigated anti-inflammatory, cytotoxic and antioxidant activities of boesenbergin A (**24**), a chalcone isolated from *B. rotunda*. The anti-inflammatory activity of boesenbergin A was significant at 12.5 to 50 µg/mL and without any significant cytotoxicity for the murine macrophage cell line RAW 264.7 at 50 µg/mL. Moreover, this compound displayed considerable antioxidant activity, when the results of ORAC assay were reported as Trolox equivalents. Boesenbergin A (20 µg/mL) and quercetin (5 µg/mL) were equivalent to a Trolox concentration of 11.91 ± 0.23 and 160.32 ± 2.75 µM, respectively [28].



24

In 2015, Ninomiya and co-workers addressed that the methanol extract from the rhizomes of *K. parviflora* revealed inhibitory effects against melanogenesis in theophylline- stimulated murine B16 melanoma 4A5 cells. Among twenty-five flavonoids and three acetophenones, several constituents including 5-hydroxy-7,3′,4′-trimethoxyflavone (**25**) (IC_{50} = 8.8 µM), 5,7,3′,4′-tetramethoxyflavone (**19**) (IC_{50} = 8.6 µM), 5,3′-dihydroxy-3,7,4′-trimethoxyflavone (**26**) (IC_{50} = 2.9 µM), and 5-hydroxy-3,7,3′,4′-tetramethoxyflavone (**23**) (IC_{50} = 3.5 µM) showed inhibitory effects without notable cytotoxicity at the effective concentrations [29].



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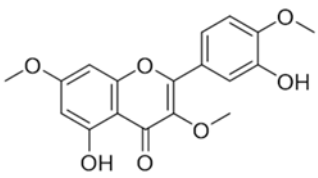
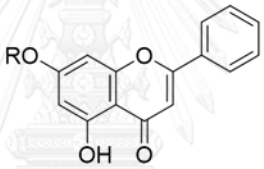


26

Furthermore, the structural modification of flavonoids has been investigated in order to enhance biological activity such as antibacterial, antifungal and anticancer. It was also well known that halogenated compounds also expressed strong biological activities. For example, in 1999 Kim and co-workers prepared eighteen chrysin derivatives and tested *in vivo* against the diabetes mellitus. Several modified compounds especially those with propyl (**27**), butyl (**28**) and octyl (**29**) exhibited hypoglycemic effect on diabetes mice [30].

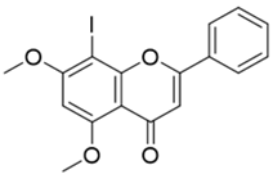


27: R= C_3H_7

28: R = C_4H_9

29: R = C_8H_{17}

In 2003, Qing and co-workers reported that a series of chrysin derivatives was tested *in vitro* against human gastric adenocarcinoma cell line (SGC-7901) and colorectal adenocarcinoma (HT-29) cells. Among these compounds, 5,7-dimethoxy-8-iodochrysin (**30**) and 8-bromo-5-hydroxy-7-methoxychrysin (**31**) exhibited the strongest activities against SGC-7901 and HT-29 cells, respectively. 5,7-Dihydroxy-8-nitrochrysin (**32**) was found to have strong activities against both SGC-7901 and HT-29 cells [31].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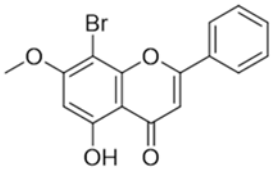
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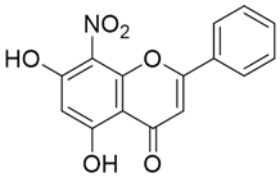
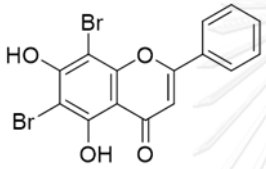

32

In 2005, Park and co-workers synthesized 6,8-disubstituted chrysin derivatives and evaluated their PGE_2 inhibitory activities. 6,8-Dibromochrysin (**33**), 6,8-diiodochrysin (**34**), 6,8-dimethylthiochrysin (**35**) and 6,8-dimethoxychrysin (**36**) showed as strong inhibitory activities of PGE_2 production from LPS-induced RAW 264.7 cells [32].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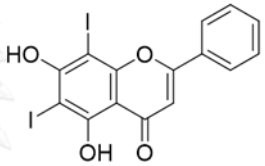
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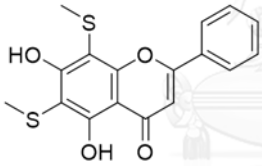
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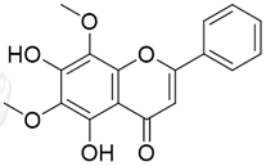
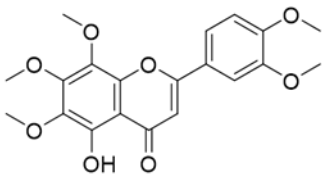

36

In 2007, Li and co-workers isolated fifteen polymethoxyflavones (PMFs) and hydroxylated PMFs from sweet orange (*Citrus sinensis*) peel and investigated for anti-carcinogenic activities. 5-Hydroxy-6,7,8,3′,4′-pentamethoxyflavone (**37**) and 5-hydroxy-3,6,7,8,3′,4′-hexamethoxyflavone (**38**) showed strong inhibitory activities against the proliferation [33].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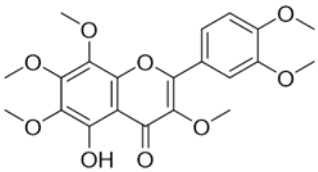
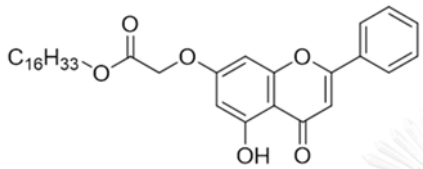

38

In 2009, Zhu and co-workers prepared a series of long chain derivatives of chrysin and evaluated their antiproliferative activities against the human liver cancer. Hexadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy) acetate (**39**) and *N*-hexadecyl 2-(5-hydroxy-4-oxo-2-phenyl-4H-chromen-7-yloxy) acetamide (**40**) displayed potent EGFR inhibitory activity with IC_{50} of 0.048 μM and 0.035 μM), comparable to the positive control erlotinib [34].



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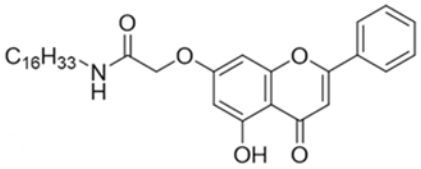
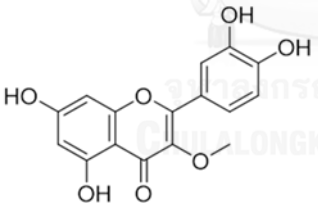


40

In 2014, Mitsunaga and co-workers synthesized quercetin derivatives. 3-*O*-Methylquercetin (**41**) and 3,4′,7-*O*-trimethylquercetin (**42**) increased melanin content more potently than the positive control theophylline, while exhibiting low cytotoxicity [35].



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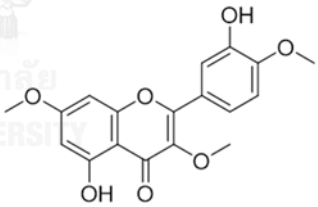


42

In 2014, Peng and co-workers synthesized twelve chrysin, diosmetin, apigenin, and luteolin alkyl derivatives and investigated their α-glucosidase inhibitory activity. The glucosidase inhibitory activity of all derivatives is higher compared with those of the positive control drugs, acarbose, and 1-deoxynojirimycin with IC_{50} < 24.396 μmol/L [36].

As mentioned above, flavonoids and their related compounds were interesting to explore biological activities. Therefore, the present study aims to

isolate flavonoids from *B. rotunda* and *K. parviflora*, synthesized the related compounds and assessed for biological activities, particularly anti-bacterial against *Propionibacterium acnes* (KCCM41747) and *Staphylococcus aureus* (ATCC25923) causing skin infections, *Streptococcus sobrinus* (KCCM11898) and *Streptococcus mutans* (ATCC25175) causing caries decay of teeth and *Salmonella typhi* (ATCC442) being the causative agent of typhoid fever, anti-tyrosinase and melanogenesis activity.

The objectives of this research could be summarized as following:

1. To extract, isolate and purify flavonoids from the rhizomes from *B. rotunda* and *K. parviflora*.

2. To synthesize the flavanone and flavone derivatives

3. To evaluate and study the relationship between flavonoids and their biological activities including anti-bacterial, anti-tyrosinase and melanogenesis activities.

CHAPTER II

EXPERIMENTAL

2.1 Instruments and equipment

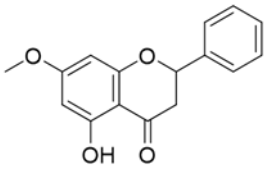
Thin layer chromatography (TLC) was performed on an aluminum sheets precoated with silica gel, Kieselgel 60 F_{254} (Merck, Germany), column chromatography was performed on silica gel no. 7734 (Merck, Germany). All NMR spectra (^{1}H and ^{13}C NMR) were performed in deuterated chloroform ($CDCl_3$) or dimethylsulfoxide-d_6 (DMSO-d_6) on a Bruker AV400 and Varain Mercury 400 plus spectrometer at 400 MHz for ^{1}H NMR and at 100 MHz for ^{13}C NMR. The chemical shifts (δ) are assigned by comparison with residue solvent protons.

2.2 Chemicals

The reagents used for synthesis were purchased from Merck chemical company or otherwise stated. All solvents used in this research were purified prior to use by standard methodology except for those which were reagent grades.

2.3 Extraction, isolation and purification of *Boesenbergia rotunda*.

The dried rhizomes of *B. rotunda* (2.5 kg) were extracted with CH_2Cl_2 at room temperature for 5 days. The extract was concentrated by rotatory evaporator. Then the CH_2Cl_2 extract (120 g) was subjected to silica gel quick column to give 7 fractions. According to TLC pattern, fractions 3, 4 and 5 contained the same major spot. After recrystallization with hexane:EtOAc (7:3), pinostrobin (**20**) as yellow solid 61.3 g (51 %) was obtained. The isolation of fraction 6 by silica gel column furnished alpinetin (**43**) as pale yellow solid 8.9 g (8 %).



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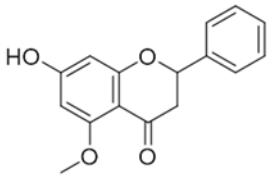


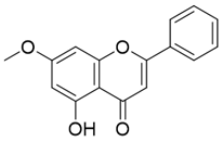
43

Pinostrobin (**20**) : yellow solid, (51 %) ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.05 (s, 1H), 7.58–7.37 (m, 5H), 6.10 (d, *J* = 3.6 Hz, 2H), 5.45 (dd, *J* = 13.0, 3.0 Hz, 1H), 3.84 (s, 3H), 3.12 (dd, *J* = 17.2, 13.0 Hz, 1H), 2.85 (dd, *J* = 17.2, 3.1 Hz, 1H).

Alpinetin (**43**) : pale yellow solid (8 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.06 (s, 1H), 7.59–7.37 (m, 5H), 6.03 (d, *J* = 1.0 Hz, 2H), 5.45 (dd, *J* = 13.0, 3.1 Hz, 1H), 3.84 (s, 3H), 3.12 (dd, *J* = 17.2, 13.0 Hz, 1H), 2.85 (dd, *J* = 17.2, 3.1 Hz, 1H).

2.4 Extraction, isolation and purification of *Kaempferia parviflora*.

The dried rhizomes of *K. parviflora* (7 kg) were extracted with CH_2Cl_2 by maceration at room temperature. The CH_2Cl_2 extract was evaporated under vacuum. Then the extract (170 g) was subjected to silica gel quick column to yield 7 fractions. The isolation of fractions 4, 5, 6 and 7 was performed by silica gel column to give 5 compounds: 5-hydroxy-3,7-dimethoxyflavone (**44**) as yellow solid (10 %), 5-hydroxy-7-methoxyflavone (**45**) as yellow solid (29 %), 5-hydroxy-3,7,4′-trimethoxyflavone (**46**) as pale brown solid (9 %), 5-hydroxy-7,4′-dimethoxyflavone (**47**) as yellow solid (0.01 %) and 3,5,7-trimethoxyflavone (**48**) as pale green solid (0.02 %).



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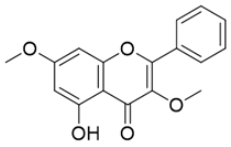

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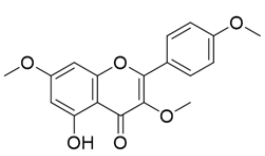

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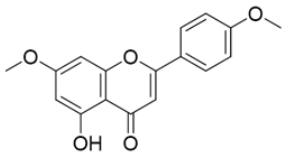

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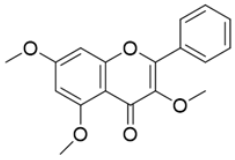


48

5-hydroxy-7-methoxyflavone (**44**): yellow solid (10 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.75 (s, 1H), 7.91 (m, 2H), 7.56 (m, 3H), 6.69 (s, 1H), 6.53 (d, *J* = 2.3 Hz, 1H), 6.41 (d, *J* = 2.3 Hz, 1H), 3.91 (s, 3H).

5-hydroxy-3,7-dimethoxyflavone (**45**): yellow solid (29 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.60 (s, 1H), 8.09 (m, 2H), 7.54 (m, 3H), 6.48 (d, *J* = 2.2 Hz, 1H), 6.39 (d, *J* = 2.2 Hz, 1H), 3.90 (s, 6H).

5-hydroxy-3,7,4′-trimethoxyflavone (**46**): pale brown solid (9 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.68 (s, 1H), 8.10 (d, *J* = 9.0 Hz, 2H), 7.05 (d, *J* = 9.0 Hz, 2H), 6.47 (d, *J* = 2.2 Hz, 1H), 6.38 (d, *J* = 2.2 Hz, 1H), 3.92 (s, 3H), 3.90 (s, 3H), 3.88 (s, 3H).

5-hydroxy-7,4′-dimethoxyflavone (**47**): yellow solid (0.01 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.82 (s, 1H), 7.84 (d, *J* = 9.0 Hz, 2H), 7.02 (d, *J* = 9.0 Hz, 2H), 6.58 (s, 1H), 6.49 (d, *J* = 2.3 Hz, 1H), 6.37 (d, *J* = 2.3 Hz, 1H), 3.89 (d, *J* = 4.9 Hz, 6H).

3,5,7-trimethoxyflavone (**48**): pale green solid (0.02 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (m, 2H), 7.49 (d, *J* = 7.5 Hz, 3H), 6.51 (d, *J* = 2.4 Hz, 1H), 6.35 (d, *J* = 2.3 Hz, 1H), 3.96 (s, 3H), 3.89 (s, 3H), 3.88 (s, 3H).

2.5 Synthesis of flavone derivatives

2.5.1 Ether derivatives of flavones

General procedure for alkylation of flavone

A solution of starting flavone (2 mmol) in dry acetone was treated with anhydrous K_2CO_3 and selected bromoalkane (2 mmol). The mixture was refluxed under N_2 atmosphere overnight, cooled to room temperature and filtered. The solid K_2CO_3 was washed with acetone. Evaporation of the combined organic solvent under reduced pressure furnished a residue, which was purified by silica gel column to give a target product [37].

General procedure for methylation of flavone

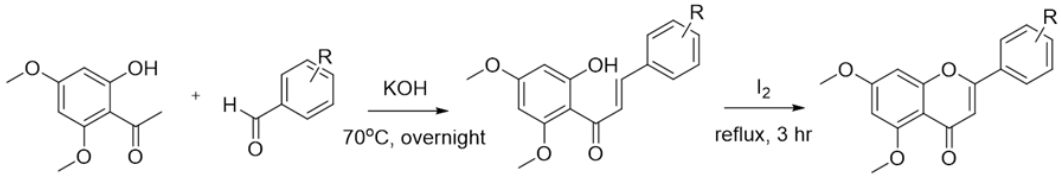
To a mixture of starting flavone (2 mmol) in 20 mL of dry acetone and anhydrous K_2CO_3, dimethyl sulfate was added slowly with stirring. The reaction mixture was refluxed for 24 hr. K_2CO_3 was removed by suction filtration. The filtrate was evaporated and purified by silica gel column [38].

2.5.2 Flavone containing halogen

To the solution of starting flavone (5 mmol) in acetone-water was added NaBr (11 mmol). After cooling, a solution of oxone in 20 mL of water was added slowly. Then a solution was stirred overnight. The reaction mixture was treated with $Na_2S_2O_3$ and solvent was removed by evaporation [38].

To the solution of starting flavone (2 mmol) in acetic acid (2 mL), I_2 (2 mmol) in CH_2Cl_2 was slowly added and stirred for 30 min at room temperature. The solution of HNO_3 in acetic acid (1 mL) was added. The reaction was stirred at room temperature for 2 hr and filtered. The solid was washed with 10 % $Na_2S_2O_3$ solution then cooled MeOH and water. The product was purified by silica gel column [39].

2.5.3 Synthesis of polymethoxyflavone



General procedure for the synthesis of 2-hydroxychalcone

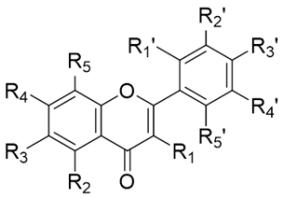
To a solution of 2-hydroxyacetophenone (1 mmol) and benzaldehyde (1 mmol) in MeOH was added 50 % KOH. The reaction mixture was heated at 70 °C overnight. Then MeOH was evaporated, and the residue was dissolved in CH_2Cl_2/H_2O (4:1). The organic layer was washed with brine and evaporated. Then the mixture was purified by silica gel column [40].

General procedure for the synthesis of flavone

To a solution of 2-hydroxychalcone (1 mmol) in DMSO was added I_2. The mixture was refluxed for 3 hr. Then the mixture was poured into water and extracted with EtOAc. The organic layer was washed with brine and the solvent was evaporated. The residue was purified by silica gel column [41].

Twenty-three synthesized flavone derivatives are displayed as shown in **Table 2.1.**

Table 2.1 Structures of flavone derivatives



Entry	Cpds	R_1	R_2	R_3	R_4	R_5	$R_{1'}$	$R_{2'}$	$R_{3'}$	$R_{4'}$	$R_{5'}$
1	49	H	OCH_3	H	OCH_3	H	H	H	H	H	H
2	50	H	OH	H	OC_2H_5	H	H	H	H	H	H
3	28	H	OH	H	OC_4H_9	H	H	H	H	H	H
4	51	H	OH	H	OC_6H_{13}	H	H	H	H	H	H
5	29	H	OH	H	OC_8H_{17}	H	H	H	H	H	H
6	52	H	OH	H	$OC_{12}H_{25}$	H	H	H	H	H	H
7	53	H	OCH_3	H	OC_2H_5	H	H	H	H	H	H
8	54	H	OCH_3	H	OC_4H_9	H	H	H	H	H	H
9	55	H	OCH_3	H	OC_6H_{13}	H	H	H	H	H	H
10	56	H	OCH_3	H	OC_8H_{17}	H	H	H	H	H	H
11	57	H	OCH_3	H	$OC_{12}H_{25}$	H	H	H	H	H	H
12	33	H	OH	Br	OH	Br	H	H	H	H	H
13	34	H	OH	I	OH	I	H	H	H	H	H
14	58	H	OH	Br	OCH_3	Br	H	H	H	H	H
15	59	H	OH	I	OCH_3	I	H	H	H	H	H
16	60	OCH_3	OH	Br	OCH_3	Br	H	H	H	H	H
17	61	OCH_3	OH	I	OCH_3	I	H	H	H	H	H
18	62	H	OCH_3	H	OCH_3	H	OCH_3	H	H	H	H
19	63	H	OCH_3	H	OCH_3	H	OCH_3	H	OCH_3	H	H
20	64	H	OCH_3	H	OCH_3	H	OCH_3	H	H	OCH_3	H
21	65	H	OCH_3	H	OCH_3	H	OCH_3	OCH_3	OCH_3	H	H
22	66	H	OCH_3	H	OCH_3	H	OCH_3	H	OCH_3	H	OCH_3
23	67	H	OCH_3	H	OCH_3	H	H	OCH_3	OCH_3	OCH_3	H

5,7-dimethoxyflavone (**49**) : yellow solid (58 %),^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 2H)8, 7.53 (m, 3H), 6.67 (s, 1H), 6.50 (d, *J* = 2.3 Hz, 1H), 6.38 (d, *J* = 2.3 Hz, 1H), 3.88 (s, 6H).

5-hydroxy-7-ethoxyflavone (**50**): yellow solid (40 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (dd, *J* = 7.9, 1.9 Hz, 2H), 7.53 (m, 3H), 6.66 (s, 1H), 6.49 (d, *J* = 2.2 Hz, 1H), 6.36 (d, *J* = 2.2 Hz, 1H), 4.11 (q, *J* = 7.0 Hz, 2H), 1.46 (t, *J* = 7.0 Hz, 3H).

5-hydroxy-7-butoxyflavone (**28**): pale yellow solid (58 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (dd, *J* = 7.7, 2.0 Hz, 2H), 7.53 (m, 3H), 6.67 (s, 1H), 6.50 (s, 1H), 6.37 (d, *J* = 2.2 Hz, 1H), 4.04 (t, *J* = 6.5 Hz, 2H), 1.58 (m, 2H), 1.25 (m, 2H), 0.99 (t, *J* = 7.4 Hz, 3H).

5-hydroxy-7-hexyloxyflavone (**51**): pale yellow solid (76 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.71 (s, 1H), 7.89 (d, *J* = 8.3 Hz, 2H), 7.54 (m, 3H), 6.67 (s, 1H), 6.50 (s, 1H), 6.37 (s, 1H), 4.03 (m, 2H), 1.81 (m, 2H), 1.39 – 1.31 (m, 6H), 0.91 (m, 3H).

5-hydroxy-7-octyloxyflavone (**29**): pale yellow solid (51 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 2H), 7.53 (m, 3H), 6.67 (s, 1H), 6.49 (d, *J* = 2.3 Hz, 1H), 6.36 (d, *J* = 2.2 Hz, 1H), 4.03 (t, *J* = 6.6 Hz, 2H), 1.81 (m, 2H), 1.45 (d, J = 7.6 Hz, 2H), 1.39 – 1.24 (m, 8H), 0.89 (m, 3H).

5-hydroxy-7-dodecyloxyflavone (**52**): pale yellow solid (56 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.89 (m, 2H), 7.53 (m, 3H), 6.67 (s, 1H), 6.50 (d, *J* = 2.2 Hz, 1H), 6.37 (d, *J* = 2.2 Hz, 1H), 4.03 (t, *J* = 6.5 Hz, 2H), 1.89 – 1.25 (m, 20H), 0.87 (m, 3H).

5-methoxy-7-ethoxyflavone (**53**): pale yellow solid (87 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 2H), 7.51 (m, 3H), 6.69 (s, 1H), 6.56 (d, *J* = 2.3 Hz, 1H), 6.38 (d, *J* = 2.3 Hz, 1H), 4.15 (q, *J* = 7.0 Hz, 2H), 3.96 (s, 3H), 1.49 (t, *J* = 7.0 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.7, 163.4, 160.9, 160.6, 159.9, 131.7 131.2, 129.1, 126.3, 109.2, 109.0, 96.5,

93.3, 64.2, 56.4, 14.6. HR-MS (ESI): caldc for $C_{18}H_{16}O_4$ $[M+Na]^+$: 319.0946, found 319.0945.

5-methoxy-7-butoxyflavone (**54**): pale yellow solid (88 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 2H), 7.47 (m, 3H), 7.09 (s, 1H), 6.58 (d, *J* = 2.2 Hz, 1H), 6.37 (d, *J* = 2.2 Hz, 1H), 4.04 (t, *J* = 6.5 Hz, 2H), 3.90 (s, 3H), 1.77 (m, 2H), 1.47 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.9, 165.1, 162.9, 161.0, 160.3, 132.1, 130.7, 129.1, 126.5, 107.8, 106.8, 97.3, 93.3, 68.7, 56.5, 29.7, 19.2, 13.7. HR-MS (ESI): caldc for $C_{20}H_{20}O_4$ $[M+Na]^+$: 347.1259, found 347.1255.

5-methoxy-7-hexyloxyflavone (**55**): pale yellow solid (81 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.91 (m, 2H), 7.53 (m, 3H), 6.92 (s, 1H), 6.60 (d, *J* = 2.2 Hz, 1H), 6.41 (d, *J* = 2.2 Hz, 1H), 4.09 (t, *J* = 6.5 Hz, 2H), 3.98 (s, 3H), 1.85 (m, 2H), 1.52 (m, 2H), 1.27 (m, 4H), 0.95 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.7, 164.1, 161.3, 160.9, 160.0, 131.4, 131.3, 129.0, 126.1, 108.6, 108.3, 96.8, 93.3, 68.8, 56.4, 31.5, 29.0, 25.6, 22.6, 14.0. HR-MS (ESI): caldc for $C_{22}H_{24}O_4$ $[M+Na]^+$: 375.1572, found 375.1577.

5-methoxy-7-octyloxyflavone (**56**): pale yellow solid (91 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 2H), 7.50 (m, 3H), 6.69 (s, 1H), 6.56 (d, *J* = 2.3 Hz, 1H), 6.38 (d, *J* = 2.2 Hz, 1H), 4.06 (t, *J* = 6.5 Hz, 2H), 3.97 (s, 3H), 1.85 (m, 2H), 1.45 – 1.22 (m, 10H), 0.91 (m, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.6, 163.6, 160.9, 160.6, 159.9, 131.6, 131.1, 128.9, 125.9,109.1, 109.0, 96.5, 93.3, 68.6, 56.4, 31.8, 29.3, 29.2, 29.0, 26.0, 22.6, 14.1. HR-MS (ESI): caldc for $C_{24}H_{28}O_4$ $[M+Na]^+$: 403.1885, found 403.1881.

5-methoxy-7-decyloxyflvone (**57**): pale yellow solid (89 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.88 (m, 2H), 7.51 (m, 3H), 6.70 (s, 1H), 6.57 (d, *J* = 2.3 Hz, 1H), 6.39 (d, *J* = 2.2 Hz, 1H), 4.06 (t, *J* = 6.5 Hz, 2H), 3.97 (s, 3H), 1.84 (q, *J* = 7.0 Hz, 2H), 1.45 – 1.12 (m, 18H), 0.89 (t, *J* = 6.8 Hz, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 177.7, 163.7, 160.9, 160.6, 159.9, 131.6, 131.1, 128.9, 125.9, 109.1, 109.0, 96.5, 93.3, 68.7, 56.4, 31.9, 29.7, 29.6,

29.6, 29.6, 29.5, 29.3, 29.0, 26.0, 22.7, 14.1. HR-MS (ESI): caldc for $C_{28}H_{36}O_4$ $[M+Na]^+$: 459.2511, found 459.2516.

6,8-dibromo-5,7-dihydroxyflavone (**33**): pale green solid (68 %), ^{1}H NMR (400 MHz, DMSO-d_6) δ 13.65 (s, 1H), 8.05 (d, *J* = 7.4 Hz, 2H), 7.56 (d, *J* = 7.5 Hz, 3H), 7.07 (s, 1H).

6,8-diiodo-5,7-dihydroxyflavone (**34**): pale yellow solid (77 %), ^{1}H NMR (400 MHz, DMSO-d_6) δ 8.18 (d, *J* = 7.4 Hz, 2H), 7.60 (d, *J* = 8.7 Hz, 3H), 7.17 (s, 1H).

6,8-dibromo-5-hydroxy-7-methoxyflavone (**58**): pale yellow solid (38 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.12 – 7.93 (m, 2H), 7.57 (d, *J* = 9.7 Hz, 3H), 6.83 (s, 1H), 4.01 (s, 3H).

6,8-diiodo-5-hydroxy-7-methoxyflavone (**59**): pale yellow solid (26 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 8.07 (d, *J* = 7.4 Hz, 2H), 7.59 (m, 3H), 6.85 (s, 1H), 3.98 (s, 3H).

6,8-dibromo-5-hydroxy-3,7-dimethoxyflavone (**60**) : pale yellow solid (25 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.36 (s, 1H), 8.18 (m, 2H), 7.49 (m, 3H), 3.94 (s, 3H), 3.85 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.6, 160.1, 157.8, 156.8, 151.5, 139.9, 131.7, 129.9, 128.9, 128.8, 109.1, 100.7, 95.3, 61.2, 60.3.

6,8-diiodo-5-hydroxy-3,7-dimethoxyflavone (**61**) : pale yellow solid (17 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 13.61 (s, 1H), 8.32 (m, 2H), 7.61 (m, 3H), 4.09 (s, 3H), 3.96 (s, 3H). ^{13}C NMR (101 MHz, $CDCl_3$) δ 178.9, 157.8, 157.7, 155.2, 154.9, 139.9, 132.0, 129.5, 128.9, 128.7, 108.5, 68.1, 67.9, 63.3, 60.4.

5,7,2′-trimethoxyflavone (**62**): pale yellow solid (88 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.87 (d, *J* = 7.9 Hz, 2H), 7.45 (t, *J* = 7.6 Hz, 2H), 7.02 (s, 1H), 6.54 (d, *J* = 2.3 Hz, 1H), 6.36 (d, *J* = 2.3 Hz, 1H), 4.01 – 3.85 (m, 9H).

5,7,2′,4′-tetramethoxyflavone (**63**): pale brown solid (92 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.85 (d, *J* = 8.7 Hz, 1H), 6.61 (dd, *J* = 8.8, 2.4 Hz, 2H), 6.53 (d, *J* = 2.6 Hz, 2H), 6.36 (d, *J* = 2.3 Hz, 1H), 3.91 (dd, *J* = 19.0, 8.5 Hz, 12H).

5,7,2′,5′-tetramethoxyflavone (**64**): pale brown solid (87 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.44 (d, *J* = 3.2 Hz, 2H), 6.99 (m, 2H), 6.57 (d, *J* = 2.3 Hz, 1H), 6.38 (d, *J* = 2.3 Hz, 1H), 4.19 – 3.75 (m, 12H).

5,7,2′,3′,4′-tetramethoxyflavone (**65**): pale brown solid (91 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.48 (d, *J* = 8.9 Hz, 1H), 6.77 (s, 1H), 6.71 (d, *J* = 8.9 Hz, 1H), 6.49 (d, *J* = 2.4 Hz, 1H), 6.35 (d, *J* = 15.5 Hz, 1H), 4.12 – 3.70 (m, 15H).

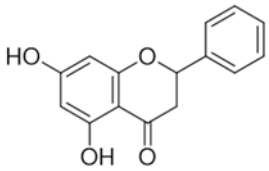
5,7,2′,4′,6′-tetramethoxyflavone (**66**): pale brown solid (78 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.49 (s, 1H), 6.38 (d, *J* = 2.2 Hz, 1H), 6.28 (d, *J* = 2.2 Hz, 1H), 6.11 (s, 2H), 3.83 (s, 3H), 3.81 (s, 3H), 3.77 (s, 3H), 3.64 (s, 6H).

5,7,3′,4′,5′-pentamethoxyflavone (**67**): brown solid (86 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 7.10 (s, 2H), 6.90 (s, 1H), 6.61 (d, *J* = 2.3 Hz, 1H), 6.40 (d, *J* = 2.2 Hz, 1H), 4.17 – 3.77 (m, 15H).

2.6 Synthesis of flavanone derivatives

2.6.1 Synthesis of pinocembrin

The solution of pinostrobin (**20**) (0.27 g, 1 mmol) and iodohexane (2.12 g, 10 mmol) in DMF was refluxed for 24 hr under N_2 atmosphere. Then the mixture was poured into water (20 mL) and extracted with EtOAc (x3). The organic layer was washed with $Na_2S_2O_3$ and brine, solvent was removed by evaporation. The compound was purified by silica gel column [42].

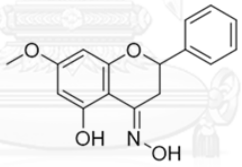


68

Pinocembrin (**68**): pale yellow solid (0.18 g, 72 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.04 (s, 1H), 7.44 (m, 2H), 7.41 (m, 3H), 6.00 (d, *J* = 1.0 Hz, 2H), 5.42 (dd, *J* = 13.1, 3.1 Hz, 1H), 3.09 (dd, *J* = 17.2, 13.0 Hz, 1H), 2.82 (dd, *J* = 17.2, 3.1 Hz, 1H).

2.6.2 Synthesis of pinostrobin oxime

Pinostrobin (**20**) (0.27 g, 1 mmol) was treated with hydroxylamine hydrochloride (77 mg, 1.1 mmol) dissolved in EtOH. The mixture was treated with $NaHCO_3$ (0.1 g, 1.1 mmol). The reaction was carried out at 60 °C for 4 hr. The mixture was treated with HCl and H_2O. The product was purified by silica gel column [43].



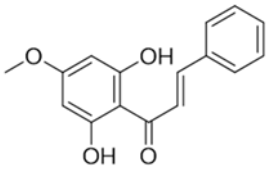
69

Pinostrobin oxime (**69**): pale yellow solid (0.07 g, 26 %), ^{1}H NMR (400 MHz, DMSO-d_6) δ 11.43 (s, 1H), 11.30 (s, 1H), 7.48 (m, 2H), 7.38 (m, 3H), 6.07 (q, *J* = 2.4 Hz, 2H), 5.19 (dd, *J* = 11.5, 3.1 Hz, 1H), 3.69 (s, 3H), 3.34 (dd, *J* = 17.1, 3.3 Hz, 1H), 2.81 (dd, *J* = 17.1, 11.6 Hz, 1H).

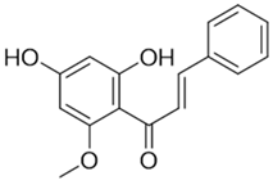
2.7 Synthesis of chalcone derivatives from flavanone

4 M KOH cooled to 0 °C in an ice bath was added to a solution of starting flavanone (1 mmol) in EtOH and then the reaction mixture was kept at room temperature for 30 min. The mixture was poured into ice-water (10 mL), adjusted to

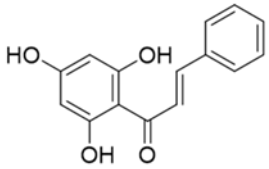
pH 3-4 with 1M HCl, and then extracted with EtOAc. The organic layer was washed with water and brine, dried over anhydrous Na_2SO_4. After evaporation, the residue was purified by silica gel column [14].



70



71



72

Pinostrobin chalcone (**70**): orange solid (65 %), ^{1}H NMR (400 MHz, $CDCl_3$) δ 12.04 (s, 1H), 8.05 (d, J = 15.6 Hz, 1H), 7.84 (d, J = 15.6 Hz, 1H), 7.64 (dd, J = 6.4, 3.2 Hz, 2H), 7.45 (m, 3H), 6.10 (d, J = 3.5 Hz, 1H), 6.01 (s, 1H), 3.84 (s, 3H).

Alpinetin chalcone (**71**): yellow solid (25 %), ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.51 (s, 1H), 10.56 (s, 1H), 8.09 (d, *J* = 15.7 Hz, 1H), 7.68 (d, *J* = 15.7 Hz, 1H), 7.66 (m, 2H), 7.43 (m, 3H), 5.83 (s, 2H).

Pinocembrin chalcone (**72**): yellow solid (21 %), ^{1}H NMR (400 MHz, DMSO-d_6) δ 12.47 (s, 2H), 10.50 (s, 1H), 8.10 (d, *J* = 15.7 Hz, 1H), 7.69 (d, *J* = 15.7 Hz, 1H), 7.66 (m, 2H), 7.43 (m, 3H), 5.83 (s, 2H).

2.8 Biological activity study

2.8.1 Anti-bacterial activity

2.8.1.1 Preliminary screening test of antibacterial activity by diffusion method

The compounds were tested against bacteria pathogens: *Propionibacterium acnes* (KCCM41747), *Staphylococcus aureus* (ATCC25923), *Streptococcus sobrinus* (KCCM11898), *Streptococcus mutans* (ATCC25175), and *Salmonella typhi* (ATCC442).

Nutrient broth was inoculated with the test organisms and incubated at 37 °C for 24 hr then 0.6 mL of the broth culture of the test organism was added to 60 mL of molten agar which has been cooled to 45 °C and mixed well and poured into a sterile Petri dish. The agar was allowed to set and harden, and required numbers of holes were cut using a sterile cork borer. The agar plugs were removed. After that, the bacterial inoculum was uniformly spread using sterile cotton swab on a sterile petri dish nutrient agar. The samples were prepared at the concentration of 1 mM and put it into the well. The plates were incubated at 37 °C for 24 hr. Antibacterial activity was evaluated by measuring the diameter in mm of the inhibition zone around the disc [44].

2.8.1.2 Determination of minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC)

Six various concentrations were used for each compound. To all wells were added 50 µL of nutrient broth (NB) and the serial dilution was performed using a multichannel pipette. Tips were discarded after using such that each well had 50 µL of the compounds in serially descending concentrations. After that, each well was added 40 µL NB and bacterial suspension 10 µL (1 to $2x10^8$ CFU/mL obtained from the 0.5 McFarland standards) was added by pipette to each well. The plates were prepared in triplicate and incubated at 37 °C for 18-24 hr. The colorimetric assay, 10 µL of 0.01% resazurin as oxidation-reduction indicator was added into each well to give blue color, then left for 10 min. The MIC was the lowest concentration of well which still had blue color. 10 µL of blue color solution were taken to put into the new plate that containing NB. Then, the plates were incubated at 37 °C for 18-24 hr. The MBC was the lowest concentration of plate that bacteria did not growth. The test was performed and concurrently with commercial antibiotic (chloramphenicol) as a positive control [45].

2.8.1.3 Determination of combined activity using checkerboard method

To all wells were added 50 µL of nutrient broth (NB) and the serial dilution was performed using a multichannel pipette. The first compound of the combination was serially diluted along the ordinate, while the second compound was dilute along the abscissa. After that, each well was bacterial suspension 10 µL (1 to $2x10^8$ CFU/mL obtained from the 0.5 McFarland standards) and 40 µL of nutrient broth. The plates were incubated at 37 ℃ for 18-24 hr. The synergistic effect has been defined as the MIC of both compounds in combination compared with each use alone, measuring the fractional inhibitory concentration index (FICI) [46].

2.8.2 Anti-tyrosinase activity

The sample (60 µL) was placed in a 96-well plate and 30 µL of mushroom tyrosinase (333 U/mL in phosphate buffer, 50 mM, pH 6.5) and 110 µL of substrates (2 mM L-tyrosine or 2 mM L-DOPA) were added. After incubation at 37 ℃ for 30 min, the absorbance was measured at 510 nm using a microplate reader. The tyrosinase activity was expressed as the half maximal inhibitory concentration (IC_{50}), which is the concentration of the samples producing 50% inhibition [35].

2.8.3 Cell culture

Murine melanoma B16-F0 cells were cultured in Dulbecco's modified Eagle medium (DMEM) supplemented with 10 % fetal bovine serum, 100,000 unit/L penicillin, and 100 mg/L streptomycin. Cells were cultured at 37 ℃ in humidified atmosphere of 5 % CO_2 [35].

2.8.4 Measurement of cellular melanin content

Confluent cultures of B16 melanoma cells were rinsed in phosphate-buffered saline (PBS) and removed using 0.25 % trypsin/EDTA. The cells were placed into a 24-well plate (5.0×10^4 cells/well) and allowed to adhere at 37 °C for 24 hr. Sample compounds were added and the cells incubated for 72 hr. Following incubation, cell medium was collected and 200 µL were loaded into a 96-well plate. The absorbance of the medium was measured at 510 nm by using a microplate reader and used as a measurement of extracellular melanin contents. The cells were washed with PBS following lysis in 600 µL of 1 M NaOH by heating at 100 °C for 30 min. A portion of the resulting lysate (250 µL) was loaded into a 96-well microplate, and the absorbance was measured at 405 nm using a microplate reader. Measured absorbance was used as an index of intracellular melanin contents [35].

2.8.5 Cell viability

The cell viability was determined using the microculture tetrazolium technique (MTT). Cultures were initiated in 24-well plates at 5.0×10^4 cells per well. After incubation with compounds, 50 µL of MTT reagent (5 mg/mL of 3-[4,5-dimethyl-2-thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide in PBS) was added to each well. The plates were incubated in a humidified atmosphere of 5 % CO_2 at 37 °C for 4 hr. After the medium was removed, 1.0 mL of *iso*propanol (containing 0.04 N HCl) was added to each well, and a 150 µL sample was added to a 96-well plate. Absorbance was measured at 590 nm by using a microplate reader. Each experiment was repeated twice [35].

CHAPTER III

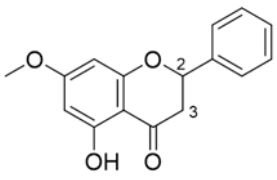
RESULTS AND DISCUSSION

Boesenbergia rotunda (L.) Mansf and *Kaempferia parviflora* Wall. ex Baker. are Thai herbs used for medicinal purposes. The main aim of this research is to explore the biological activities of the isolated compounds from these two plants. In addition, certain synthesized derivatives were conducted to investigate the structure-activity relationship (SAR). The examined biological activities included anti-bacterial activity against *Propionibacterium acnes* (KCCM41747), *Staphylococcus aureus* (ATCC25923), *Streptococcus sobrinus* (KCCM11898), *Streptococcus mutans* (ATCC25175), and *Salmonella typhi* (ATCC442), anti-tyrosinase activity, and melanogenesis activity.

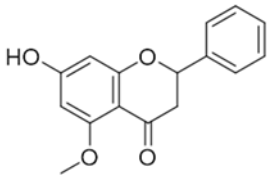
3.1 Isolation of flavones and flavanones from *Boesenbergia rotunda* and *Kaempferia parviflora*.

3.1.1 Isolation of flavanones from *B. rotunda*

The CH_2Cl_2 extract from the rhizomes of *B. rotunda* was subjected to silica gel quick column leading to the isolation of pinostrobin (**20**) as yellow solid (51 %) and alpinetin (**43**) as pale yellow solid (8 %). The ^{1}H NMR spectra of flavanones generally display a doublet-doublet signal at δ 5.45 ppm for the proton at H-2 and two doublet-doublet signals at δ 2.85 and 3.12 ppm for protons at H-3.



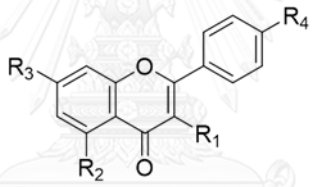
20



43

3.1.2 Isolation of flavones from *K. parviflora*

The separation of the CH_2Cl_2 extract from the rhizomes of *K. parviflora* by silica gel column yielding three major flavones: 5-hydroxy-7-methoxyflavone (**44**) as yellow solid (10 %), 5-hydroxy-3,7-dimethoxyflavone (**45**) as yellow solid (29 %), 5-hydroxy-3,7,4′-trimethoxyflavone (**46**) as pale brown solid (9 %) and two minor flavones: 5-hydroxy-7,4′-dimethoxyflavone (**47**) as yellow solid (0.01 %) and 3,5,7-trimethoxyflavone (**48**) as pale green solid (0.02 %). These two minor compounds were obtained in small amount, so no further biological activity study has been carried out. The ^{1}H NMR spectra of flavones generally show a signal around δ 6.58-6.69 ppm for the proton at H-3. The aromatic protons were observed around δ 8.16-7.02 ppm.



44: R_1=R_4=H, R_2=OH, R_3=OCH_3
45: R_1=R_3=OCH_3, R_2=OH, R_4=H
46: R_1=R_3=R_4=OCH_3, R_2=OH
47: R_1=H, R_2=OH, R_3=R_4=OCH_3
48: R_1=R_2=R_3=OCH_3, R_4=H

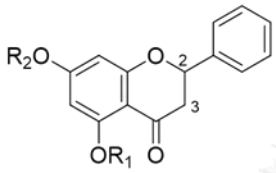
3.2 Anti-bacterial activity of isolated flavonoids and their derivatives

The relationship between anti-bacterial activities and structures of flavonoids was observed. Twenty-three tested flavonoids including five isolated from plants and eighteen related compounds were investigated for their anti-bacterial activities against *P. acnes* (KCCM41747), *S. aureus* (ATCC25923), *S. sobrinus* (KCCM11898), *S. mutans* (ATCC25175), and *S. typhi* (ATCC442) by disc diffusion method. The data of

anti-bacterial activity was presented as zone of inhibition (mm). All tested flavonoids could be classified into four subgroups as:

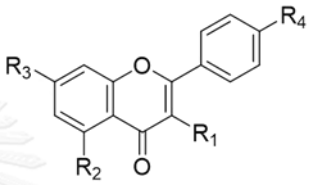
3.2.1 Anti-bacterial activity of natural compounds from *B. rotunda* and *K. parviflora*

Five natural compounds (**20**, **43**-**46**) were investigated for their anti-bacterial activity. The inhibition zones of these compounds are shown in **Table 3.1**.



20: R_1= H, R_2= CH_3

43: R_1= CH_3, R_2= H



44: R_1=R_4=H, R_2=OH, R_3=OCH_3

45: R_1=R_3=OCH_3, R_2=OH, R_4=H

46: R_1=R_3=R_4=OCH_3, R_2=OH

Table 3.1 Anti-bacterial activity of natural compounds from *B. rotunda* and *K. parviflora*

Entry	Cpds	Inhibition zone (mm)				
		P. acnes KCCM41747	*S. aureus* ATCC25923	*S. sobrinus* KCCM11898	*S. mutans* ATCC25175	*S. typhi* ATCC442
1	**20**	9.00 ±0.00	10.00±0.82	10.67±0.94	10.33±0.47	11.00±0.00
2	**43**	9.33±0.47	11.67±0.47	11.33±0.47	11.67±0.47	11.33±0.47
3	**44**	8.67±0.47	10.67±0.47	9.67±0.47	10.67±0.94	8.00±0.00
4	**45**	8.00±0.00	9.00±0.00	11.00±0.82	11.67±0.47	11.00±0.82
5	**46**	9.00±0.00	9.00±0.00	11.00±0.00	9.33±0.47	9.67±0.47
6	C*	25.00±0.00	26.00±0.82	26.00±0.00	29.33±0.47	12.00±0.00

*C: chloramphenicol (positive control)

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

Flavanones (**20** and **43**) from *B. rotunda* and flavones (**44**-**46**) from *K. parviflora* showed moderate to good activity against five bacteria. Pinostrobin (**20**, 5-hydroxy-7-methoxyflavanone) and 5-hydroxy-7-methoxyflavone (**44**) were similar in structure except the double bond between C-2 and C-3, showed similar activities with moderate activity against *P. acnes* and good activity against *S. aureus* and *S. mutans*. For other bacteria, compound **20** exhibited good activity while compound **44** showed moderately active against *S. sobrinus* and weak activity against *S. typhi*. Pinostrobin (**20**) and 5-hydroxy-7-methoxyflavone (**44**) had been recently reported to possess anti-bacterial activity. Compound **20** showed good activity against *Helicobacter pylori* (gram-negative bacteria) activity in the same range as that of drug currently used in the treatment of peptic ulcer [26]. Compound **44** exhibited moderate activity against *Bacillus subtilis* [47]. In recent years, the related compounds of these flavonoids have been studied on their biological activities including antimicrobial, antioxidant and anti-inflammatory. Nevertheless, no report of the anti-bacterial activity against these bacteria was addressed. Therefore, next examination was focused on these related compounds and their anti-bacterial activity.

3.2.2 Anti-bacterial activity of related compounds

In this series, the synthesized compounds (**49**, **68**-**72**) which related to natural flavonoids were evaluated for their anti-bacterial activity compared with starting flavonoids: chrysin and pinostrobin (**20**). 5,7-Dimethoxyflavone (**49**) or 5,7-dimethoxychrysin was derived by methylation of hydroxy groups at C-5 and C-7. The ^{1}H NMR spectrum displayed two methoxy groups at δ 3.88 ppm. Pinocembrin (**68**) and pinostrobin oxime (**69**) were related to pinostrobin (**20**) by demethylation with iodohexane [42] and treating with hydroxylamine hydrochloride [43], respectively. Comparing the ^{1}H NMR spectrum of pinocembrin (**68**) with that of pinostrobin (**20**),

there was no signal belonging to the methoxy group at C-7 (δ 3.84 ppm) in the spectrum of the former. The ^{1}H NMR spectrum of pinostrobin oxime (**69**) displayed significant protons of C-3 at δ 3.34 and 2.81 ppm. The chalcone derivatives (**33**-**35**) were prepared by treating flavanones (**20**, **43** and **68**) with KOH [14]. Two olefinic-proton signals of the products were observed at δ 7.43-7.84 ppm.

The appearance and % yield of synthesized related compounds are displayed in **Table 3.2**. The ^{1}H NMR chemical shift assignment was accumulated as presented in **Table 3.3**.

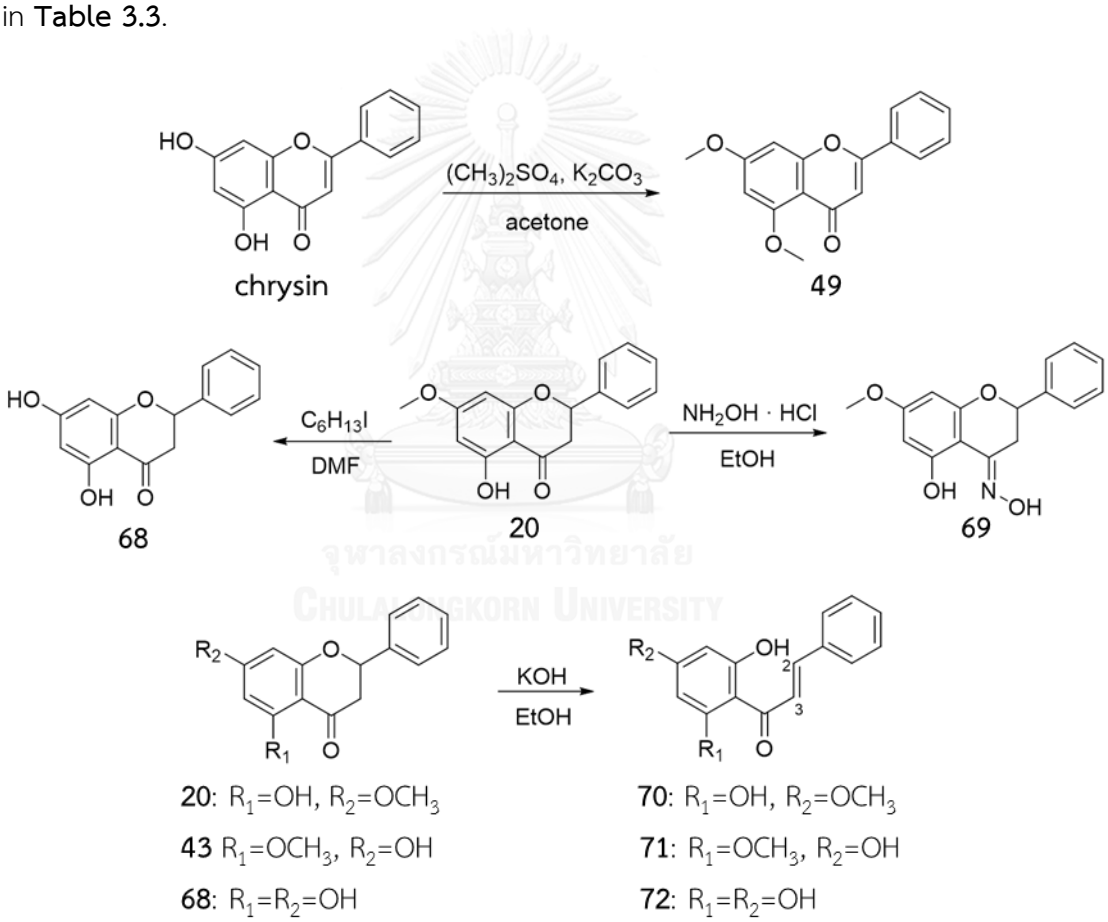


Table 3.2 The appearance and % yield of synthesized related compounds

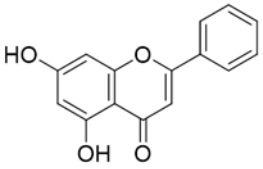
Entry	Cpds	Appearance	% yield
1	**49**	yellow solid	58
2	**68**	pale yellow solid	72
3	**69**	pale yellow solid	26
4	**70**	orange solid	65
5	**71**	yellow solid	25
6	**72**	yellow solid	21

Table 3.3 The chemical shift assignments of ^{1}H and ^{13}C NMR spectra of compounds **49, 68-72**

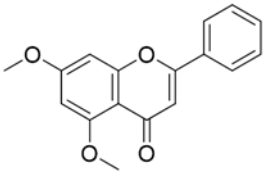
Position	**49**[a]	**68**[a]	**69**[b]	**70**[a]	**71**[b]	**72**[b]
2	-	5.42 (dd, *J* = 13.1, 3.1 Hz, 1H)	5.19 (dd, *J* = 11.5, 3.1 Hz, 1H)	8.05 (d, J = 15.6 Hz, 1H)	8.09 (d, *J* = 15.7 Hz, 1H)	8.10 (d, *J* = 15.7 Hz, 1H)
3	6.67 (s, 1H)	3.09 (dd, *J* = 17.2, 13.0 Hz, 1H), 2.82 (dd, *J* = 17.2, 3.1 Hz, 1H)	3.34 (dd, *J* = 17.1, 3.3 Hz, 1H), 2.81 (dd, *J* = 17.1, 11.6 Hz, 1H)	7.84 (d, J = 15.6 Hz, 1H)	7.68 (d, *J* = 15.7 Hz, 1H)	7.69 (d, *J* = 15.7 Hz, 1H)
6	6.38 (d, *J* = 2.3 Hz, 1H)	6.00 (d, *J* = 1.0 Hz, 2H)	6.07 (q, *J* = 2.4 Hz, 2H)	6.01 (s, 1H)	5.83 (s, 2H)	5.83 (s, 2H)
8	6.50 (d, *J* = 2.3 Hz, 1H)	6.00 (d, *J* = 1.0 Hz, 2H)	6.07 (q, *J* = 2.4 Hz, 2H)	6.10 (d, J = 3.5 Hz, 1H)	5.83 (s, 2H)	5.83 (s, 2H)
2´	7.88 (m, 2H)	7.44 (m, 2H)	7.48 (m, 2H)	7.64 (dd, J = 6.4, 3.2 Hz, 2H)	7.66 (m, 2H)	7.66 (m, 2H)
3´	7.53 (m, 3H)	7.41 (m, 3H)	7.38 (m, 2H)	7.45 (m, 3H)	7.43 (m, 3H)	7.43 (m, 3H)
4´	7.53 (m, 3H)	7.41 (m, 3H)	7.38 (m, 2H)	7.45 (m, 3H)	7.43 (m, 3H)	7.43 (m, 3H)
5´	7.53 (m, 3H)	7.41 (m, 3H)	7.38 (m, 2H)	7.45 (m, 3H)	7.43 (m, 3H)	7.43 (m, 3H)
6´	7.88 (m, 2H)	7.44 (m, 2H)	7.48 (m, 2H)	7.64 (dd, J = 6.4, 3.2 Hz, 2H)	7.66 (m, 2H)	7.66 (m, 2H)
5-OMe	3.88 (s, 6H)	-	-	-	-	-
7-OMe	3.88 (s, 6H)	-	3.69 (s, 3H)	3.84 (s, 3H)	-	-

The spectra were recorded in [a]$CDCl_3$ and [b]DMSO-d_6

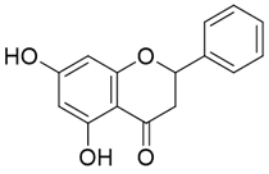
Seven compounds including chrysin, 5,7-dimethoxyflavone (**49**) and five related compounds (**68-72**) of pinostrobin (**20**) were evaluated for anti-bacterial activity. The inhibition zone of these compounds was tabulated in **Table 3.4**.



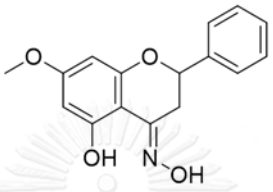
chrysin



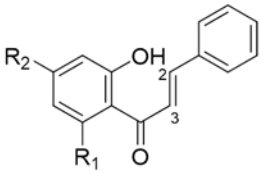
49



68



69



70: R_1=OH, R_2=OCH_3

71: R_1=OCH_3, R_2=OH

72: R_1=R_2=OH

Table 3.4 Anti-bacterial activity of related compounds

Entry	Cpds	Inhibition zone (mm)				
		P. acnes KCCM41747	*S. aureus* ATCC25923	*S. sobrinus* KCCM11898	*S. mutans* ATCC25175	*S. typhi* ATCC442
1	**chrysin**	10.33±0.47	11.00±0.82	13.67±0.94	15.33±0.47	11.67±0.47
2	**49**	9.33±0.47	9.33±0.94	12.33±0.47	11.33±0.47	8.00±0.00
3	**68**	8.33±0.94	9.33±0.47	8.00±1.41	8.00±0.82	7.00±1.41
4	**69**	11.00±0.82	11.33±1.25	15.33±0.47	9.67±0.94	8.00±0.82
5	**70**	10.00±0.52	9.33±0.94	12.33±1.70	9.33±0.47	10.67±1.70
6	**71**	7.67±0.47	9.00±0.00	10.33±0.94	8.33±0.47	9.33±0.47
7	**72**	8.00±0.00	9.00±0.82	10.33±0.94	10.33±0.94	8.33±0.47
8	**C***	25.00±0.00	26.00±0.82	26.00±0.00	29.33±0.47	12.00±0.00

*C: chloramphenicol (positive control)

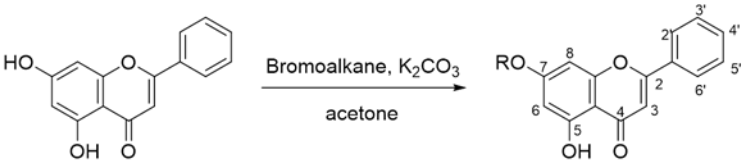
Key to the inhibition zone activity (mm): inhibition zone >15.0: excellent, 13.1-15.0: very good, 10.1-13.0: good, 8.1-10.0: moderate, 6.1-8.0: weak, ≤6.0: no activity

Comparing between chrysin and 5,7-dimethoxyflavone (**49**), chrysin exhibited higher activity than compound **49**. It showed good to excellent activity against five bacteria, especially *S. mutans*, with inhibition zone about 15 mm while compound **49** showed weak activity against *S. typhi*. For these flavones, the hydroxy groups at C-5 and C-7 may play an important role for this anti-bacterial activity.

Considering the activity of compounds **68**-**72**, pinocembrin (**68**) and pinostrobin oxime (**69**) were related to pinostrobin (**20**). Pinocembrin (**68**) showed slightly active than pinostrobin (**20**) while pinostrobin oxime (**69**) exhibited excellent activity against *S. sobrinus*. Chalcone derivatives exhibited similar potent activity. Most of them showed moderate to good activity against five bacteria except alpinetin chalcone (**71**) and pinocembrin chalcone (**72**) exhibited weak activity against *P. acnes*. In 2008, Ávila and co-workers found that the C-4′ hydroxyl group, a C-4 oxygenated substituent or a C-3′ isoprenoid side chain revealed anti-bacterial activity against gram-positive bacteria, while the C-2 hydroxyl group might have importance for the stability of the molecule [48].

3.2.3 Anti-bacterial activity of ether derivatives of chrysin

Five compounds were prepared by alkylation of chrysin using bromoalkane. The appearance and % yield of synthesized compounds are shown in **Table 3.5**. The ^{1}H NMR chemical shift assignment was accumulated as presented in **Table 3.6**.



Chrysin

50: R=C_2H_5

28: R=C_4H_9

51: R=C_6H_{13}

29: R=C_8H_{17}

52: R=$C_{12}H_{25}$

Table 3.5 The appearance and % yield of synthesized ether derivatives

Entry	Cpds	Appearance	% yield
1	**50**	yellow solid	40
2	**28**	pale yellow solid	58
3	**51**	pale yellow solid	76
4	**29**	pale yellow solid	51
5	**52**	pale yellow solid	56

Table 3.6 The chemical shift assignments of ^{1}H NMR spectra of compounds **28-29**, **50-52**

Position	**50**	**28**	**51**	**29**	**52**
3	6.66 (s, 1H)	6.67 (s, 1H)	6.67 (s, 1H)	6.67 (s, 1H)	6.67 (s, 1H)
6	6.49 (d, *J* = 2.2 Hz, 1H)	6.50 (s, 1H)	6.50 (s, 1H)	6.49 (d, *J* = 2.3 Hz, 1H)	6.50 (d, *J* = 2.2 Hz, 1H)
8	6.36 (d, *J* = 2.2 Hz, 1H)	6.37 (d, *J* = 2.2 Hz, 1H)	6.37 (s, 1H)	6.36 (d, *J* = 2.2 Hz, 1H)	6.37 (d, *J* = 2.2 Hz, 1H)
2´	7.88 (dd, *J* = 7.9, 1.9 Hz, 2H)	7.89 (dd, *J* = 7.7, 2.0 Hz, 2H)	7.89 (d, *J* = 8.3 Hz, 2H)	7.88 (m, 2H)	7.89 (m, 2H)
3´	7.53 (m, 3H)	7.53 (m, 3H)	7.54 (m, 3H)	7.53 (m, 3H)	7.53 (m, 3H)
4´	7.53 (m, 3H)	7.53 (m, 3H)	7.54 (m, 3H)	7.53 (m, 3H)	7.53 (m, 3H)
5´	7.53 (m, 3H)	7.53 (m, 3H)	7.54 (m, 3H)	7.53 (m, 3H)	7.53 (m, 3H)
6´	7.88 (dd, *J* = 7.9, 1.9 Hz, 2H)	7.89 (dd, *J* = 7.7, 2.0 Hz, 2H)	7.89 (d, *J* = 8.3 Hz, 2H)	7.88 (m, 2H)	7.89 (m, 2H)

The chemical shift of the side chain at C-7 of

50: 4.11 (q, *J* = 7.0 Hz, 2H), 1.46 (t, *J* = 7.0 Hz, 3H)

28: 4.04 (t, *J* = 6.5 Hz, 2H), 1.58 (m, 2H), 1.25 (m, 2H), 0.99 (t, *J* = 7.4 Hz, 3H)

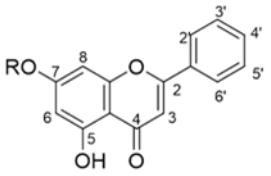
51: 4.03 (m, 2H), 1.81 (m, 2H), 1.39 – 1.31 (m, 6H), 0.91 (m, 6H)

29: 4.03 (t, *J* = 6.6 Hz, 2H), 1.81 (m, 2H), 1.45 (d, *J* = 7.6 Hz, 2H), 1.39 – 1.24 (m, 8H), 0.89 (m, 3H).

52: 4.03 (t, *J* = 6.5 Hz, 2H), 1.89 – 1.25 (m, 20H), 0.87 (m, 3H)

The spectra were recorded in $CDCl_3$

All synthesized compounds were characterized by ^{1}H NMR. The ^{1}H NMR spectra of these compounds showed aromatic protons around δ 7.47-7.94 ppm. The protons of alkoxyl group at C-7 were displayed at δ 0.87-4.11 ppm. These compounds were investigated their anti-bacterial activities. The inhibition zones are tabulated in **Table 3.7**.



50: R=C_2H_5
28: R=C_4H_9
51: R=C_6H_{13}
29: R=C_8H_{17}
52: R=$C_{12}H_{25}$

Table 3.7 Anti-bacterial activity of synthesized ether derivatives

Entry	Cpds	Inhibition zone (mm)				
		P. acnes KCCM41747	*S. aureus* ATCC25923	*S. sobrinus* KCCM11898	*S. mutans* ATCC25175	*S. typhi* ATCC442
1	50	7.00±0.00	6.00±0.00	8.67±0.47	7.00±0.00	6.00±0.00
2	28	8.67±0.47	9.67±0.47	10.67±0.47	9.67±0.47	10.67±0.47
3	51	9.00±0.00	9.33±0.94	11.67±0.47	13.00±0.00	9.00±0.00
4	29	9.00±0.00	10.33±0.47	13.33±0.47	11.33±0.47	8.67±0.94
5	52	9.67±0.47	9.00±0.00	8.00±0.00	10.33±0.47	8.67±0.47
6	C*	25.00±0.00	26.00±0.82	26.00±0.00	29.33±0.47	12.00±0.00

*C: Chloramphenicol (positive control)

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

The chain length of alkoxyl group was varied by increasing the length of carbon atom at 7-position as -OC_2H_5, -OC_4H_9, -OC_6H_{13}, -OC_8H_{17} and -$OC_{12}H_{25}$. There was however no clear trend in structure-activity relationship. For *P. acnes*, they showed weak to moderate activity while compound 52 exhibited the highest activity. For *S. aureus*, compound 29 revealed the highest activity and compound 50 containing 2 carbon atoms in side chain showed no activity. For *S. sorbinus*,

compounds **28**, **29** and **51** indicated moderate to good activity with inhibition zone about 10-13 mm. Compounds **29**, **51** and **52** displayed good activity against *S. mutans* with inhibition zone 10-13 mm. On the other hand, they exhibited moderate activity against *S. typhi* with inhibition zone 8-9 mm. Therefore, the effect of length of carbon atom at 7-position was specific with bacteria stains. In 1999, alkyl derivatives of chrysin were synthesized and examined their effect on glucose blood level. Compounds with propyl, butyl, octyl and tolyl groups expressed hypoglycemic effect on diabetic mice and did not show toxicity with test animal at the maximum dose [30]. However, these compounds did not reveal the anti-bacterial activity against those bacteria.

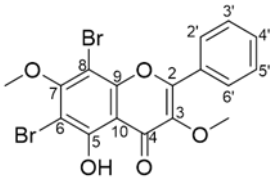
3.2.4 Anti-bacterial activity of halogenated flavones

Six halogenated flavones were synthesized using NaBr/oxone [38] or I_2 [39]. The appearance and % yield of these compounds are displayed in **Table 3.8**.

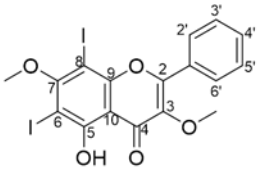
Table 3.8 The appearance and % yield of halogenated flavones

Entry	Cpds	Appearance	% yield
1	**33**	pale green solid	68
2	**34**	pale yellow solid	77
3	**58**	pale yellow solid	38
4	**59**	pale yellow solid	26
5	**60**	pale yellow solid	25
6	**61**	pale yellow solid	17

All synthesized compounds were characterized by ^{1}H NMR. The aromatic protons of ring A at C-6 and C-8 of starting flavone (δ 6.3-6.5 ppm) were disappeared. Two halogenated flavones (**60** and **61**) were disclosed to be new compounds. Therefore, the structures of these compounds were characterized using various spectroscopic techniques including ^{1}H and ^{13}C NMR.



60



61

The ^{1}H NMR spectra of compounds **60** and **61** showed almost the same pattern as 5-hydroxy-3,7-dimethoxyflavone (**45**), except for the disappearance of aromatic protons at C-6 and C-8 of starting flavone (δ 6.39-6.48 ppm). The ^{13}C NMR spectra showed a signal belonging to a carbonyl group (C-4) at 178.6 ppm. The aromatic carbons were observed in the range of 128.7-132.0 ppm. The carbon signals at C-6 and C-8 of compound **60** were detected at 100.7 and 95.3 ppm, while the signals of C-6 and C-8 of compound **61** were visualized around 71 ppm. The ^{1}H and ^{13}C NMR spectra of compounds **60** and **61** are displayed in **Figures 3.1-3.4**.

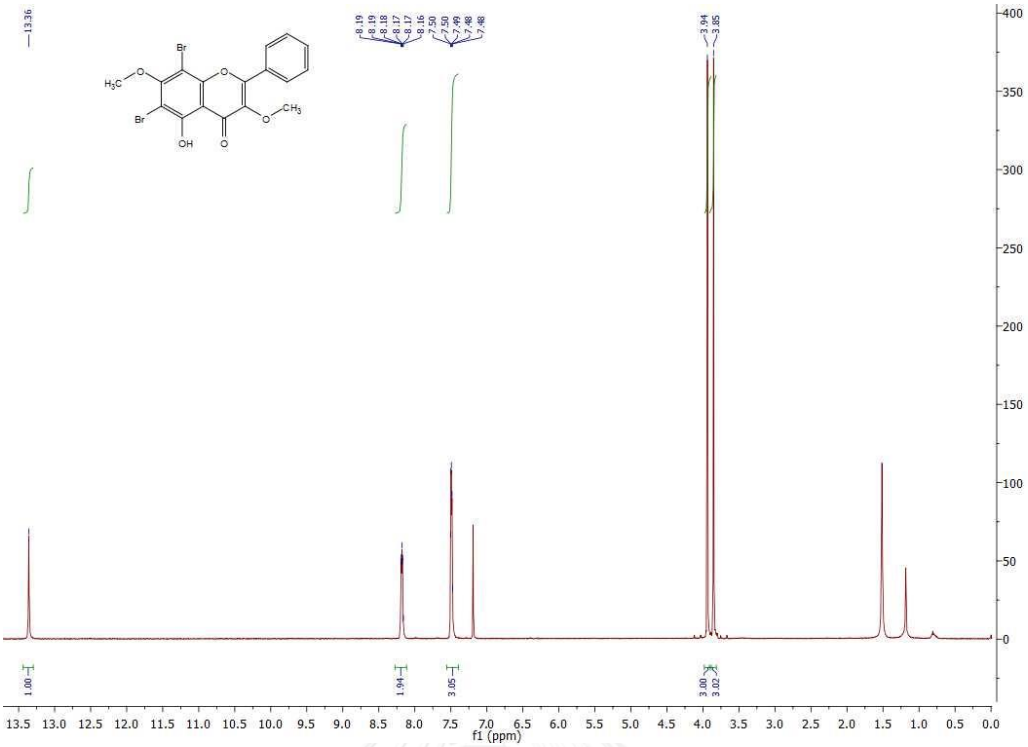


Figure 3.1 The ^{1}H NMR (400 MHz) spectrum of compound **60** ($CDCl_3$)

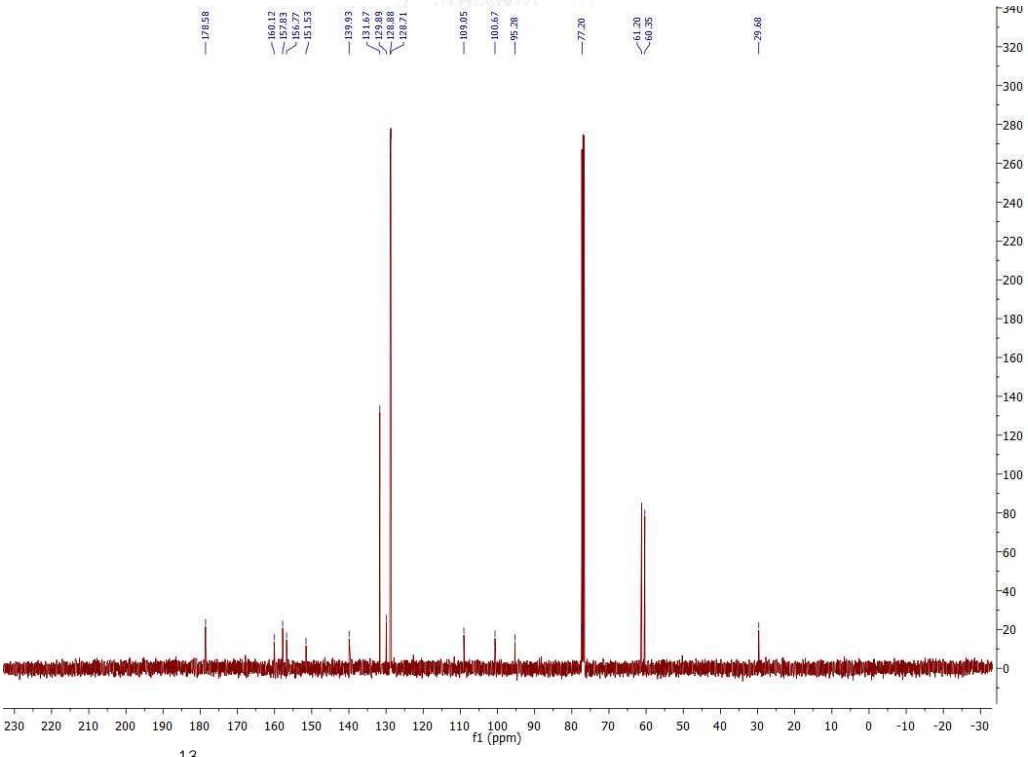


Figure 3.2 The ^{13}C NMR (100 MHz) spectrum of compound **60** ($CDCl_3$)

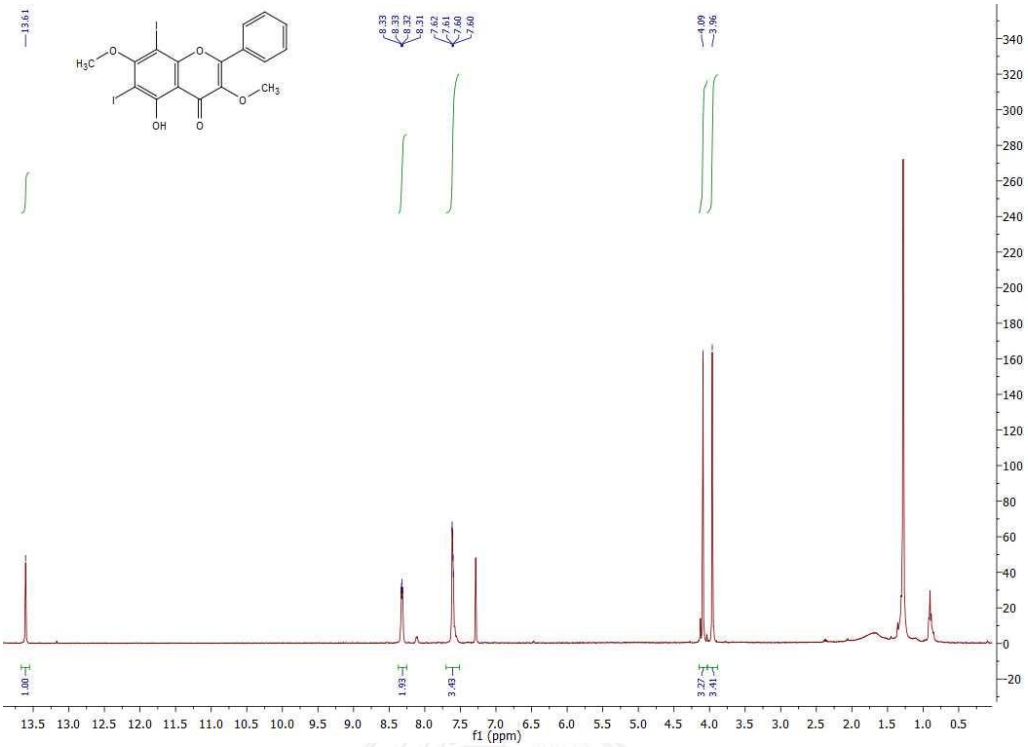


Figure 3.3 The ^{1}H NMR (400 MHz) spectrum of compound **61** ($CDCl_3$)

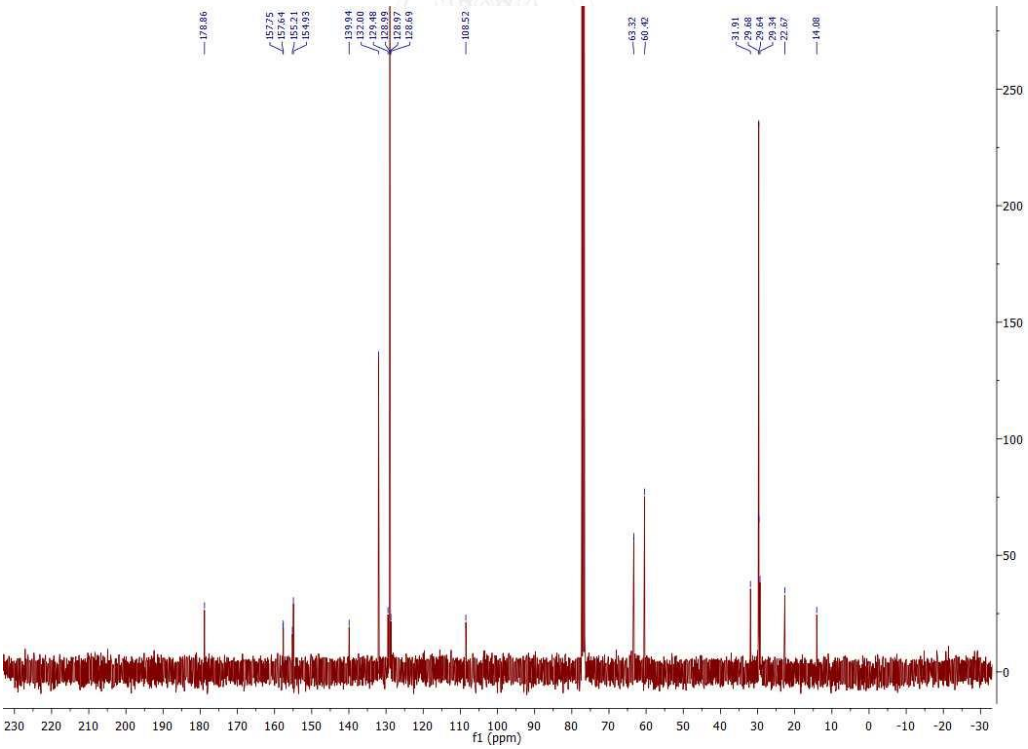


Figure 3.4 The ^{13}C NMR (100 MHz) spectrum of compound **61** ($CDCl_3$)

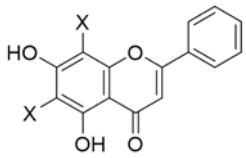
The chemical shift assignments of ^{1}H and ^{13}C NMR spectra of compounds **60** and **61** are displayed in **Table 3.9**.

Table 3.9 The chemical shift assignments of ^{1}H and ^{13}C NMR spectra of compounds **60** and **61**

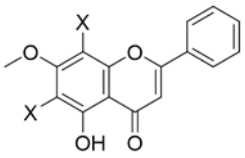
Position	Compound **60**		Compound **61**	
	δ_H	δ_C	δ_H	δ_C
2	-	156.8	-	155.2
3	-	139.9	-	139.9
4	-	178.6	-	178.9
5	-	157.8	-	157.7
6	-	100.7	-	68.0
7	-	160.1	-	157.8
8	-	95.3	-	67.9
9	-	151.5	-	154.9
10	-	109.1	-	108.5
1´	-	131.7	-	132.0
2´	8.18 (m, 2H)	128.8	8.32 (m, 2H)	128.7
3´	7.49 (m, 3H)	129.9	7.61 (m, 3H)	129.5
4´	7.49 (m, 3H)	128.9	7.61 (m, 3H)	128.9
5´	7.49 (m, 3H)	129.9	7.61 (m, 3H)	129.5
6´	8.18 (m, 2H)	128.8	8.32 (m, 2H)	128.7
3-OMe	3.85 (s, 3H)	60.3	3.96 (s, 3H)	60.4
8-OMe	3.94 (s, 3H)	61.2	4.09 (s, 3H)	63.3
6-OH	13.36 (s, 1H)	-	13.61 (s, 1H)	-

The spectra were recorded in $CDCl_3$

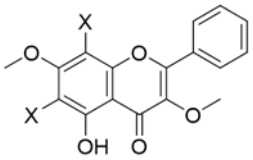
Six synthesized halogenated flavones were subjected to anti-bacterial test and the inhibition zones are tabulated in **Table 3.10**.



33 X= Br
34 X= I



58 X= Br
59 X= I



60 X= Br
61 X= I

Table 3.10 Anti-bacterial activity of synthesized halogenated flavones

Entry	Cpds	Inhibition zone (mm)				
		P. acnes KCCM41747	*S. aureus* ATCC25923	*S. sobrinus* KCCM11898	*S. mutans* ATCC25175	*S. typhi* ATCC442
1	**33**	21.00±0.82	20.00±0.82	19.67±0.47	19.67±0.47	19.67±0.47
2	**34**	20.33±0.94	20.67±0.47	18.67±0.47	18.67±1.25	18.33±1.25
3	**58**	8.33±0.23	9.67±1.84	9.00±1.41	8.67±0.62	9.75±1.24
4	**59**	9.33±1.04	8.33±0.47	8.50±0.71	9.33±0.62	10.33±1.32
5	**60**	8.33±0.47	8.33±1.25	8.00±1.41	8.33±0.47	8.33±1.25
6	**61**	8.33±0.47	8.00±0.82	10.33±0.47	9.00±0.82	8.67±1.25
7	C*	25.00±0.00	26.00±0.82	26.00±0.00	29.33±0.47	12.00±0.00

*C: Chloramphenicol (positive control)

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

For the series containing halogen substituents at C-6 and C-8 of flavone derivatives, 6,8-dibromo-5,7-dihydroxyflavone (**33**) and 6,8-diiodo-5,7-dihydroxyflavone (**34**) showed excellent activity against all bacteria, while other compounds exhibited moderate activity. Thus, for flavone, the hydroxy groups at C-5 and C-7 may play an important role for this activity as mentioned in **3.2.2**. Comparing between bromo- and iodo- substituents of 5,7-dihydroxyflavone, compound **33** showed slightly higher anti-bacterial activity than compound **34** except *S. aureus*. This result indicated that bromo- substituent affected to anti-bacterial activity more than iodo- substituent. Furthermore, 6,8-dibromo-5,7-dihydroxyflavone (**33**) and 6,8-

diiodo-5,7-dihydroxyflavone (**34**) have been reported for their biological activities. Compounds **33** and **34** revealed better inhibitory activity against SGC-7901 cancer cells than against HT-29 cells [31] and strong inhibitory activities of PGE_2 production from LPS-induced RAW 264.7 cells [39]. On the contrary, compounds **33** and **34** did not improve any significant positive effect on anti-inflammatory activity using the model of carrageenan induced mice paw edema [38]. Nevertheless, these compounds have not been addressed for the anti-bacterial activity against these tested bacteria.

From the above results, two compounds: 6,8-dibromo-5,7-dihydroxyflavone (**33**) and 6,8-diiodo-5,7-dihydroxyflavone (**34**) were selected to further evaluate minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) using micro dilution method. The results are tabulated in **Table 3.11.**

Table 3.11 The MIC, MBC and MIC index of compounds **33** and **34**

Bacterial strain	Cpds	MIC (μM)	MBC (μM)	MIC index (MBC/MIC)	Indication
P. acnes	**33**	31.25	250	8	Bacteriostatic
KCCM41747	**34**	31.25	250	8	Bacteriostatic
S. aureus	**33**	31.25	250	8	Bacteriostatic
ATCC25923	**34**	31.25	250	8	Bacteriostatic
S. sobrinus	**33**	62.5	250	4	Bactericidal
KCCM11898	**34**	62.5	250	4	Bactericidal
S. mutans	**33**	62.5	250	4	Bactericidal
ATCC25175	**34**	62.5	250	4	Bactericidal
S. typhi	**33**	62.5	250	4	Bactericidal
ATCC442	**34**	62.5	250	4	Bactericidal

MIC was determined as the minimum concentration that inhibited bacterial growth while MBC was the lowest concentration that can kill bacterial. Five bacteria were suppressed by compounds **33** and **34** with MIC as 31.25 μM for *P. acnes* and *S. aureus* and 62.5 μM for *S. sobrinus, S. mutans* and *S. typhi*. The MBC of these compounds against all bacterial was 250 μM.

The MIC index was calculated by MBC/MIC to determine if the compounds possessed bactericidal or bacteriostatic properties [49]. When MIC index is ≤ 4, the compound is bactericidal while the MIC index > 4, the compound is bacteriostatic. For *P. acnes* and *S. aureus*, the MIC index of compounds **33** and **34** were 8. Thus, for these bacteria, both compounds were bacteriostatic agent which inhibited the growth of bacterial at low concentration. Moreover, the MIC index on *S. sobrinus, S. mutans* and *S. typhi* of both compounds were 4. The MIC index revealed that these compounds were bactericidal agent.

3.2.5 The synergistic effects between flavones 33 and 34 and known antibiotics

Nowadays, more pathogenic bacteria have become resistant to known antibiotic agents. The new antibiotics are studied for use in treatment of serious bacterial infections. The combination of two or more antibiotics for enhancing the activity is one of the methods to search for new antibacterial agents. The synergistic effect is defined as the combination exhibits the greater effect compared with the antibiotic alone. The synergistic effect between flavones (**33** and **34**) and four known antibiotics including chloramphenicol, tetracycline, streptomycin and ampicillin were determined using checkerboard method. The results of the combination of antibiotic and flavones (**33** and **34**) are shown in **Table 3.12**

Table 3.12 Antibacterial result from the combinations of antibiotics and flavone (**33** and **34**)

Mix[a]	*P. acne*			*S. aureus*			*S. sobrinus*			*S. mutans*			*S. typhi*		
	MIC		Activity[b]	MIC		Activity[b]	MIC		Activity[b]	MIC		Activity[b]	MIC		Activity[b]
	alone	mix		alone	mix		alone	mix		alone	mix		alone	mix	
33	31.25	0.488	S	31.25	0.488	S	62.5	0.977	S	62.5	0.977	S	62.5	0.977	S
CHMP	15.625	1.953		62.5	7.8125		15.625	1.953		15.625	1.953		15.625	1.953	
33	31.25	0.488	S	31.25	0.488	S	62.5	0.977	S	62.5	0.977	S	62.5	3.906	S
TETRA	7.8125	0.977		3.906	0.488		1.953	0.244		0.976	0.122		0.488	0.122	
33	31.25	0.488	S	31.25	0.488	S	62.5	0.977	S	62.5	0.977	S	62.5	0.977	S
STREP	15.625	0.977		15.625	1.953		3.906	0.244		1.953	0.122		3.906	0.488	
33	31.25	1.953	S	31.25	0.977	S	62.5	0.977	S	62.5	0.977	S	62.5	0.977	S
AMP	62.5	7.813		125	15.625		125	15.625		125	15.625		125	15.625	
34	31.25	0.997	S	31.25	1.953	S	62.5	0.977	S	62.5	1.953	S	62.5	15.625	S
CHMP	15.625	1.953		62.5	3.906		15.625	1.953		15.625	1.953		15.625	1.953	
34	31.25	3.906	S	31.25	0.488	S	62.5	3.906	S	62.5	1.953	S	62.5	3.906	S
TETRA	7.8125	0.977		3.906	0.488		1.953	0.244		0.976	0.122		0.488	0.122	
34	31.25	0.488	S	31.25	3.906	S	62.5	0.977	S	62.5	0.977	S	62.5	1.953	S
STREP	15.625	0.977		15.625	1.953		3.906	0.488		1.953	0.244		3.906	0.488	
34	31.25	0.977	S	31.25	3.906	S	62.5	1.953	S	62.5	1.953	S	62.5	7.8125	S
AMP	62.5	7.813		125	15.625		125	15.625		125	15.625		125	15.625	

[a]CHMP: chloramphenicol, TETRA: tetracycline, STREP: streptomycin, AMP: Ampicillin, [b]S: synergy

As the result, all combination exhibited synergism. The combination of streptomycin with 6,8-dibromo-5,7-dihydroxyflavone (**33**) had the most synergistic effect against all bacteria. The increased anti-bacterial activity of streptomycin in combination was eight folds against *S. aureus* and *S. typhi* and sixteen folds against *P. acnes*, *S. aureus* and *S. mutans*. The lowest MIC of streptomycin was 0.122 μM. Other combination, pair of 6,8-diiodo-5,7-dihydroxyflavone (**34**) and streptomycin had synergistic effect against *P. acnes*, with the rate in increasing of activity of streptomycin was sixteen folds in combination compared to the activity of streptomycin alone. Furthermore, the combination of **34** and chloramphenicol showed synergism against *S. aureus* with the rate of anti-bacterial activity in combination sixteen folds compared with the activity of chloramphenicol being tested alone.

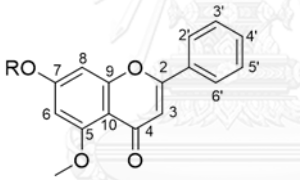
In conclusion, for anti-bacterial activity, the hydroxy groups at C-5 and C-7 play an important role. Bromo- substituent affected to anti-bacterial activity more than iodo- substituent. Among these compounds, 6,8-dibromo-5,7-dihydroxyflavone (**33**) and 6,8-diiodo-5,7-dihydroxyflavone (**34**) showed the highest anti-bacterial activity. For *P. acnes* and *S. aureus*, these compounds were bacteriostatic agent. On the other hand, both were bactericidal agent for *S. sobrinus, S. mutans* and *S. typhi*. The combination of flavones **33** and **34** and four known-antibiotics were exhibited as all combination showed synergistic effect.

3.3 Anti-tyrosinase and melanogenesis activities of flavone derivatives

Seventeen flavone derivatives, including chrysin, six ether flavones, four halogenated flavones and six polymethoxyflavones, were investigated for their anti-tyrosinase and melanogenesis activities.

3.3.1 Synthesis of ether derivatives of 5-methoxyflavones

Five new ether derivatives were prepared by reacting chrysin with selected bromoalkane or dimethyl sulfate and K_2CO_3 in acetone. The mixture was refluxed for overnight under N_2 atmosphere to furnish the desired compounds. These new flavones were characterized by ^{1}H, ^{13}C NMR and HR-MS. The appearance and % yield of synthesized compounds are displayed in **Table 3.13.**



53: R=C_2H_5
54: R=C_4H_9
55: R=C_6H_{13}
56: R=C_8H_{17}
57: R=$C_{12}H_{25}$

Table 3.13 The appearance and % yield of synthesized compounds

Entry	Cpds	Appearance	% yield
1	**53**	pale yellow solid	87
2	**54**	pale yellow solid	88
3	**55**	pale yellow solid	81
4	**56**	pale yellow solid	91
5	**57**	pale yellow solid	89

The ^{1}H NMR spectra of compounds **53**-**57** showed similar pattern. The aromatic protons at C-3, C-6 and C-8 were observed around δ 6.39-6.97 ppm. The protons of alkoxyl group at C-7 were displayed at δ 0.87-4.15 ppm. The ^{13}C NMR spectra showed a signal belonging to a carbonyl group (C-4) at 177.6-177.9 ppm. The

carbons of alkoxyl group at C-7 were observed at 64.2-68.8 ppm. The ^{1}H and ^{13}C NMR spectra of compounds **53**-**57** are displayed in **Figures 3.5-3.14.**

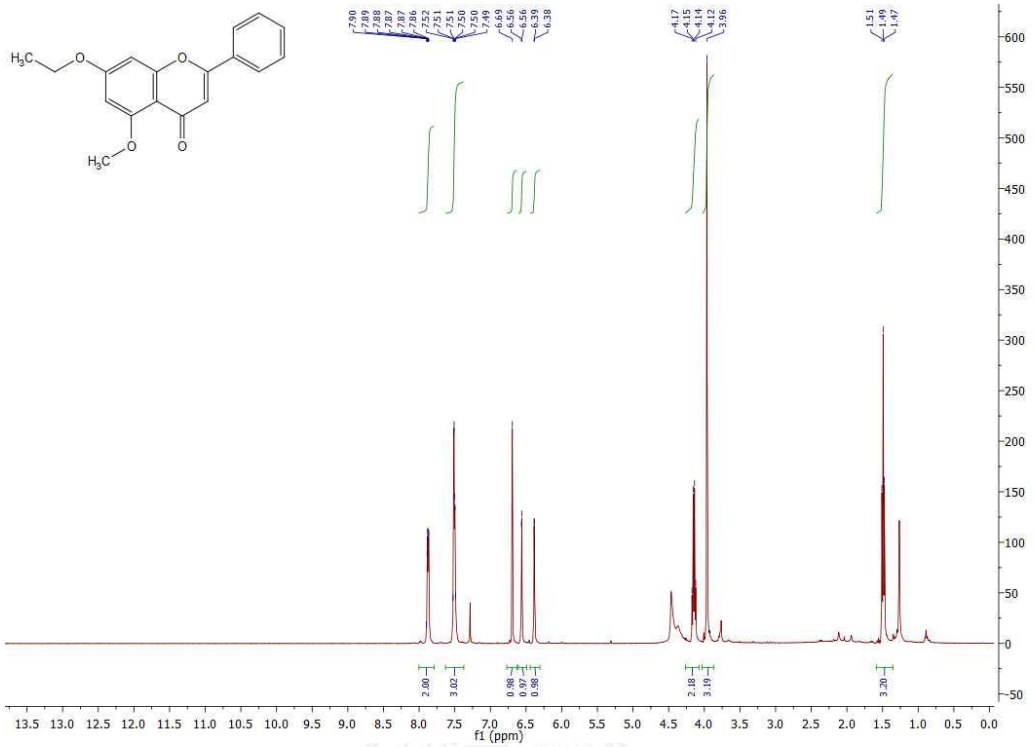


Figure 3.5 The ^{1}H NMR (400 MHz) spectrum of compound **53** ($CDCl_3$)

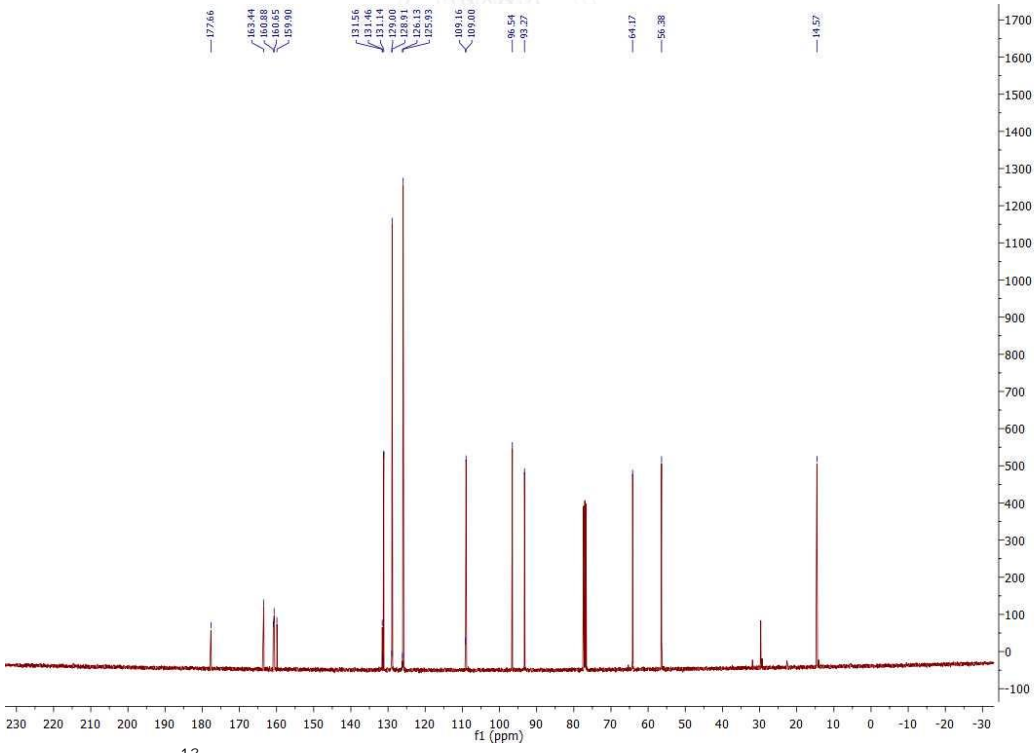


Figure 3.6 The ^{13}C NMR (100 MHz) spectrum of compound **53** ($CDCl_3$)

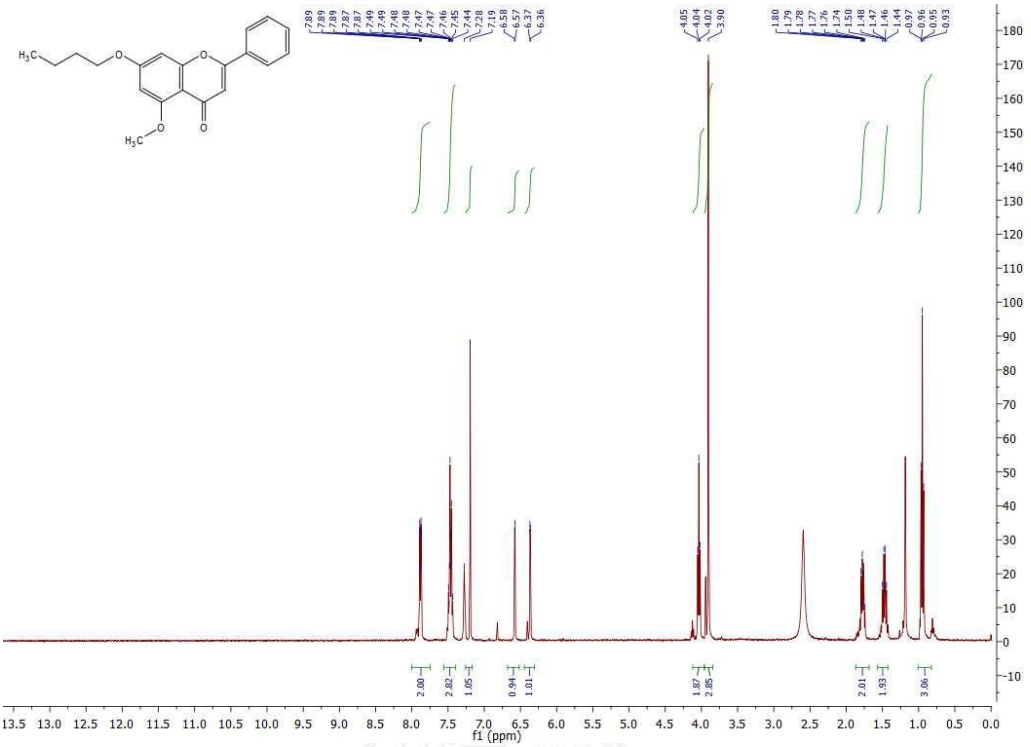


Figure 3.7 The ^{1}H NMR (400 MHz) spectrum of compound **54** ($CDCl_3$)

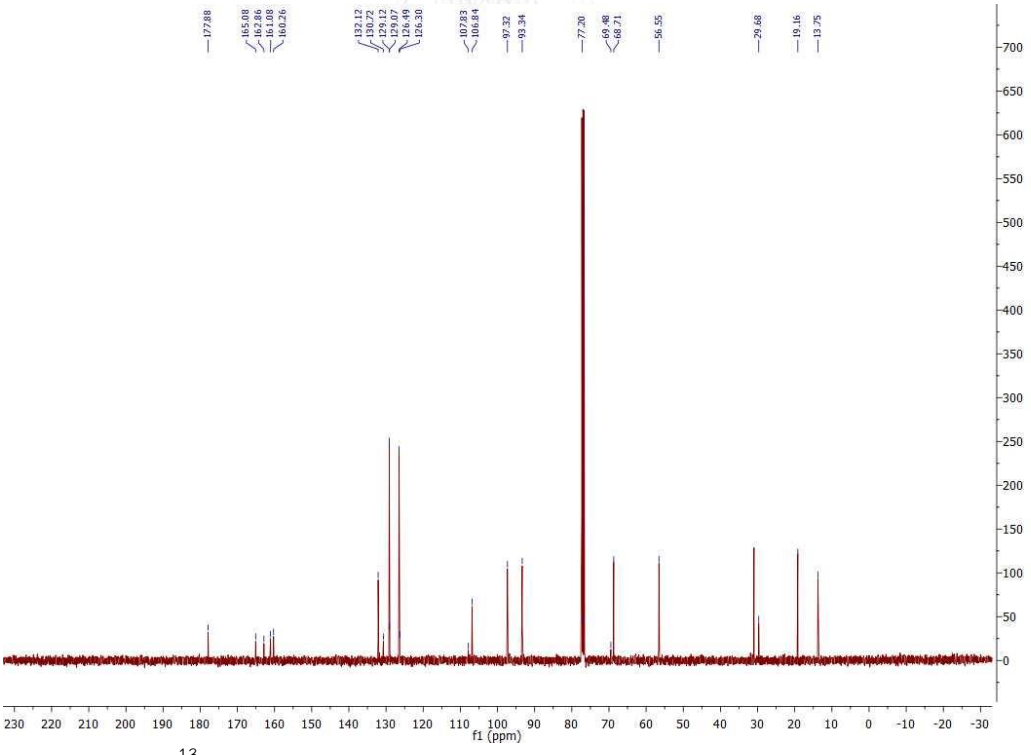


Figure 3.8 The ^{13}C NMR (100 MHz) spectrum of compound **54** ($CDCl_3$)

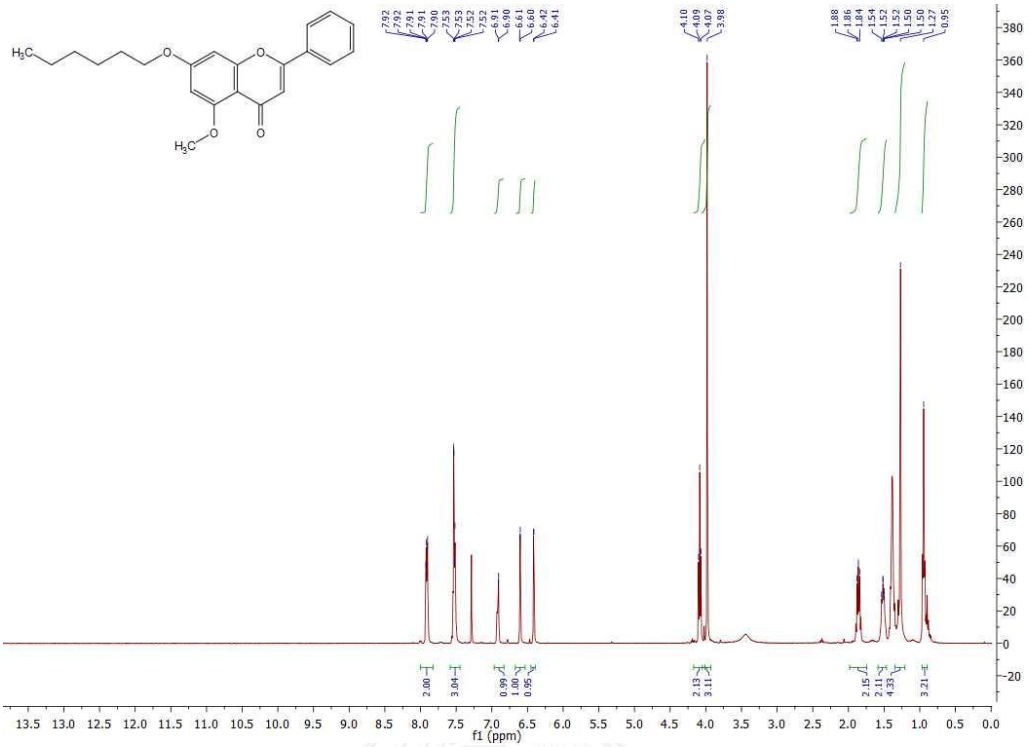


Figure 3.9 The ^{1}H NMR (400 MHz) spectrum of compound **55** ($CDCl_3$)

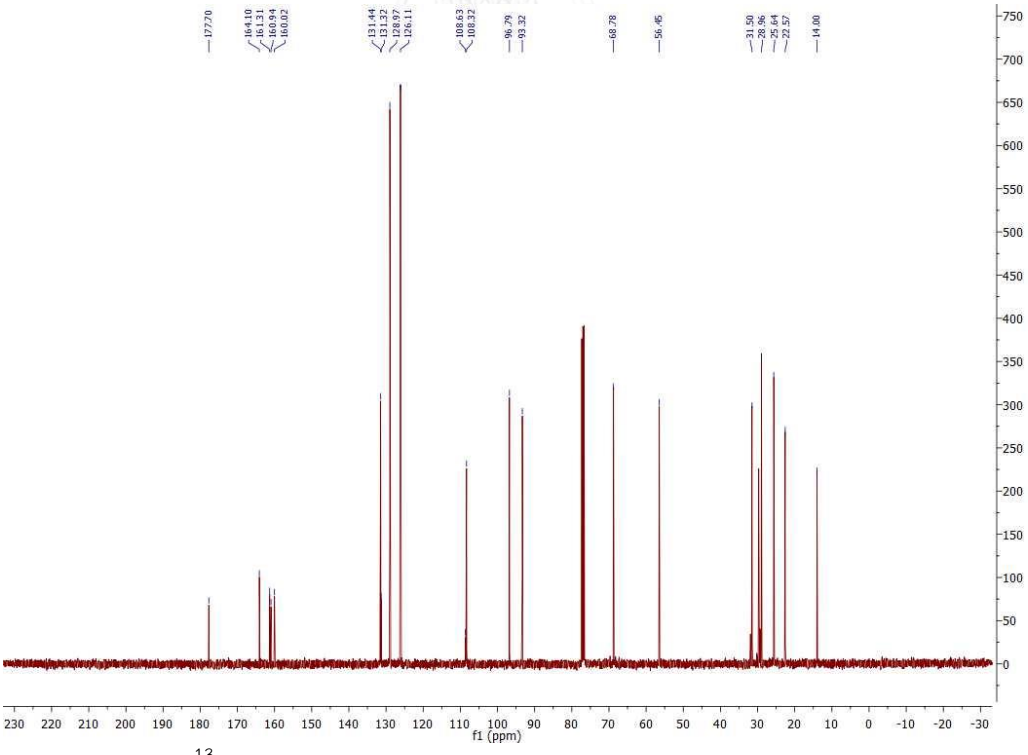


Figure 3.10 The ^{13}C NMR (100 MHz) spectrum of compound **55** ($CDCl_3$)

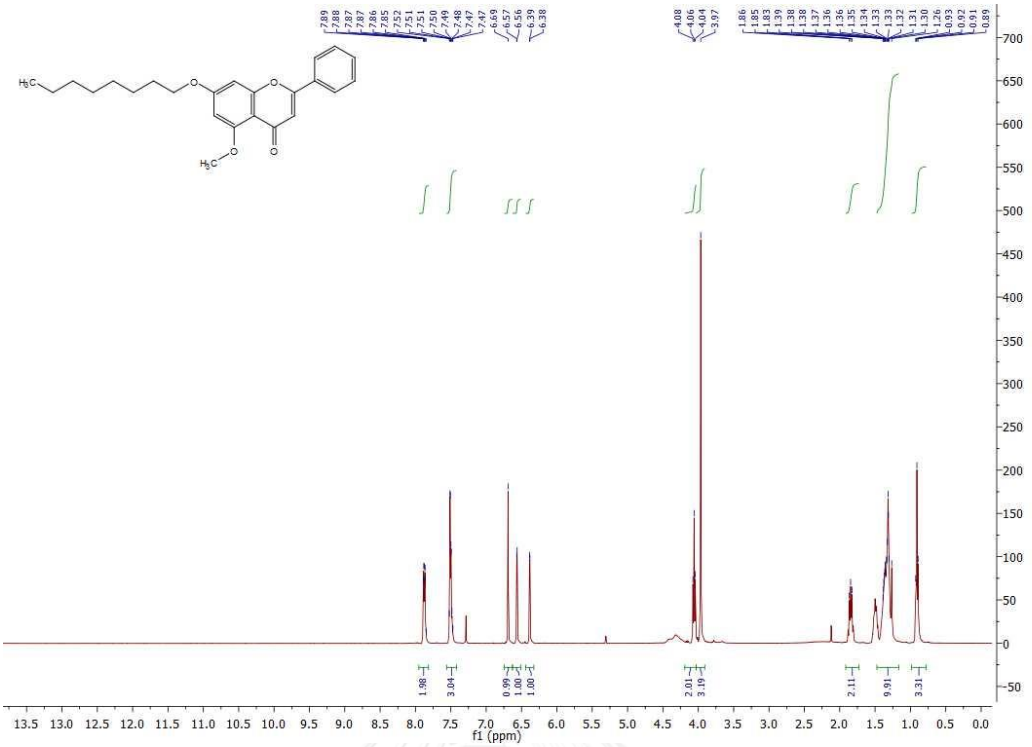


Figure 3.11 The ^{1}H NMR (400 MHz) spectrum of compound **56** ($CDCl_3$)

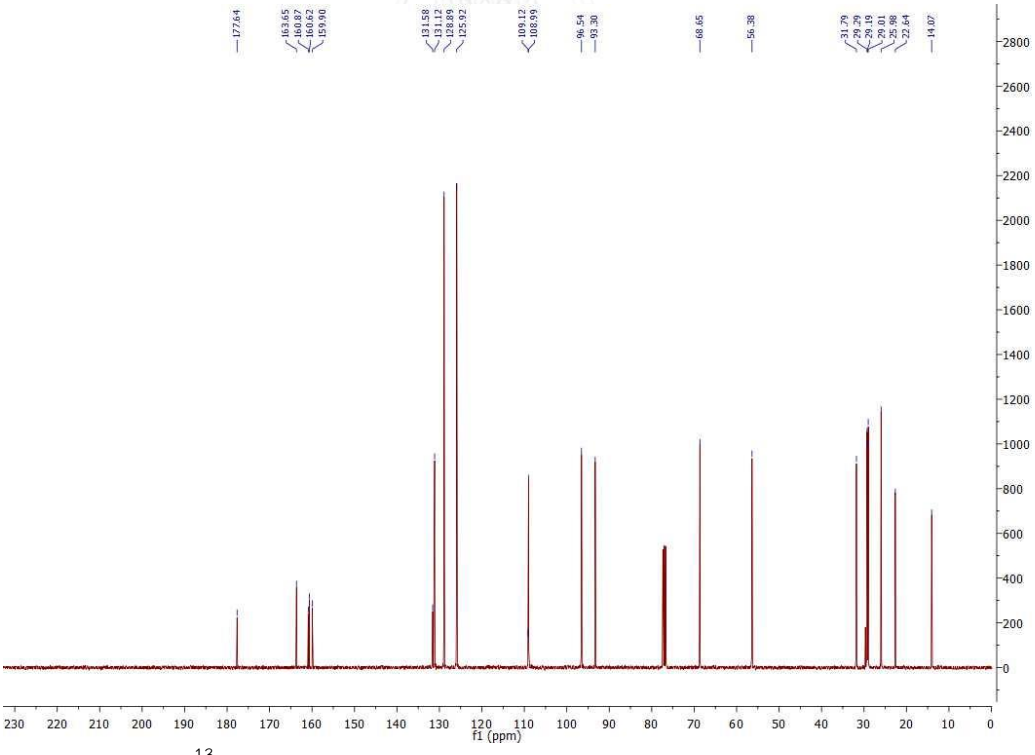


Figure 3.12 The ^{13}C NMR (100 MHz) spectrum of compound **56** ($CDCl_3$)

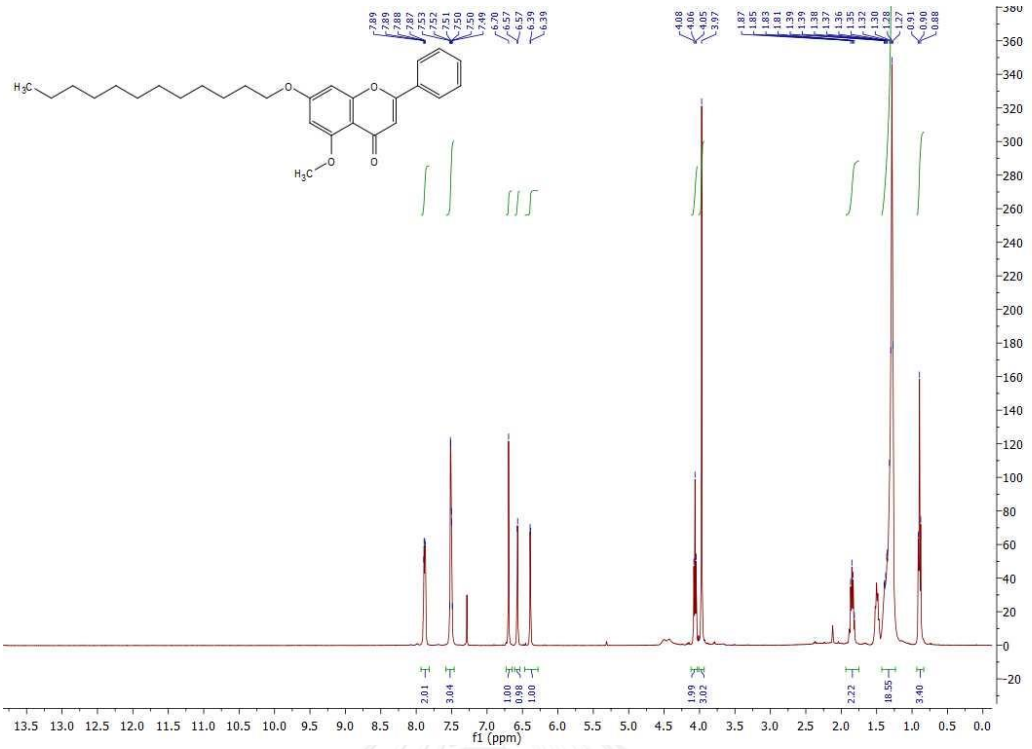


Figure 3.13 The ^{1}H NMR (400 MHz) spectrum of compound **57** ($CDCl_3$)

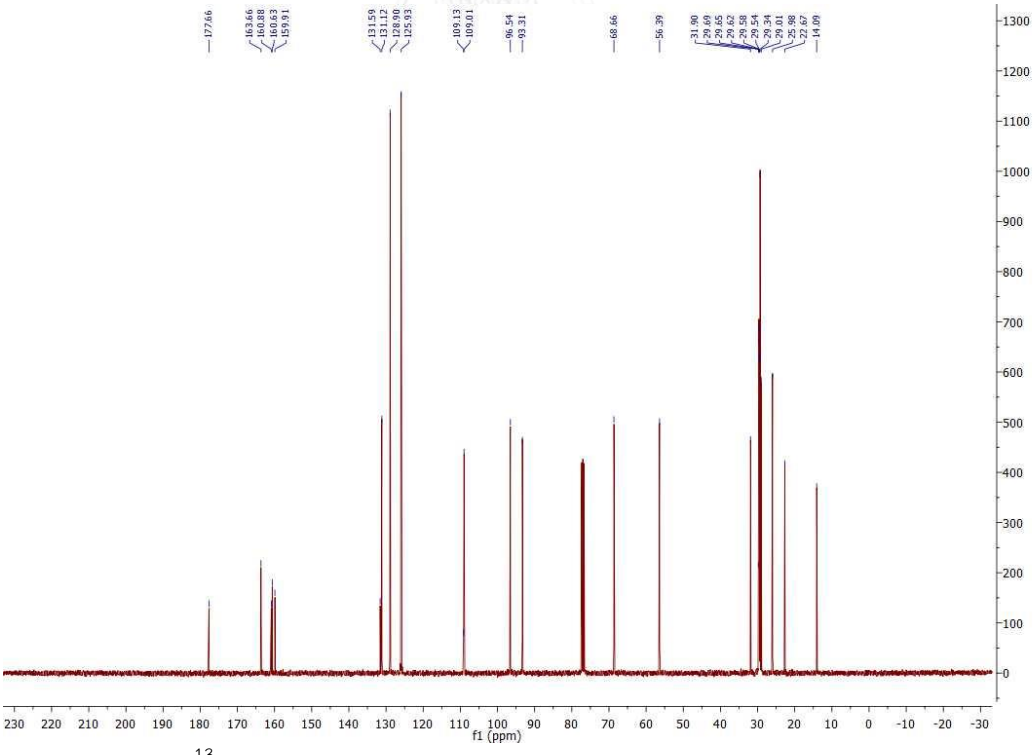


Figure 3.14 The ^{13}C NMR (100 MHz) spectrum of compound **57** ($CDCl_3$)

The chemical shift assignments of ^{1}H and ^{13}C NMR spectra of compounds **53**-**57** are displayed in **Tables 3.14-3.15**.

Table 3.14 The chemical shift assignments of ^{1}H NMR spectra of compounds **53**-**57**

Position	**53**	**54**	**55**	**56**	**57**
3	6.69 (s, 1H)	7.09 (s, 1H)	6.92 (s, 1H)	6.69 (s, 1H)	6.70 (s, 1H)
6	6.56 (d, *J* = 2.3 Hz, 1H)	6.58 (d, *J* = 2.2 Hz, 1H)	6.60 (d, *J* = 2.2 Hz, 1H)	6.56 (d, *J* = 2.3 Hz, 1H)	6.57 (d, *J* = 2.3 Hz, 1H)
8	6.38 (d, *J* = 2.3 Hz, 1H)	6.37 (d, *J* = 2.2 Hz, 1H)	6.41 (d, *J* = 2.2 Hz, 1H)	6.38 (d, *J* = 2.2 Hz, 1H)	6.39 (d, *J* = 2.2 Hz, 1H)
2´	7.88 (m, 2H)	7.88 (m, 2H)	7.91 (m, 2H)	7.88 (m, 2H)	7.88 (m, 2H)
3´	7.51 (m, 3H)	7.47 (m, 3H)	7.53 (m, 3H)	7.50 (m, 3H)	7.51 (m, 3H)
4´	7.51 (m, 3H)	7.47 (m, 3H)	7.53 (m, 3H)	7.50 (m, 3H)	7.51 (m, 3H)
5´	7.51 (m, 3H)	7.47 (m, 3H)	7.53 (m, 3H)	7.50 (m, 3H)	7.51 (m, 3H)
6´	7.88 (m, 2H)	7.88 (m, 2H)	7.91 (m, 2H)	7.88 (m, 2H)	7.88 (m, 2H)
3-OMe	3.96 (s, 3H)	3.90 (s, 3H)	3.98 (s, 3H)	3.97 (s, 3H)	3.97 (s, 3H)

The chemical shift of the side chain of

53: 4.15 (q, *J* = 7.0 Hz, 2H), 1.49 (t, *J* = 7.0 Hz, 3H)

54: 4.04 (t, *J* = 6.5 Hz, 2H), 1.77 (m, 2H), 1.47 (m, 2H), 0.95 (t, *J* = 7.4 Hz, 3H)

55: 4.09 (t, *J* = 6.5 Hz, 2H), 1.85 (m, 2H), 1.52 (m, 2H), 1.27 (m, 4H), 0.95 (m, 3H)

56: 4.06 (t, *J* = 6.5 Hz, 2H), 1.85 (m, 2H), 1.45 – 1.22 (m, 10H), 0.91 (m, 3H)

57: 4.06, (t, *J* = 6.5 Hz, 2H), 1.84 (q, *J* = 7.0 Hz, 2H), 1.45 – 1.12 (m, 18H), 0.89 (t, *J* = 6.8 Hz, 3H)

The spectra were recorded in $CDCl_3$

Table 3.15 The chemical shift assignments of ^{13}C NMR spectra of compounds **53**-**57**

Position	**53**	**54**	**55**	**56**	**57**
2	160.9	162.9	161.3	160.9	160.9
3	109.1	107.8	108.6	109.1	109.1
4	177.7	177.9	177.7	177.6	177.7
5	160.6	161.0	160.9	160.6	160.6
6	96.5	97.3	96.8	96.5	96.5
7	163.4	165.1	164.1	163.6	163.7
8	93.3	93.3	93.3	93.3	93.3
9	159.9	160.3	160.0	159.9	159.9
10	109.0	106.8	108.3	109.0	109.0
1′	131.2	130.7	131.3	131.1	131.1
2′	126.3	126.5	126.1	125.9	125.9
3′	129.1	129.1	129.0	128.9	128.9
4′	131.7	132.1	131.4	131.6	131.6
5′	129.1	129.1	129.0	128.9	128.9
6′	126.3	126.5	126.1	125.9	125.9
3-OMe	56.4	56.6	56.4	56.4	56.4

The chemical shift of the side chain:

53: 64.2, 14.6

54: 68.7, 29.7, 19.2, 13.7

55: 68.8, 31.5, 29.0, 25.6, 22.6, 14.0

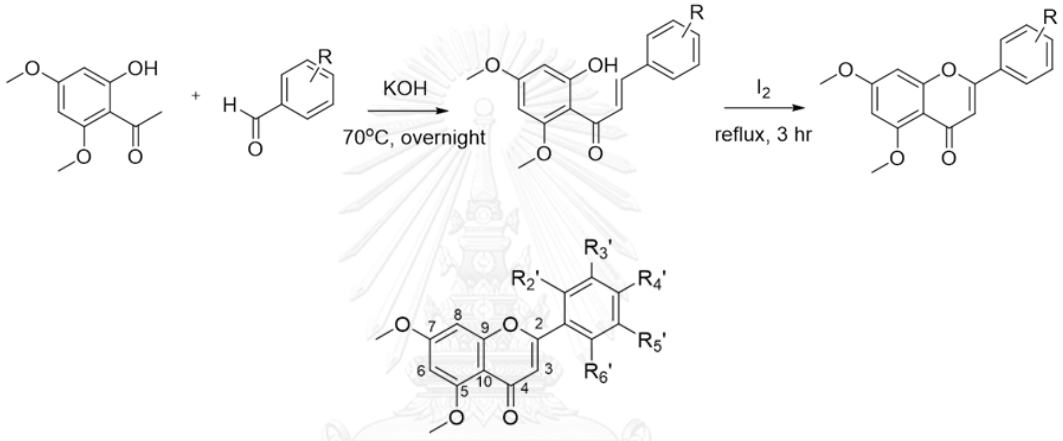
56: 68.6, 31.8, 29.3, 29.2, 29.0, 26.0, 22.6, 14.1

57: 68.7, 31.9, 29.7, 29.6, 29.6, 29.6, 29.5, 29.3, 29.0, 26.0, 22.7, 14.1

The spectra were recorded in $CDCl_3$

3.3.1.2 Synthesis of polymethoxyflavones

Polymethoxyflavones were prepared *via* chalcone. To illustrate this, 2-hydroxy-4,6-dimethoxyacetophenone and methylated benzaldehyde were treated with KOH. The mixture was heated at 70°C overnight to achieve 2-hydroxy-4,6-dimethoxychalcone. The reaction of I_2 and prepared chalcones was refluxed for 3 hr to furnish six polymethoxyflavones [40], [41]. The appearance and % yield of synthesized polymethoxyflavones are displayed in **Table 3.16.**



62: $R_{2'}$= OCH_3 $R_{3'}$= $R_{4'}$= $R_{5'}$= $R_{6'}$= H

63: $R_{2'}$= $R_{4'}$= OCH_3 $R_{3'}$= $R_{5'}$= $R_{6'}$= H

64: $R_{2'}$= $R_{5'}$= OCH_3 $R_{3'}$= $R_{4'}$= $R_{6'}$= H

65: $R_{2'}$= $R_{3'}$= $R_{4'}$= OCH_3 $R_{5'}$= $R_{6'}$= H

66: $R_{2'}$= $R_{4'}$= $R_{6'}$= OCH_3 $R_{3'}$= $R_{5'}$= H

67: $R_{3'}$= $R_{4'}$= $R_{5'}$= OCH_3 $R_{2'}$= $R_{6'}$= H

Table 3.16 The appearance and % yield of synthesized polymethoxyflavone

Entry	Cpds	Appearance	% yield
1	**62**	pale yellow solid	88
2	**63**	pale brown solid	92
3	**64**	pale brown solid	87
4	**65**	pale brown solid	91
5	**66**	pale brown solid	78
6	**67**	brown solid	86

These compounds were characterized by ^{1}H NMR. The spectra showed protons of methoxy groups around δ 4.19-3.62 ppm and other protons on benzene rings were detected approximately at δ 7.87-6.11 ppm. The signal of –OH (δ 12.28-14.54 ppm) and protons at C2 (δ 7.65-8.31 ppm) of chalcone were disappeared. The ^{1}H NMR chemical shift assignment was accumulated as presented in **Table 3.17**.

Table 3.17 The chemical shift assignments of ^{1}H NMR spectra of compounds **62-67**

Position	**62**	**63**	**64**	**65**	**66**	**67**
3	7.02 (s, 1H)	6.53 (d, *J* = 2.6 Hz, 2H)	6.57 (d, *J* = 2.3 Hz, 1H),	6.49 (d, *J* = 2.4 Hz, 1H)	6.28 (d, *J* = 2.2 Hz, 1H)	6.40 (d, *J* = 2.2 Hz, 1H)
6	6.36 (d, *J* = 2.3 Hz, 1H)	6.36 (d, *J* = 2.3 Hz, 1H)	6.38 (d, *J* = 2.3 Hz, 1H)	6.35 (d, *J* = 15.5 Hz, 1H)	6.38 (d, *J* = 2.2 Hz, 1H)	6.61 (d, *J* = 2.3 Hz, 1H)
8	6.54 (d, *J* = 2.3 Hz, 1H)	6.61 (dd, *J* = 8.8, 2.4 Hz, 2H)	6.99 (m, 2H)	6.77 (s, 1H)	7.49 (s, 1H)	6.90 (s, 1H)
2´	-	-	-	-	-	7.10 (s, 2H)
3´	7.45 (t, *J* = 7.6 Hz, 2H)	6.53 (d, *J* = 2.6 Hz, 2H)	7.44 (d, *J* = 3.2 Hz, 2H)	-	6.11 (s, 2H)	-
4´	7.87 (d, *J* = 7.9 Hz, 2H)	-	6.99 (m, 2H)	-	-	-
5´	7.45 (t, *J* = 7.6 Hz, 2H)	6.61 (dd, *J* = 8.8, 2.4 Hz, 2H)	-	6.71 (d, *J* = 8.9 Hz, 1H)	6.11 (s, 2H)	-
6´	7.87 (d, *J* = 7.9 Hz, 2H)	7.85 (d, *J* = 8.7 Hz, 1H)	7.44 (d, *J* = 3.2 Hz, 2H)	7.48 (d, *J* = 8.9 Hz, 1H)	-	7.10 (s, 2H)

The chemical shift of methoxy groups:

62: 4.01 – 3.85 (m, 9H)

63: 3.91 (dd, *J* = 19.0, 8.5 Hz, 12H)

64: 4.19 – 3.75 (m, 12H)

65: 4.12 – 3.70 (m, 15H)

66: 3.83 (s, 3H), 3.81 (s, 3H), 3.77 (s, 3H), 3.64 (s, 6H)

67: 4.17 – 3.77 (m, 15H)

The spectra were recorded in $CDCl_3$

3.3.2 Anti-tyrosinase activity

Tyrosinase is a key enzyme in melanin biosynthesis that involved in determining the color of skin and hair. It catalyzes oxidation of both L-tyrosine and L-DOPA, following another oxidation of L-DOPA to dopaquinone, and finally oxidative polymerization *via* several dopaquinone derivatives to melanin [50]. Seventeen flavones, including chrysin, six ether flavones, four halogenated flavones and six polymethoxyflavones were evaluated their anti-tyrosinase activity using L-tyrosine or L-DOPA as substrates and kojic acid as positive control. The data of anti-tyrosinase activity were expressed as IC_{50} (μM) as presented in **Table 3.18.**

Table 3.18 IC_{50} values of anti-tyrosinase activity of tested flavones

Entry	Cpds	IC_{50} (μM)	
		L-tyrosine	L-DOPA
1	chrysin	>100	>100
2	49	>100	>100
3	53	>100	>100
4	54	>100	>100
5	55	>100	>100
6	56	>100	>100
7	57	>100	>100
8	33	>100	>100
9	34	>100	>100
10	58	>100	>100
11	59	>100	>100
12	62	>100	>100
13	63	>100	>100
14	64	>100	>100
15	65	>100	>100
16	66	>100	>100
17	67	>100	>100
18	kojic acid	0.0001	0.0015

The IC_{50} values of synthesized flavones (**49, 33**-**34, 53**-**57** and **58**-**67**) including chrysin were more than 100 μM. Thus, none of the compounds showed inhibitory activities when using either L-tyrosine or L-DOPA as substrates. This suggests that tyrosinase inhibition is barely involved in the mechanism of action of these compounds.

3.3.3 Melanogenesis activity

Melanin is a heterogeneous, polyphenol-like biopolymer with a complex structure with color varying from yellow to black. The important role of melanin is to protect the skin from UV damage by absorbing UV light. Melanin is secreted from melanocytes distributed in the basal layer of the dermis by keratinocytes, existing on the skin surface, produce messengers such as α-MSH (α-melanocyte-stimulating hormone) to melanocytes after getting stimulated by UV irradiation. Then melanocyte biosynthesizes melanin in melanosome and transports them to keratinocytes [51].

The melanogenesis-stimulating activities of synthesized flavones were determined by measuring both intra- and extracellular melanin content in B16 melanoma cells. The data of cell viability and the melanogenesis activity of B16 melanoma cells were expressed as mean ± SD values. Seventeen derivatives of flavones in this examination were classified into three subgroups as:

3.3.3.1 Melanogenesis activity of ether derivatives

Six ether derivatives (**49**, **53**-**57**) and chrysin were investigated for cell viability and melanogenesis activity as shown in **Figure 3.15.**

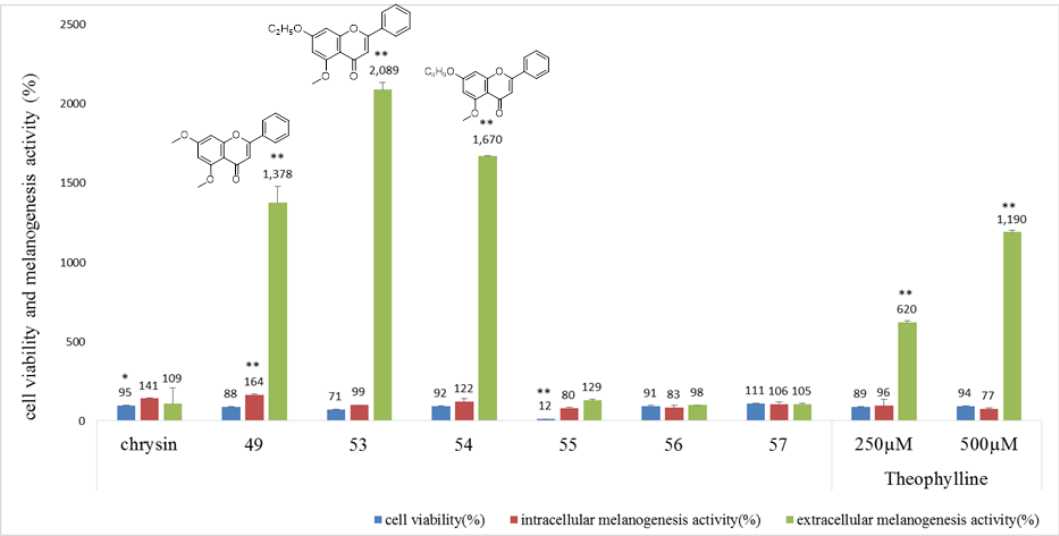


Figure 3.15 % Cell viability and melanogenesis activity on B16 melanoma cells of chrysin**,** **49** and **53-57**

Figure 3.1 shows % cell viability and melanogenesis activity. The blue, red and green columns represent % cell viability, % intracellular melanin content and % extracellular melanin content at 25 μM, respectively. Theophylline was used as a positive control at 250 and 500 μM. For this series, all compounds were not toxic to B16 melanoma cell, except compound **55** which showed 12 % cell viability. Intracellular melanin content was determined by measuring melanin in cell while extracellular melanin content was measured the melanin that was transported to the medium. Compound **53** showed the highest extracellular melanogenesis activity, followed by compounds **54** and **49**. On the other hand, intracellular melanin content of compound **53** exhibited the lowest. If the extracellular melanin content was high, the intracellular melanogenesis activity would be low. In comparison of extracellular melanin content, 5,7-dimethoxyflavone (**49**), 5-methoxy-7-ethoxyflavone (**53**) and 5-methoxy-7-butoxyflavone (**54**) showed higher activity more than two folds of theophylline although the concentration of compounds were less than ten folds of a positive control.

In this examination, the effect of size of alkoxyl group which has not been explored before, was focused by increasing the number of carbon atom. Very interestingly, 5-methoxy-7-ethoxyflavone (**53**) was disclosed to express the strongest activity, followed by 5-methoxy-7-butoxyflavone (**54**) and 5,7-dimethoxyflavone (**49**), respectively. Nevertheless, if the number of carbon chain at 7-*O* position was more than 4, there was no stimulatory effect on the extracellular melanin levels. These results pointed out that the activity was greatly depended on number of carbon at 7-*O* position.

In addition, comparing chrysin whose structure contains dihydroxy groups at C-5 and C-7, with 5,7-dimethoxyflavone (**49**), it was observed that compound **49** showed higher activity than chrysin. Thus, the methoxy groups on A-ring also played an important role in melanogenesis activity.

3.3.3.2 Melanogenesis activity of halogenated flavones

Four halogenated flavones (**33**-**34** and **58**-**59**) were comparatively studied. % Cell viability and melanogenesis activity are shown in **Figure 3.16.**

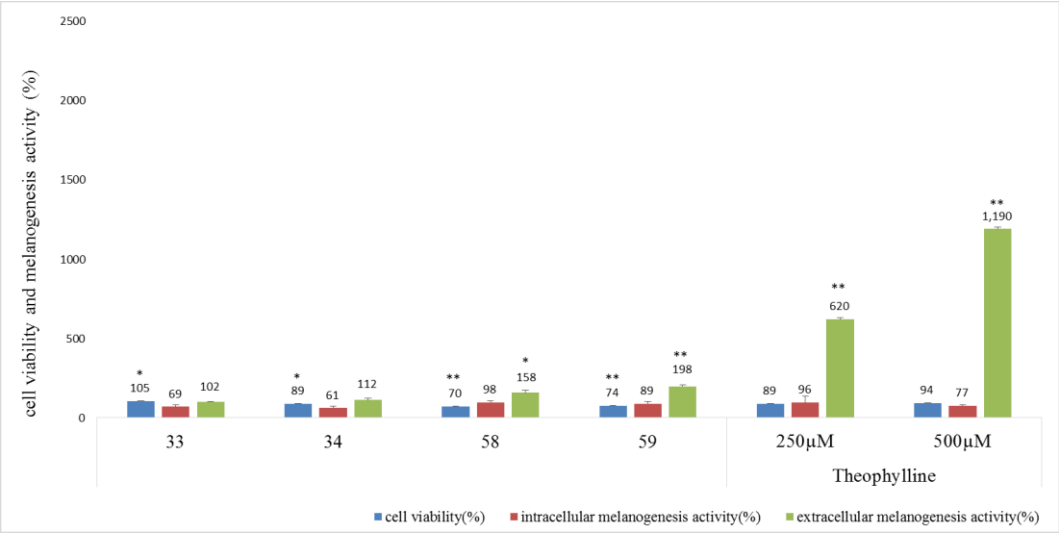


Figure 3.16 % Cell viability and melanogenesis activity on B16 melanoma cells of **33**-**34** and **58**-**59**

At 25 μM of extracellular melanin content, all four compounds (**33-34** and **58-59**) showed little melanogenesis stimulatory activity. Nevertheless, 5-hydroxy-7-methoxyflavone analogues (**58** and **59**) were noticed to show slightly higher activity than 5,7-dihydroxyflavone analogues (**33** and **34**). 6,8-Diiodoflavones (**34** and **59**) expressed higher activity than 6,8-dibromoflavones (**33** and **58)**. Thus, the methoxy group on A-ring may play the important role as mentioned in **3.3.3.1** and iodo- had effect on melanogenesis stimulatory than bromo substituent.

3.3.3.3 Melanogenesis activity of polymethoxyflavones

To extent the exploration on the effect of the number of methoxy groups of polymethoxyflavones *vs* melanogenesis activity, methoxy, dimethoxy and trimethoxy groups at various positions on B-ring of parent 5,7-dimethoxyflavone were investigated. % Cell viability and melanogenesis activity are shown in **Figure 3.17**.

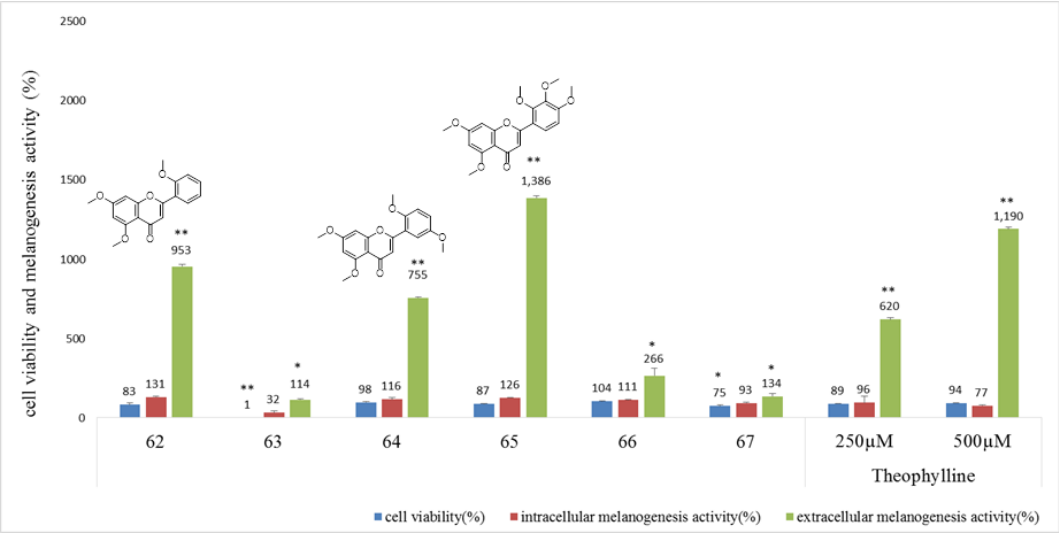


Figure 3.17 % Cell viability and melanogenesis activity on B16 melanoma cells of **62-67**

5,7,2′,3′,4′-Pentamethoxyflavone (**65**) showed the strongest melanogenesis stimulation at 25 μM with % extracellular melanin content more than two fold of a positive control at 250 μM, followed by 5,7,2′-trimethoxyflavone (**62**) and 5,7,2′,5′-tetramethoxyflavone (**64**), respectively. 5,7,2′,4′,6′-Pentamethoxyflavone (**66**) and 5,7,3′,4′,5′-pentamethoxyflavone (**67**) showed little activity. On the other hand, 5,7,2′,4′-tetramethoxyflavone (**63**) showed toxicity at 25 μM.

5,7,2′,3′,4′-Pentamethoxyflavone (**65**) was selected to examine the relationship between concentration and activity. At extracellular melanogenesis activity, the melanin content was decreased when decreasing the concentration. In comparison, at the lowest concentration compound **65** still exhibited activity more than theophylline both intra- and extracellular melanin content despite the concentration was less than forty folds. The results are shown in **Figure 3.18.**

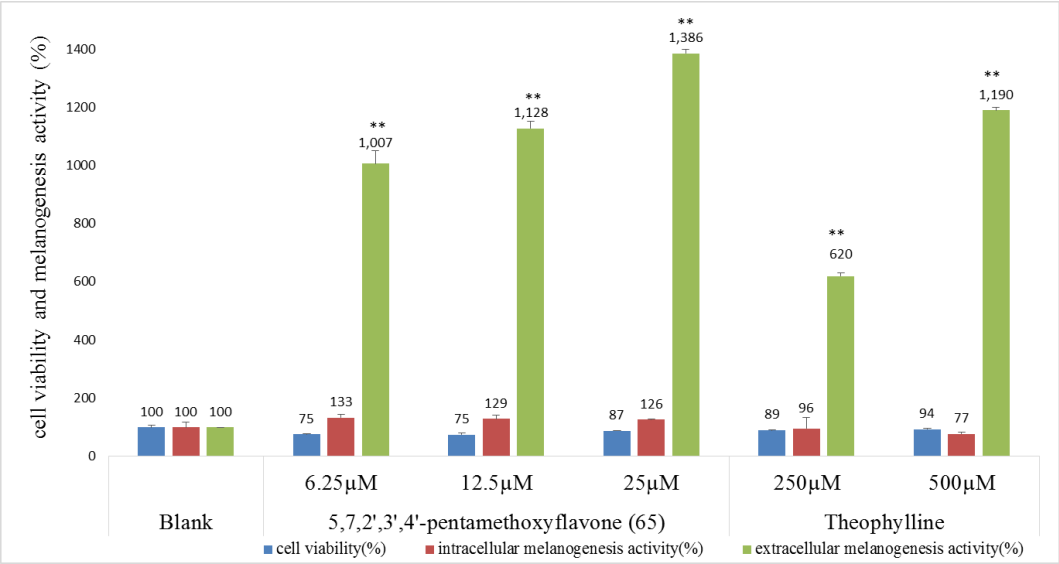


Figure 3.18 % Cell viability and melanogenesis activity on B16 melanoma cells of **65**

Another intriguing observation could be found for 5,7,3´,4´,5´-pentamethoxyflavone (**67**, **Figure 3.19**). This compound showed strong activity at low concentration (6.25 µM) with % activity was about 1600. At 3.125 µM, compound **67** still exhibited activity more than almost two folds of theophylline at 250 µM. Interestingly, when its concentration was increased, the extracellular melanin content was decreased.

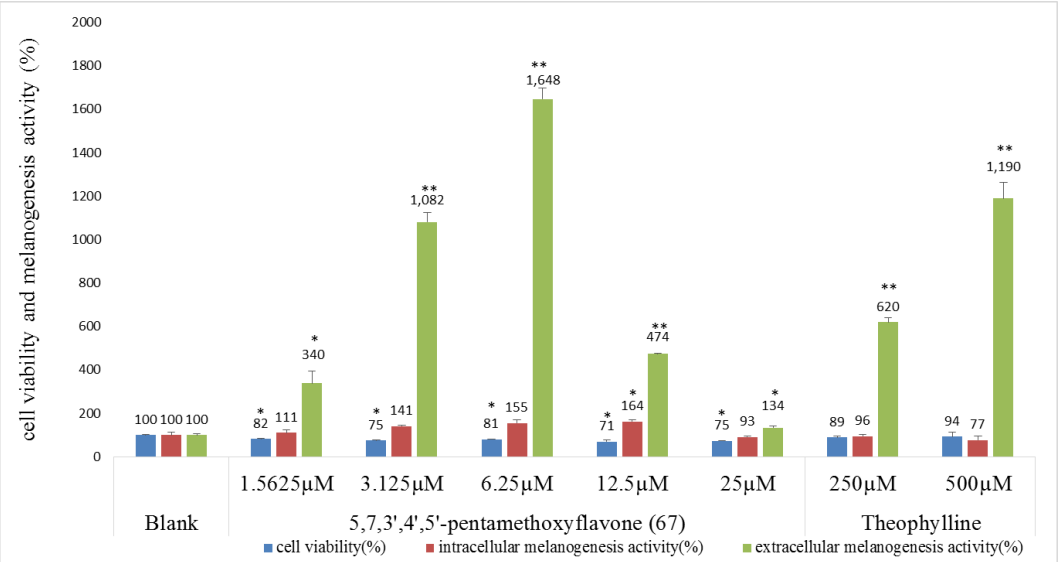


Figure 3.19 % Cell viability and melanogenesis activity on B16 melanoma cells of **67**

However, the influence of methoxy groups at various positions on B-ring on melanogenesis activity did not show the clear trend in structure-activity relationship. Only 2′,3′,4′-trimethoxy group showed higher activity than 2′-methoxy and 2′,5′-dimethoxy groups. 2′,4′-Dimethoxy group showed less % cell viability, while 3′,4′,5′-trimethoxy group showed strong activity at low concentration (6.25 μM).

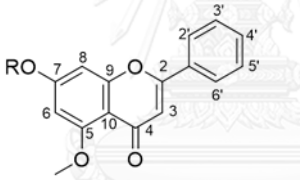
In conclusion, seventeen flavones, including chrysin, six ether flavones, four halogenated flavones and six polymethoxyflavones, did not show tyrosinase inhibitory activities when using either L-tyrosine or L-DOPA as substrates. For melanogenesis activities, 5,7-dimethoxyflavone (**49**), 5-methoxy-7-ethoxyflavone (**53**), 5-methoxy-7-butoxyflavone (**54**) and 5,7,2′,3′,4′-pentamethoxyflavone (**65**) showed higher extracellular melanin content more than two folds of theophylline although the concentration of compounds were less than ten folds of a positive control. For this activity, the methoxy groups on A-ring played an important role. Interestingly, 5,7,3′,4′,5′-pentamethoxyflavone (**67**) showed strong activity at low concentration

(6.25 μM) and when its concentration was increased, the extracellular melanin content was decreased.

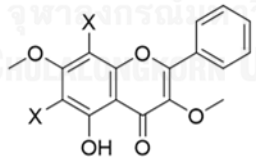
CHAPTER IV

CONCLUSION

Two flavanones and five flavones from the CH_2Cl_2 extracts of *B. rotunda* and *K. parviflora* were repeatedly isolated by chromatographic techniques. Twenty-eight flavonoid derivatives were synthesized and confirmed their structure by ^{1}H-NMR. Among them, five ether derivatives of 5-methoxyflavone (**53-57**) and two halogenated flavones, 6,8-dibromo-5-hydroxy-3,7-dimethoxyflavone (**60**) and 6,8-diiodo-5-hydroxy-3,7-dimethoxyflavone (**61**), have not previously been reported. These compounds were characterized using spectroscopic techniques (^{1}H, ^{13}C-NMR and HR-MS), and their structures are shown below:



53: R=C_2H_5
54: R=C_4H_9
55: R=C_6H_{13}
56: R=C_8H_{17}
57: R=$C_{12}H_{25}$



60 X= Br
61 X= I

As the results of anti-bacterial activity against *P. acnes* (KCCM41747), *S. aureus* (ATCC25923), *S. mutans* (KCCM11898), *S. sobrinus* (ATCC25175), and *S. typhi* (ATCC442), the hydroxy groups at C-5 and C-7 played an important role in this anti-bacterial activity. Among tested compounds, 6,8-dibromo-5,7-dihydroxyflavone (**33**) and 6,8-diiodo-5,7-dihydroxyflavone (**34**) exhibited the highest activity against all bacteria with MIC 31.25-62.5 μM. Moreover, these compounds were bacteriostatic agents against *P. acnes* and *S. aureus.* For *S. mutans, S. sobrinus* and *S. typhi*, both were bactericidal agents. The combination of flavones **33** and **34,** and four known-

antibiotics including chloramphenicol, tetracycline, streptomycin and ampicillin were determined using checkerboard method. The results exhibited that all combination showed synergistic effect.

Seventeen flavones, including chrysin, six ether flavones, four halogenated flavones and six polymethoxyflavones, were investigated for their anti-tyrosinase and melanogenesis activities. For anti-tyrosinase activity, all tested compounds did not exhibit tyrosinase inhibitory activity. For melanogenesis-stimulating activities, the methoxy group on A-ring played an important role in melanogenesis activity. 5,7-Dimethoxyflavone (**49**), 5-methoxy-7-ethoxyflavone (**53**), 5-methoxy-7-butoxyflavone (**54**) and 5,7,2′,3′,4′-pentamethoxyflavone (**65**) showed higher extracellular melanin content more than two folds of theophylline although the concentration of compounds were less than ten folds of a positive control. Interestingly, 5,7,3′,4′,5′-pentamethoxyflavone (**67**) displayed strong activity at low concentration, at 3.125 μM, 67 still exhibited activity more than two fold of positive control.

The collection of all isolated and synthesized flavonoids is summarized in **Figure 4.1**

Suggestion for future work

The possible future work related to this research would be the study on the relationship between halogenated flavones and other bacteria such as *Escherichia coli*. In addition, the combination of flavones with antibiotics should be carried on to find the best condition for future use. Furthermore, polymethoxyflavones had a promising tendency for further study on their stimulating-melanogenesis activities to find the active site and the effect of position of methoxy groups. Other biological activities of flavones were attractive for investigation such as anti-inflammatory or anticancer.

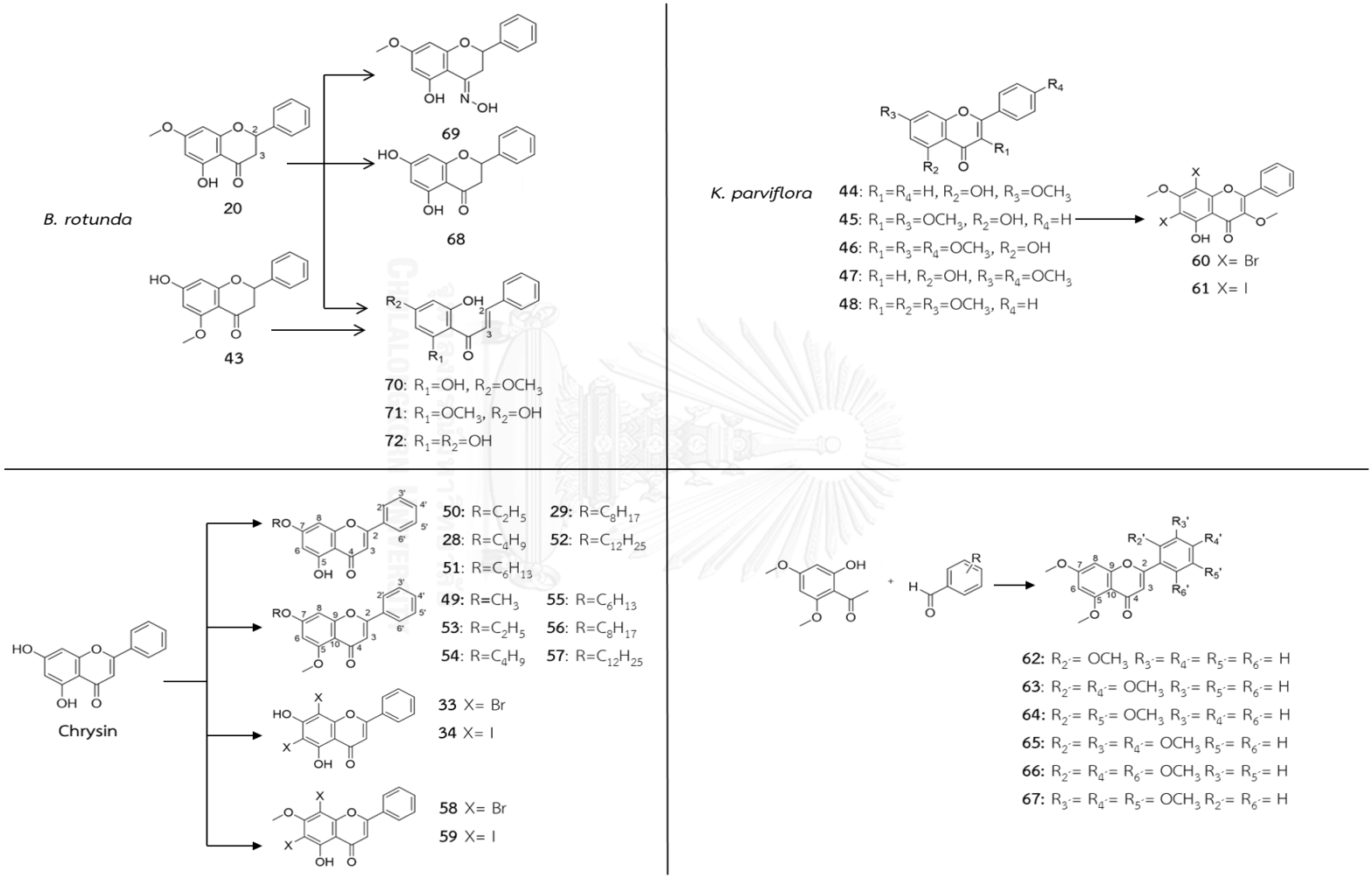


Figure 4. 1 The collection of isolated and synthesized flavonoids from this research

REFERENCES

[1] Rice, C., Miller, N., and Paganga, G. Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radical Biology and Medicine 20(7) (1996): 933-956.

[2] Orhan, D., Özçelik, B., Özgen, S., and Ergun, F. Antibacterial, antifungal, and antiviral activities of some flavonoids. Microbiological Research 165(6) (2010): 496-504.

[3] Ravishankar, D., Rajora, A., Greco, F., and Osborn, H. Flavonoids as prospective compounds for anti-cancer therapy. The International Journal of Biochemistry and Cell Biology 45(12) (2013): 2821-2831.

[4] Belo, S., Gaspar, L., Campos, P., and Marty, J. Skin Penetration of Epigallocatechin-3-Gallate and Quercetin from Green Tea and *Ginkgo biloba* Extracts Vehiculated in Cosmetic Formulations. Skin Pharmacology and Physiology 22(6) (2009): 299-304.

[5] Temkitthawon, P., Hindsb, T., Beavob, J., Viyochc, J., Suwanboriruxd, K., Pongamornkule, W., Sawasdeef, P., and Ingkaninan, K. *Kaempferia parviflora*, a plant used in traditional medicine to enhance sexual performance contains large amounts of low affinity PDE5 inhibitors. Journal of Ethnopharmacology 137(3) (2011): 1437-1441.

[6] Chong, T., Kee, L., Fei, C., Han, H., Ming, W., Ping, C., Teck, F., Khalid, N., Rahman, N., Karsani, S., Othman, S., Othman, R., and Yusof, R. *Boesenbergia rotunda*: From Ethnomedicine to Drug Discovery. Evidence-Based Complementary and Alternative Medicine 2012 (2012): 25.

[7] Wu, D., Kong, Y., Han, C., Chen, J., Hu, L., Jiang, H., and Shen, X. D-Alanine:D-alanine ligase as a new target for the flavonoids quercetin and apigenin. International Journal of Antimicrobial Agents 32(5) (2008): 421-426.

[8] Mughal, E., Ayaz, M., Hussain, Z., Hasan, A., Sadiq, A., Riaz, M., Malik, A., Hussain, S., and Choudhary, M. Synthesis and antibacterial activity of

substituted flavones, 4-thioflavones and 4-iminoflavones. Bioorganic and Medicinal Chemistry 14(14) (2006): 4704-4711.

[9] Li, M. and Xu, Z. Quercetin in a lotus leaves extract may be responsible for antibacterial activity. Archives of Pharmacal Research 31(5) (2008): 640-644.

[10] Pereira, F., Madureira, A., Sancha, S., Mulhovo, S., Luo, X., Duarte, A., and Ferreira, M. Cleistochlamys kirkii chemical constituents: Antibacterial activity and synergistic effects against resistant *Staphylococcus aureus* strains. Journal of Ethnopharmacology 178 (2016): 180-187.

[11] Biva, I., Ndi, C., Griesser, H., and Semple, S. Antibacterial constituents of *Eremophila alternifolia*: An Australian aboriginal traditional medicinal plant. Journal of Ethnopharmacology 182 (2016): 1-9.

[12] Slominski, A., Zmijewski, M., and Pawelek, J. L-tyrosine and L-DOPA as hormone-like regulators of melanocytes functions. Pigment cell and melanoma research 25(1) (2012): 14-27.

[13] D'Orazio, J., Jarrett, S., Ortiz, A., and Scott, T. UV Radiation and the Skin. International Journal of Molecular Sciences 14 (2013): 12222-12248.

[14] Jun, N., Hong, G., and Jun, K. Synthesis and evaluation of 2′,4′,6′-trihydroxychalcones as a new class of tyrosinase inhibitors. Bioorganic and Medicinal Chemistry 15(6) (2007): 2396-2402.

[15] Lan, W., Tzeng, C., Lin, C., Yen, F., and Ko, H. Prenylated flavonoids from *Artocarpus altilis*: Antioxidant activities and inhibitory effects on melanin production. Phytochemistry 89 (2013): 78-88.

[16] Wang, Y., Zhang, G., Yan, J., and Gong, D. Inhibitory effect of morin on tyrosinase: Insights from spectroscopic and molecular docking studies. Food Chemistry 163 (2014): 226-233.

[17] Zheng, Z., Zhang, Y., Zhang, S., and Chen, J. One-pot green synthesis of 1,3,5-triarylpentane-1,5-dione and triarylmethane derivatives as a new class of tyrosinase inhibitors. Bioorganic and Medicinal Chemistry Letters 26(3) (2016): 795-798.

[18] Yoshida M, Takahashi, Y., and Inoue, S. Histamine induces melanogenesis and morphologic changes by protein kinase A activation via H2 receptors in

human normal melanocytes. Journal of Investigative Dermatology 114(2) (2000): 334-342.

[19] Mitsunaga, T., and Yamauchi, K. Effect of quercetin derivatives on melanogenesis stimulation of melanoma cells. Journal of Wood Science 61(4) (2015): 351–363.

[20] hguchi, K., Akao, Y., and Nozawa, Y. Stimulation of Melanogenesis by the Citrus Flavonoid Naringenin in Mouse B16 Melanoma Cells. Bioscience, Biotechnology, and Biochemistry 70(6) (2006): 1499-1501.

[21] An, S., Kim, H., Kim, J., and Boo, Y. Flavonoids, taxifolin and luteolin attenuate cellular melanogenesis despite increasing tyrosinase protein levels. Phytotherapy Research 22(9) (2008): 1200-1207.

[22] Fujii, T. and Saito, M. Inhibitory Effect of Quercetin Isolated from Rose Hip (*Rosa canina* L.) against Melanogenesis by Mouse Melanoma Cells. Bioscience, Biotechnology and Biochemistry 73(9) (2009): 1989-1993.

[23] Horibe, I., Satoh, Y., Kumagai, A., Horike, N., Takemori, H., Uesato, S., Sugie, S., Obata, K., Kawahara, H., and Nagaoka, Y. Induction of melanogenesis by 4′-O-methylated flavonoids in B16F10 melanoma cells. Journal of Natural Medicines 67(4) (2013): 705-710.

[24] Takekoshi, S., Nagata, H., and Kitatani, K. Flavonoids Enhance Melanogenesis in Human Melanoma Cells. The Tokai journal of experimental and clinical medicine 39 (2014): 116-121.

[25] Yenjai, C., Prasanphen, K., Daodee, S., Wongpanich, V., and Kittakoop, P. Bioactive flavonoids from *Kaempferia parviflora.* Fitoterapia 75(1) (2004): 89-92.

[26] Bhamarapravati, S., Juthapruth, S., Mahachai, W., and Mahady, G. Antibacterial activity of *Boesenbergia rotunda* (L.) Mansf. and *Myristica fragrans* Houtt. agains H*elicobacter pylori.* Songklanakarin Journal of Science and Technology 28 (2006): 157-163.

[27] Tewtrakul, S., Subhadhirasakul, S., Karalai, C., Ponglimanont, C., and Cheenpracha, S. Anti-inflammatory effects of compounds from *Kaempferia*

parviflora and *Boesenbergia pandurata*. Food Chemistry 115(2) (2009): 534-538.

[28] Isa, M., Abdelwahab, S., Mohan, S., Abdul, A., Sukari, M., Taha, M., Syam, S., Narrima, P., Cheah, S., Ahmad, S., and Mustafa, M. In vitro anti-inflammatory, cytotoxic and antioxidant activities of boesenbergin A, a chalcone isolated from *Boesenbergia rotunda* (L.) (fingerroot). Brazilian Journal of Medical and Biological Research 45(6) (2012): 524-530.

[29] Ninomiya, K., Matsumoto, T., Chaipech, S., Miyake, S., Katsuyama, Y., Tsuboyama, A. Simultaneous quantitative analysis of 12 methoxyflavones with melanogenesis inhibitory activity from the rhizomes of *Kaempferia parviflora*. Journal of Natural Medicines 70(2) (2016): 179-189.

[30] hin, J., Kim, K., Kim, M., Jeong, J., and Kim, B. Synthesis and hypoglycemic effect of chrysin derivatives. Bioorganic and Medicinal Chemistry Letters 9(6) (1999): 869-874.

[31] heng, X., Meng, W., Xu, Y., Cao, J., and Qing, F. Synthesis and anticancer effect of chrysin derivatives. Bioorganic and Medicinal Chemistry Letters 13(5) (2003): 881-884.

[32] Park, H., Dao, T., and Kim, H. Synthesis and inhibition of PGE_2 production of 6,8-disubstituted chrysin derivatives. European Journal of Medicinal Chemistry 40 (2005): 943–948

[33] Li, S., Pan, M., Lai, C., Lo, C., Dushenkov, S., and Ho, C. Isolation and syntheses of polymethoxyflavones and hydroxylated polymethoxyflavones as inhibitors of HL-60 cell lines. Bioorganic and Medicinal Chemistry 15(10) (2007): 3381-3389.

[34] Lv, P., Wang, K., Li, Q., Chen, J., Sun, J., and Zhu, H. Design, synthesis and biological evaluation of chrysin long-chain derivatives as potential anticancer agents. Bioorganic and Medicinal Chemistry 18(3) (2010): 1117-1123.

[35] Yamauchi, K., Mitsunaga, T., Inagaki, M., and Suzuki, T. Synthesized quercetin derivatives stimulate melanogenesis in B16 melanoma cells by influencing the expression of melanin biosynthesis proteins MITF and p38 MAPK. Bioorganic and Medicinal Chemistry 22(13) (2014): 3331-3340.

[36] Cheng, N., Yi, W., Wang, Q., Peng, S., and Zou, X. Synthesis and α-glucosidase inhibitory activity of chrysin, diosmetin, apigenin, and luteolin derivatives. Chinese Chemical Letters 25 (2014): 1094–1098.

[37] Nguyen, T., Nguyen, K., Kamounah, F., Zhang, W., and Hansen, P. NMR of a series of novel hydroxyflavothiones. Magnetic resonance in chemistry 47: 1043-1054.

[38] Do, T., Vo, P., and Tran, T. Synthesis and Comparison of Anti-inflammatory Activity of Chrysin Derivatives. 13rd international electronic conference on synthetic organic chemistry (ECSOC-13), 1-30 November 2009 (2009).

[39] Park, H., Dao, T., and Kim, H. Synthesis and inhibition of PGE_2 production of 6,8-disubstituted chrysin derivatives. European Journal of Medicinal Chemistry 40(9) (2005): 943-948.

[40] Boumendjel, A., Boccard, J., Carrupt, P., Nicolle, E., Blanc, M., Geze, A., Choisnard, L., Wouessidjewe, D., Matera, E., and Dumontet, C. Antimitotic and Antiproliferative Activities of Chalcones: Forward Structure–Activity Relationship. Journal of Medicinal Chemistry 51(7) (2008): 2307-2310.

[41] Cabrera, M., Simoens, M., Falchi, G., Lavaggi, M., Piro, O., Castellano, E., Vidal, A., Azqueta, A., Monge, A., Cera´in, A., Sagrera G., Seoane, G., Cerecettoa, H., and Gonza´lez, M. Synthetic chalcones, flavanones, and flavones as antitumoral agents: Biological evaluation and structure–activity relationships. Bioorganic and Medicinal Chemistry 15(10) (2007): 3356-3367.

[42] Zuo, L., Yao, S., Wang, W., and Duan, W. An efficient method for demethylation of aryl methyl ethers. Tetrahedron Letters 49(25) (2008): 4054-4056.

[43] Kul'magambetova, E., Yamovoi, V., Kusainova, D., Pak, R., Kulyyasov, A., Turdybekov, K., Adekenov, S., and Gatilov, Y. Synthesis and Structure of Pinostrobin Oxime and Its Biological Activity. Chemistry of Natural Compounds 38(6) (2002): 527-531.

[44] Rahman, A., Choudhary, M., and Thomson, W. Bioassay techinques for drug development Hardwood Academic Publishers 19 (2001).

[45] Chuah, E., Zakaria, Z., Suhaili, Z., Bakar, S., and Desa, M. Antimicrobial Activities of Plant Extracts against Methicillin-Susceptible and Methicillin-Resistant *Staphylococcus aureus*. Journal of Microbiology Research 4 (2014): 6-13.

[46] Tran, T., Do, T., Tran, N., Ngo, T., Huynh, T., Tran, C., and Thai, K. Synthesis and anti Methicillin resistant *Staphylococcus aureus* activity of substituted chalcones alone and in combination with non-beta-lactam antibiotics. Bioorganic and Medicinal Chemistry Letters 22(14) (2012): 4555-4560.

[47] Salleh, W., Ahmad, F., and Yen, K. Chemical constituents from *Piper caninum* and antibacterial activity. Journal of Applied Pharmaceutical Science 5 (2015): 20-25.

[48] Ávila, H., Smânia, E., Monache, F., and Smânia, A. Structure–activity relationship of antibacterial chalcones. Bioorganic and Medicinal Chemistry 16(22) (2008): 9790-9794.

[49] Fernandez, K., Sudhakaran, S., and Jegathambigai, R. Antimicrobial activity of the crude extract of Piper sarmentosum against methicilin-resistant Staphylococcus aureus (MRSA), Escherichia coli, Vibrio cholera and Streptococcus pneumoniae. European Review for Medical and Pharmacological Sciences 16 (2012): 105-111.

[50] Roberts, N., Curtis, S., Milan, A., and Ranganath, L. The Pigment in Alkaptonuria Relationship to Melanin and Other Coloured Substances: A Review of Metabolism, Composition and Chemical Analysis. Journal of Inherited Metabolic Disease 24 (2015): 51-66.

[51] Kumar, K., Yang, J., Chu, F., Chang, S., and Wang, S. Lucidone, a novel melanin inhibitor from the fruit of Lindera erythrocarpa Makino. Phytotherapy Research 24(8) (2010): 1158-1165.

APPENDIX

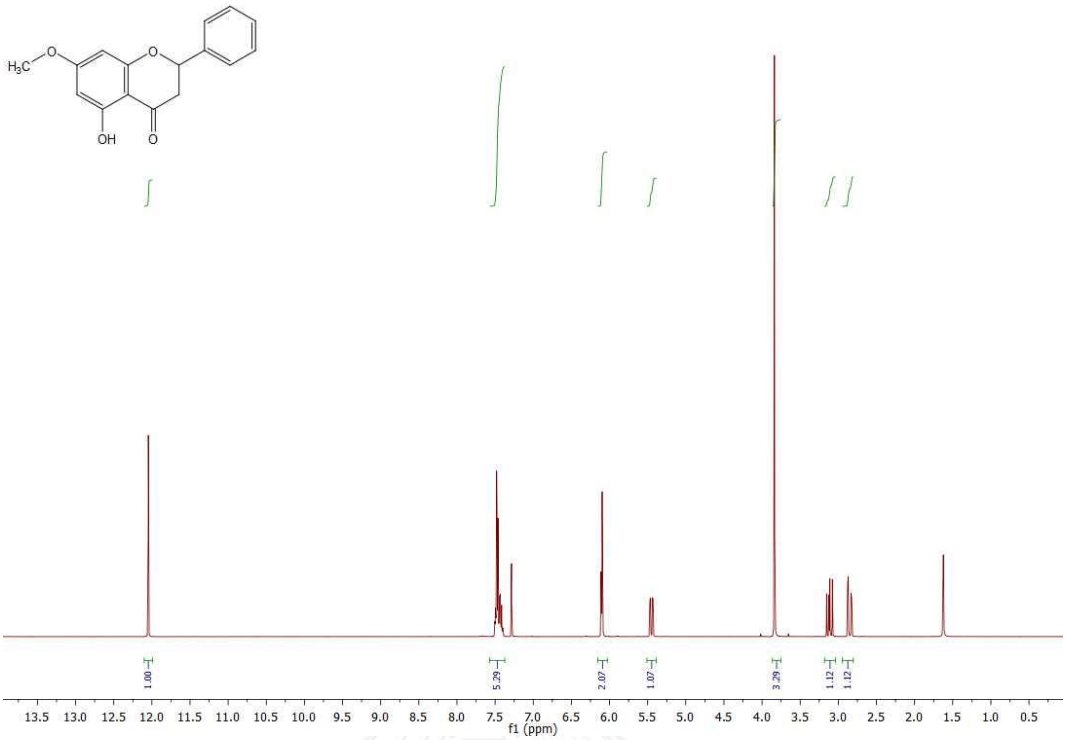


Figure A.1 ^{1}H-NMR (400 MHz) spectrum of compound **20** ($CDCl_3$)

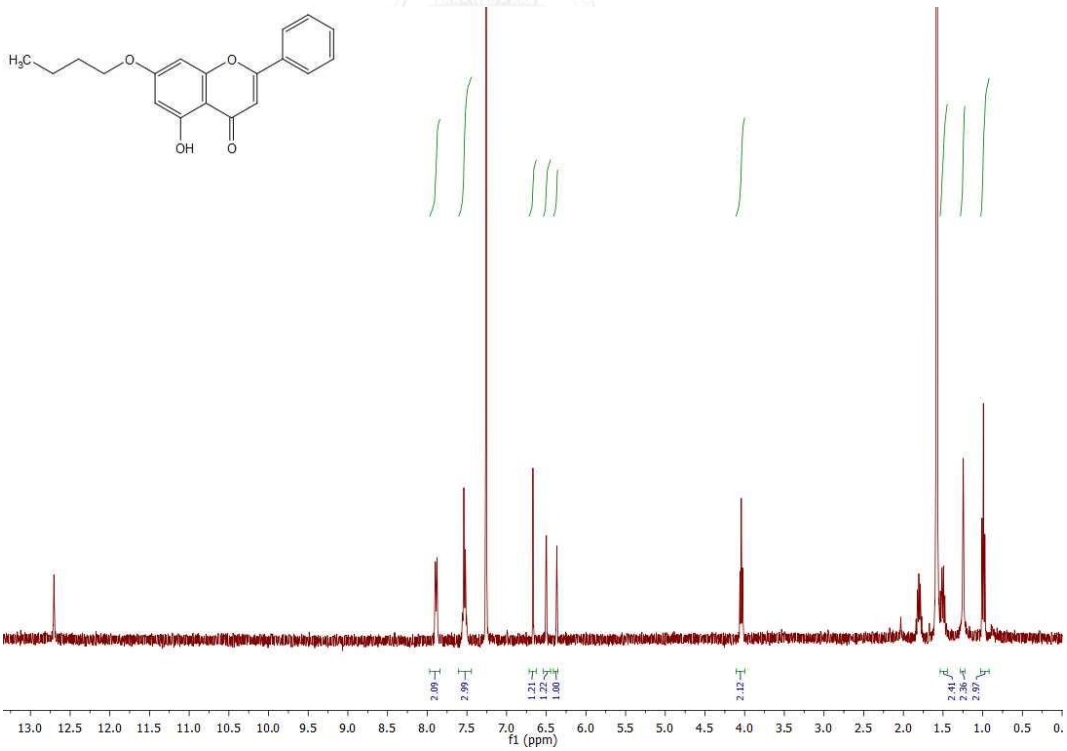


Figure A.2 ^{1}H-NMR (400 MHz) spectrum of compound **28** ($CDCl_3$)

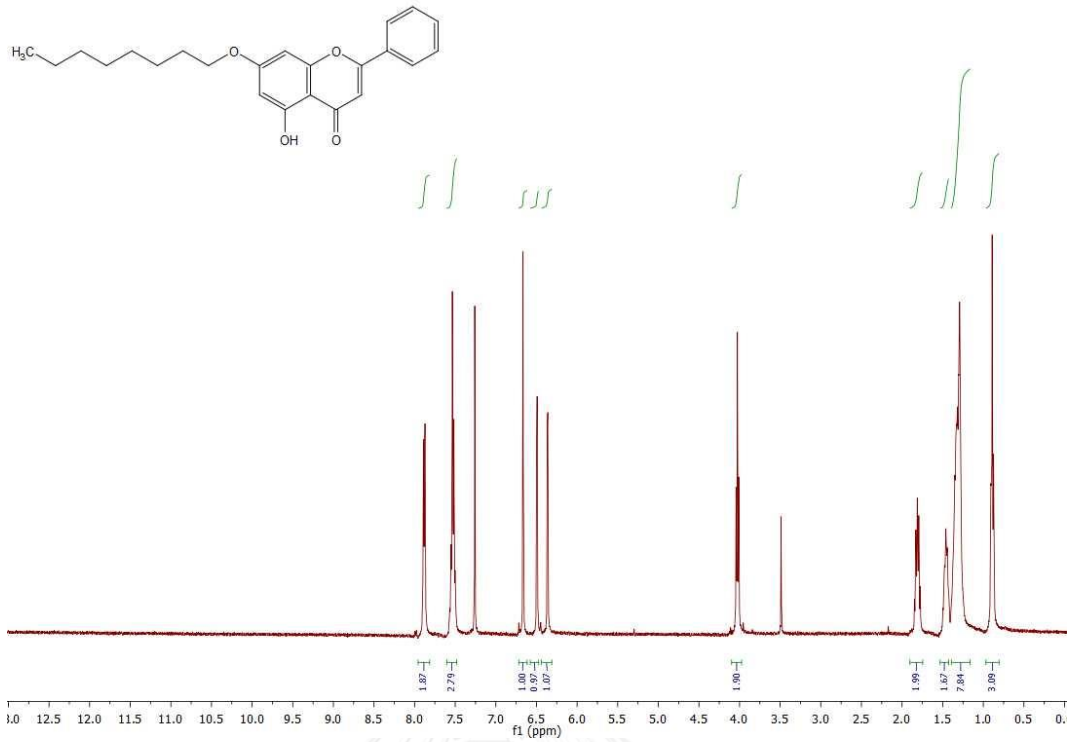


Figure A.3 ^{1}H-NMR (400 MHz) spectrum of compound **29** ($CDCl_3$)

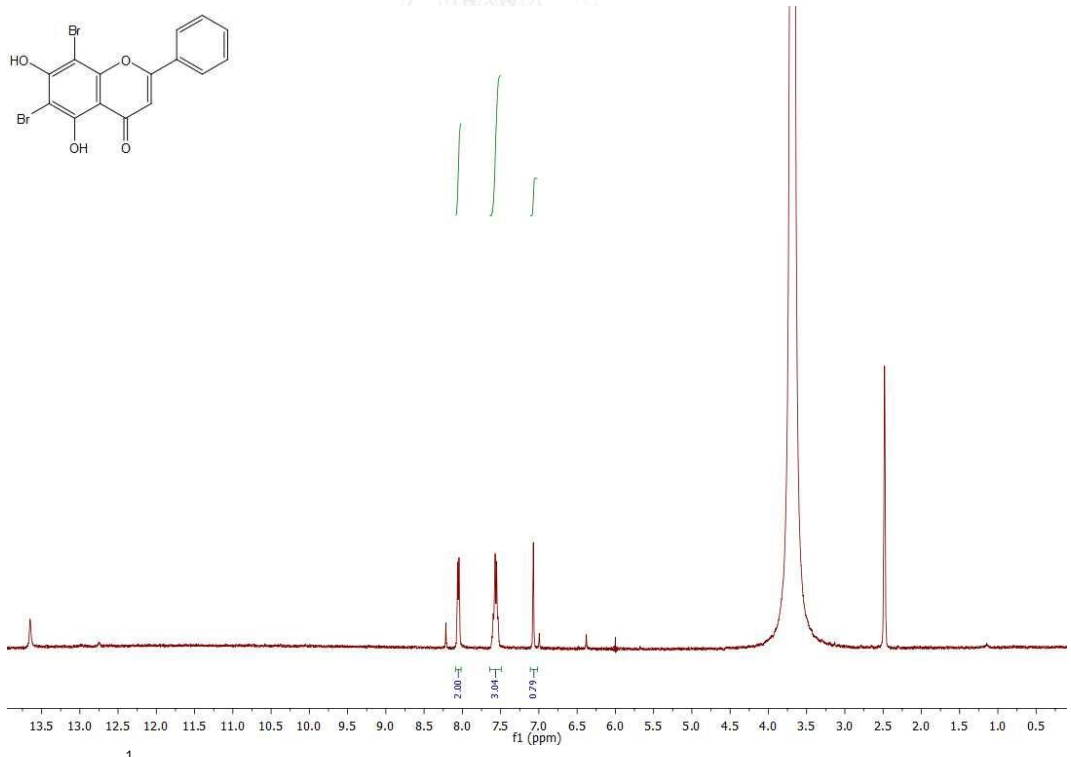


Figure A.4 ^{1}H-NMR (400 MHz) spectrum of compound **33** (DMOS-d_6)

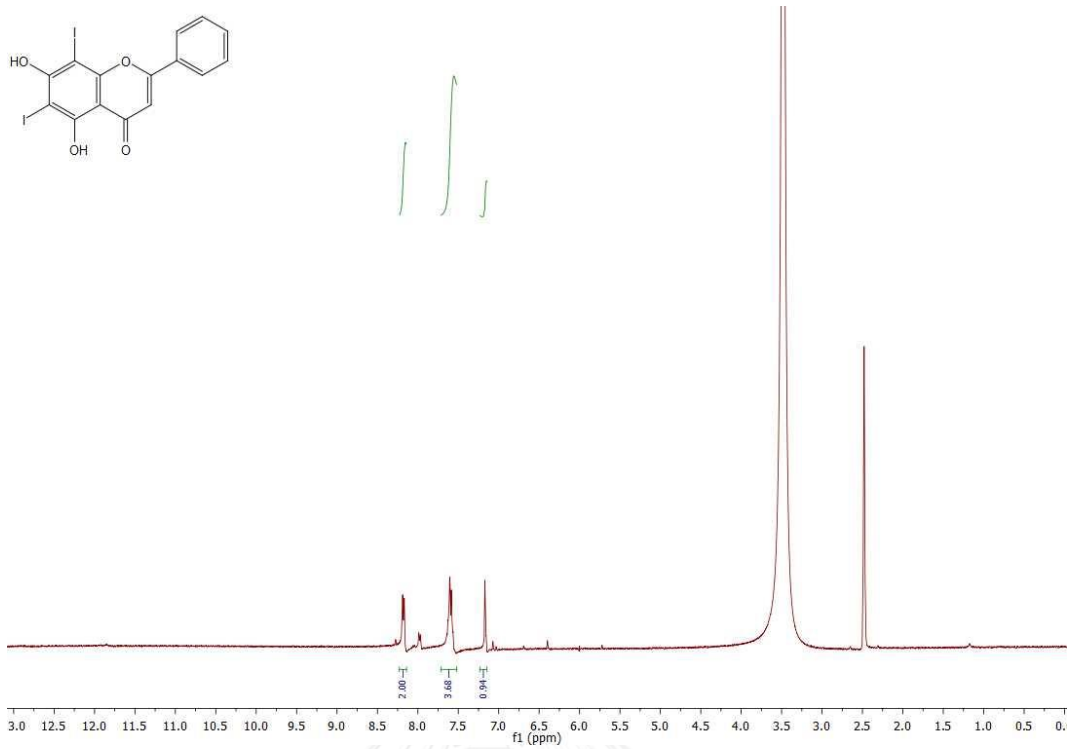


Figure A.5 ^{1}H-NMR (400 MHz) spectrum of compound **34** (DMOS-d_6)

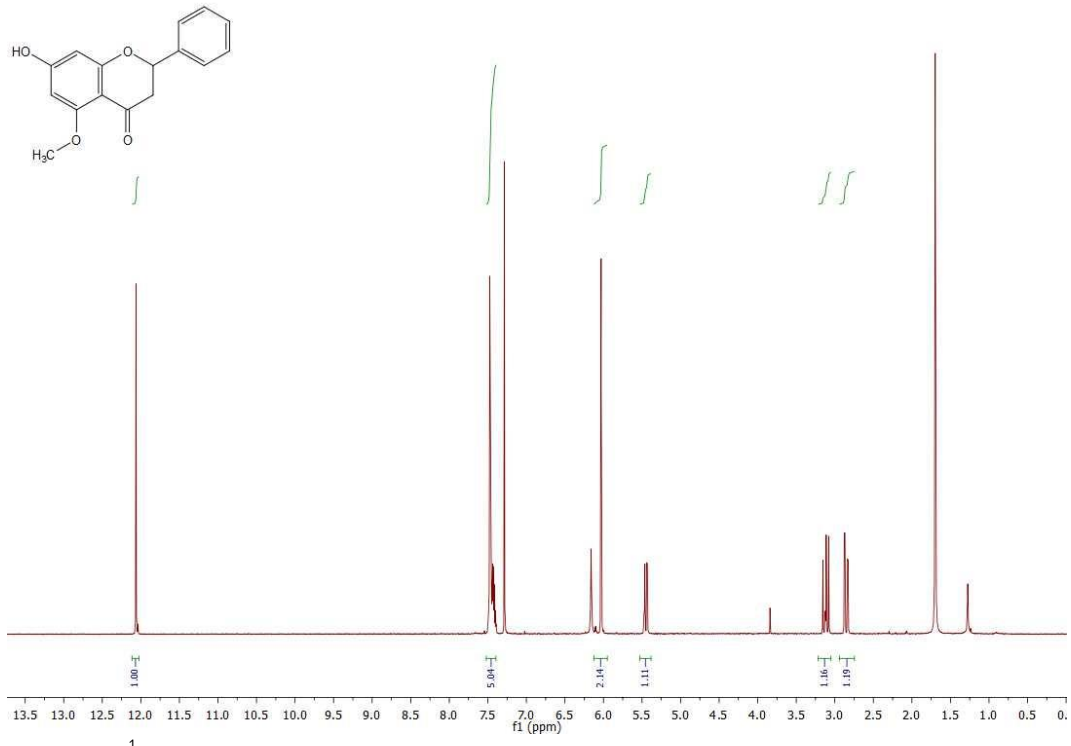


Figure A.6 ^{1}H-NMR (400 MHz) spectrum of compound **43** ($CDCl_3$)

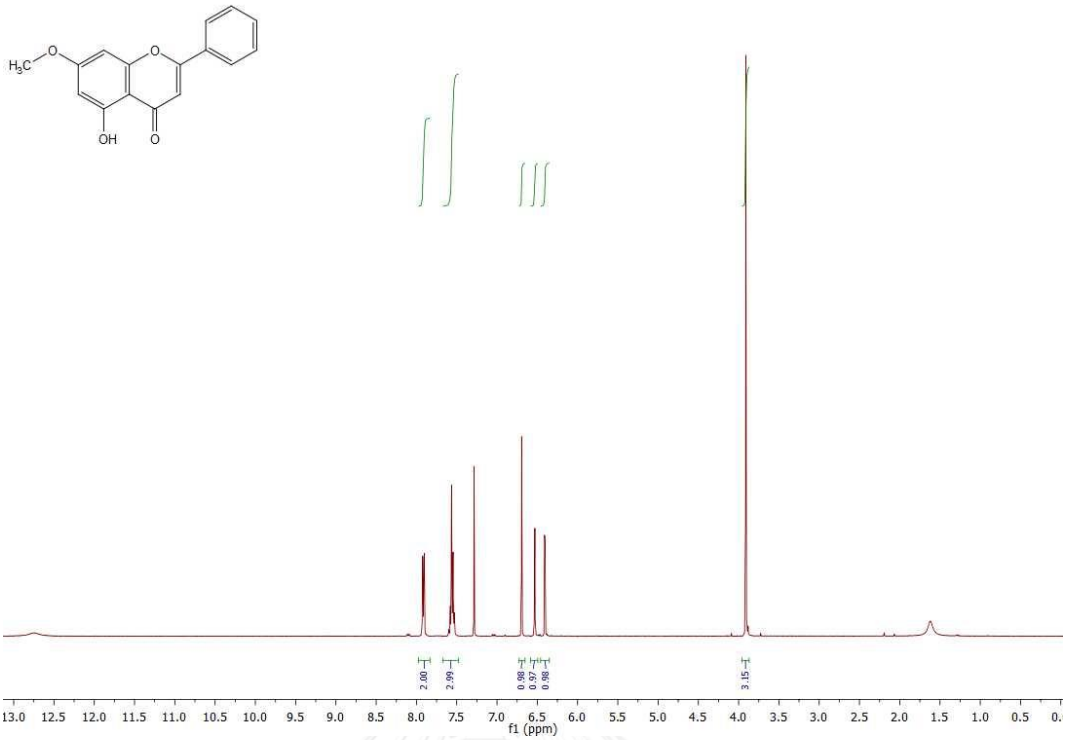


Figure A.7 ^{1}H-NMR (400 MHz) spectrum of compound **44** ($CDCl_3$)

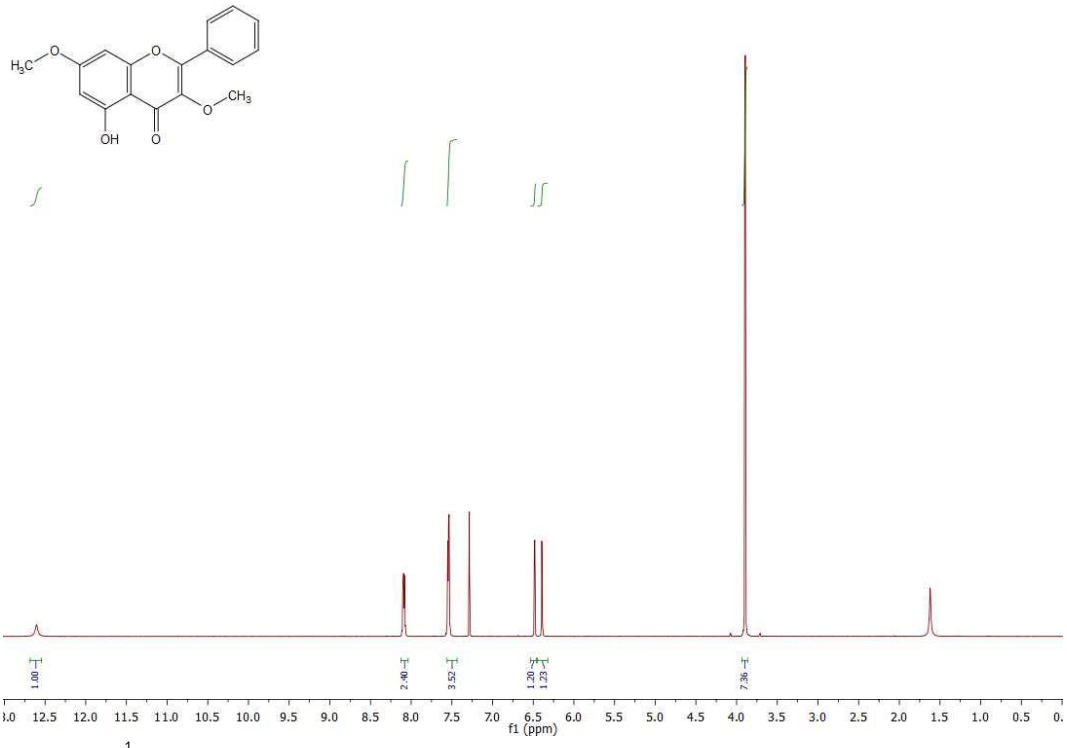


Figure A.8 ^{1}H-NMR (400 MHz) spectrum of compound **45** ($CDCl_3$)

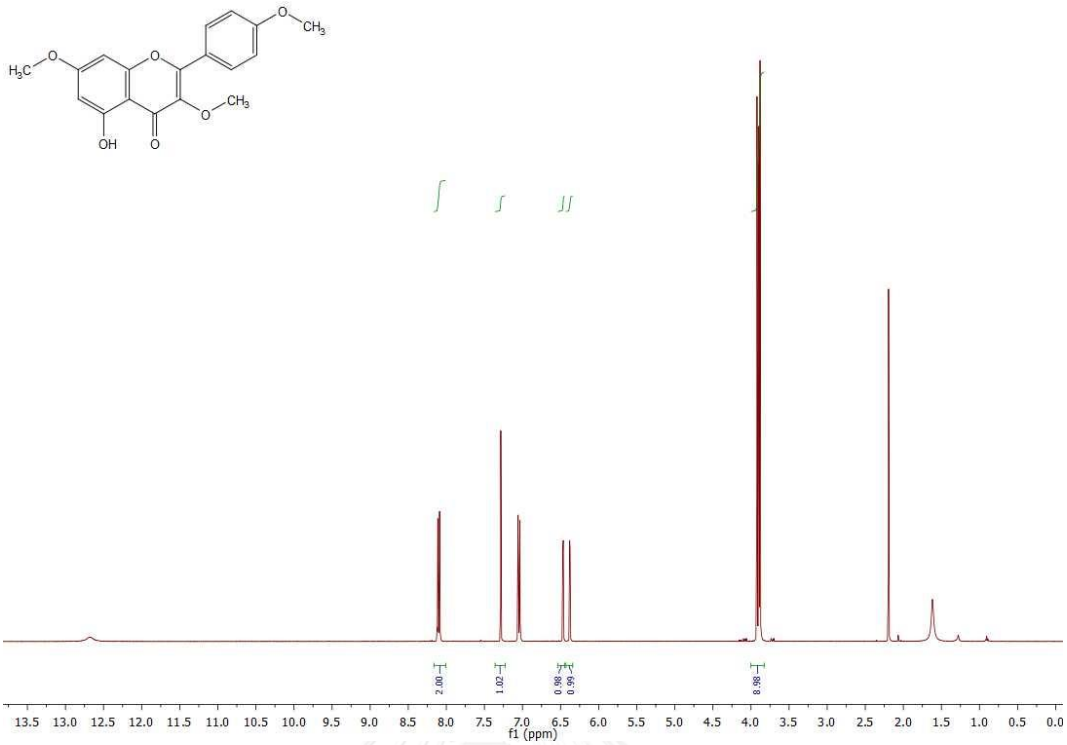


Figure A.9 ^{1}H-NMR (400 MHz) spectrum of compound **46** ($CDCl_3$)

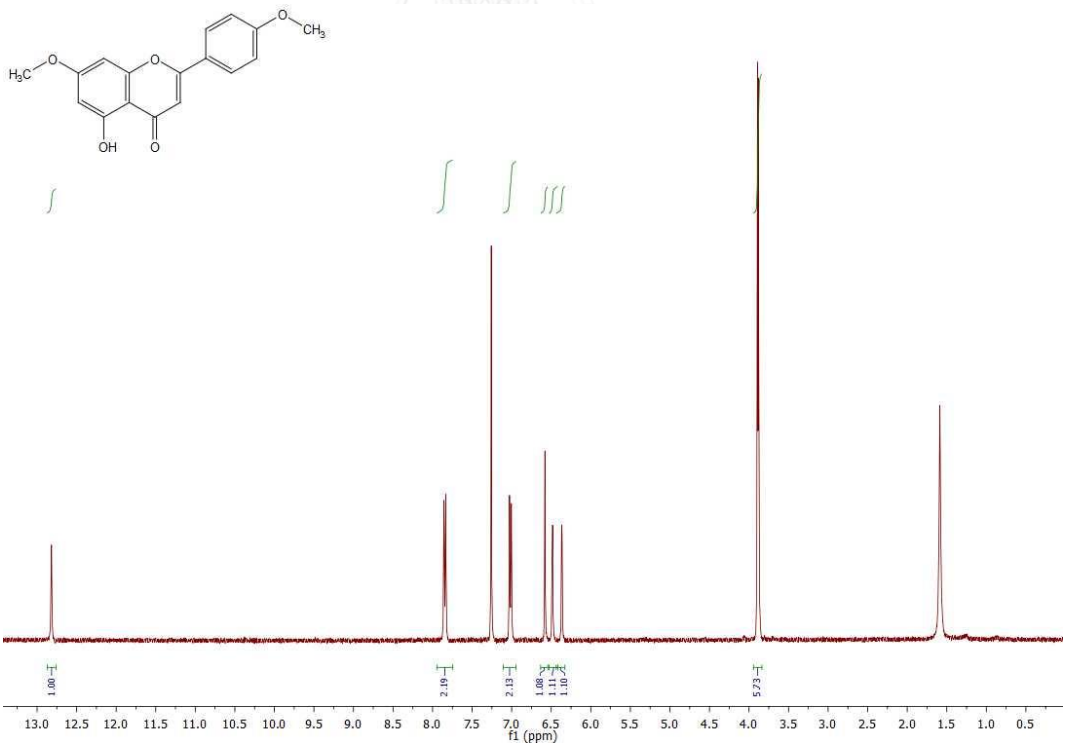


Figure A.10 ^{1}H-NMR (400 MHz) spectrum of compound **47** ($CDCl_3$)

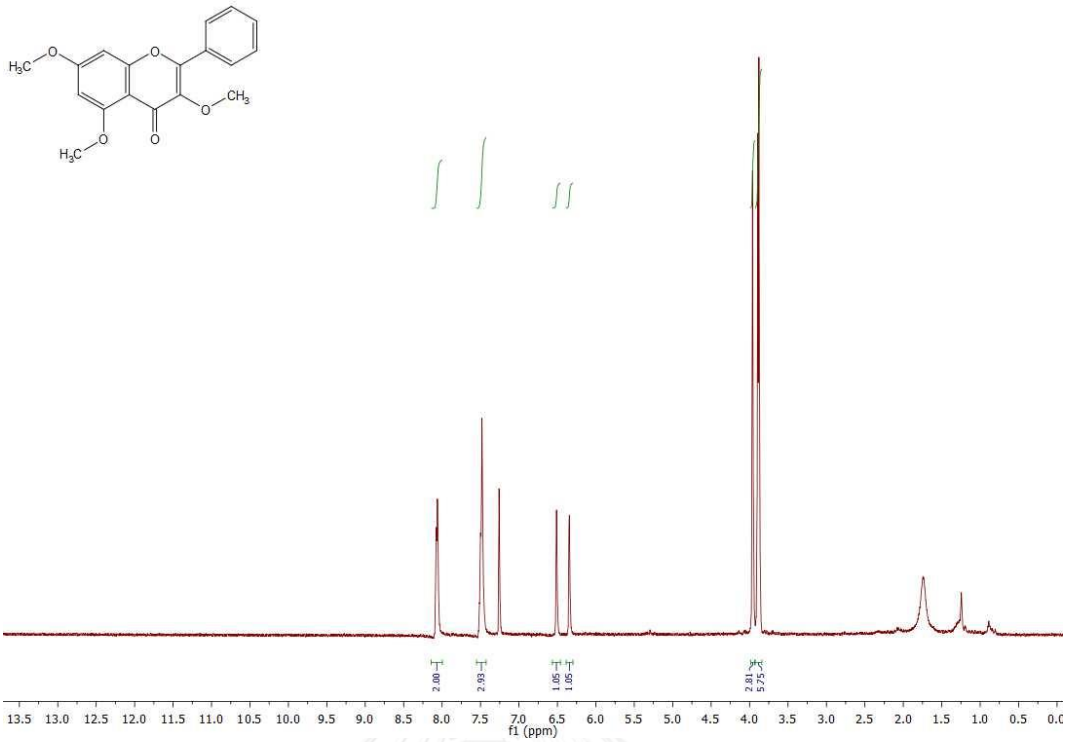


Figure A.11 ^{1}H-NMR (400 MHz) spectrum of compound **48** ($CDCl_3$)

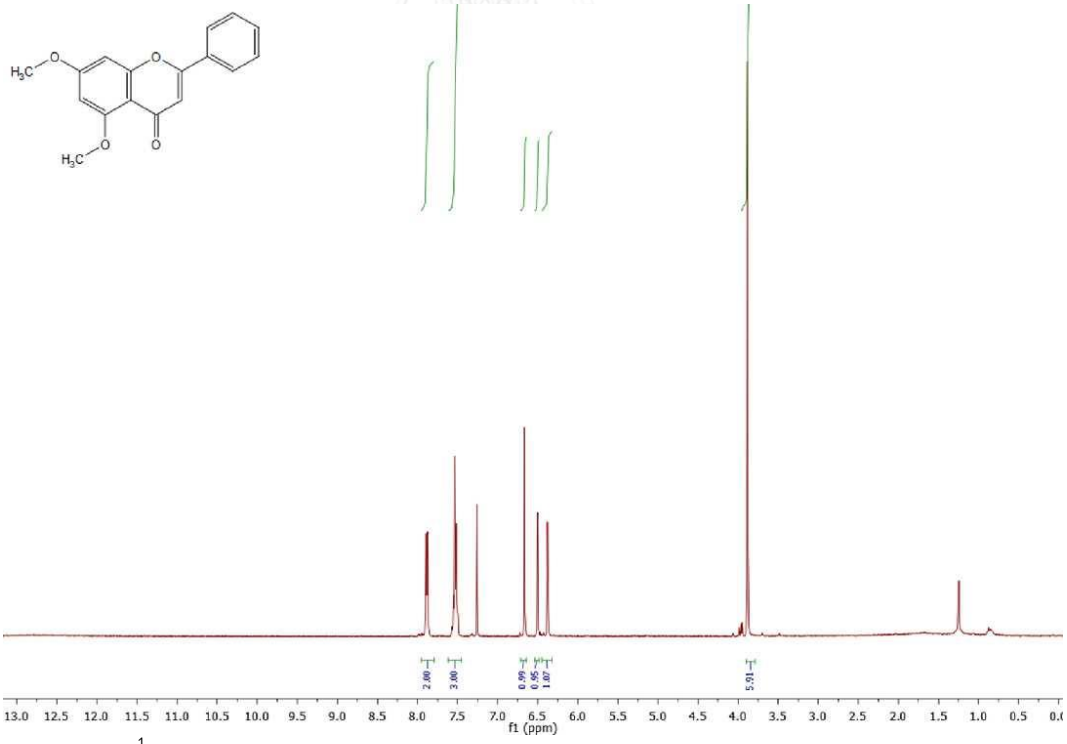


Figure A.12 ^{1}H-NMR (400 MHz) spectrum of compound **49** ($CDCl_3$)

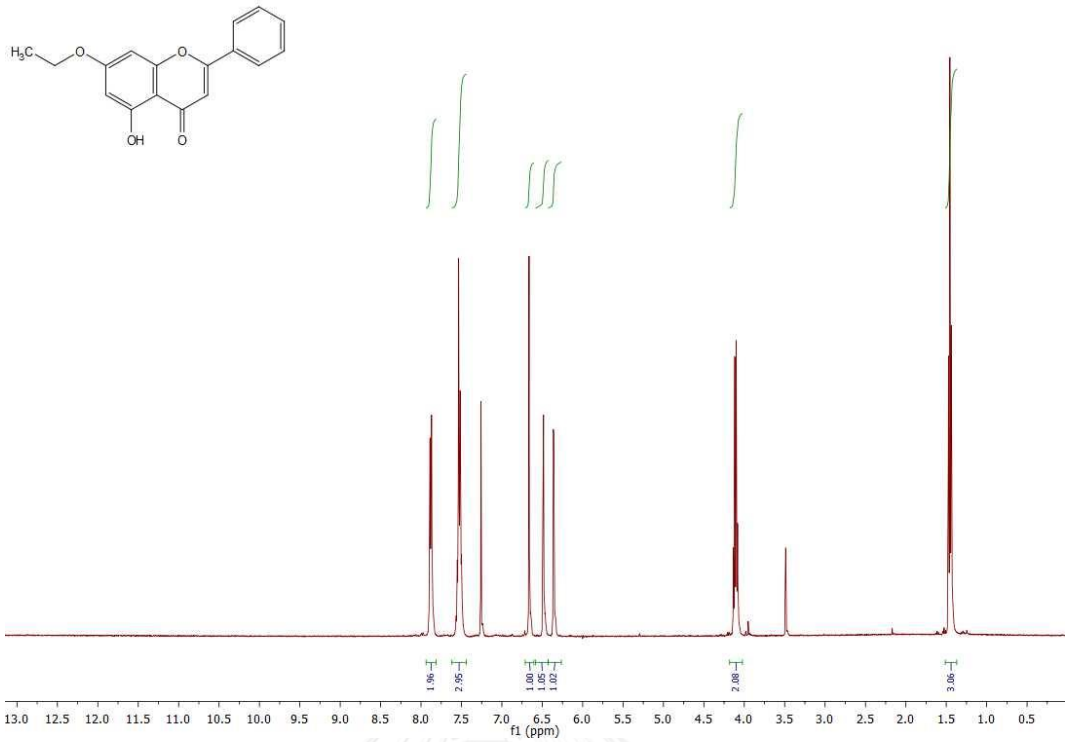


Figure A.13 ^{1}H-NMR (400 MHz) spectrum of compound **50** ($CDCl_3$)

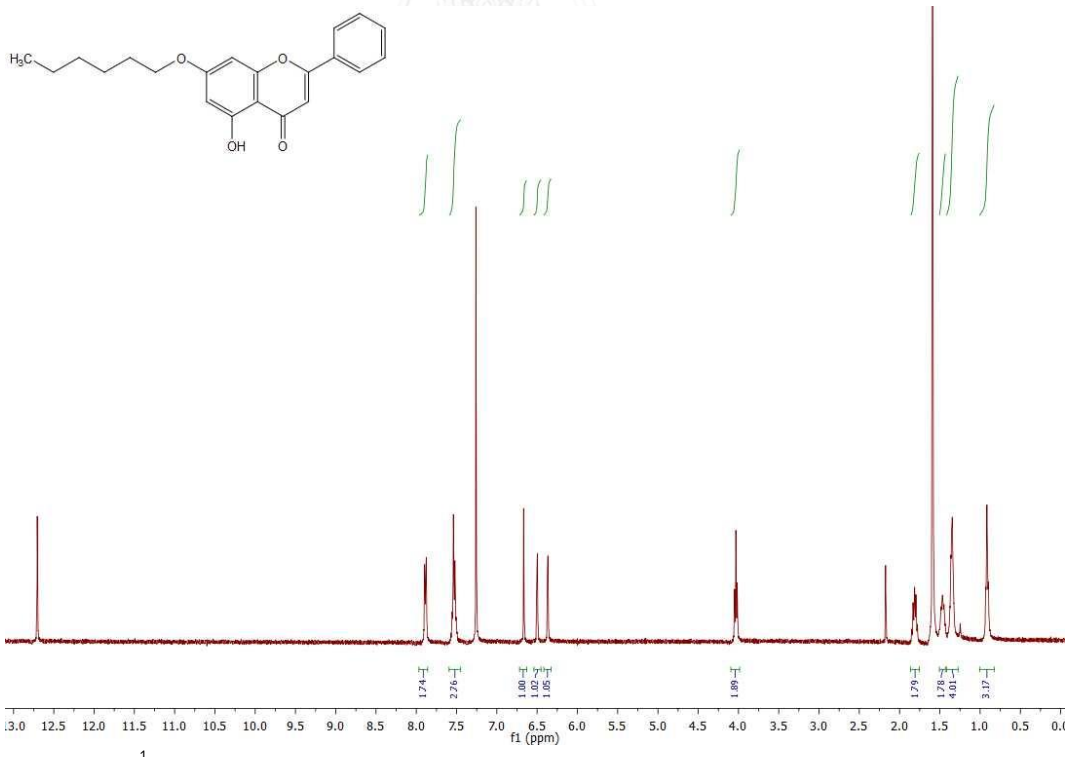


Figure A.14 ^{1}H-NMR (400 MHz) spectrum of compound **51** ($CDCl_3$)

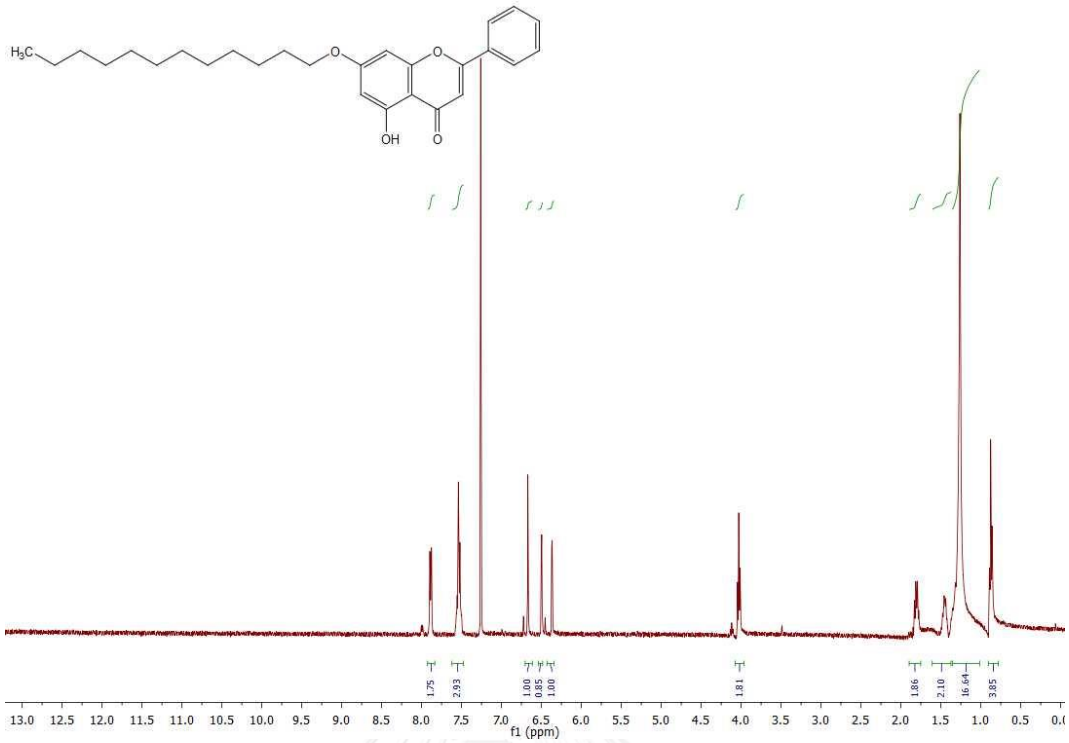


Figure A.15 ^{1}H-NMR (400 MHz) spectrum of compound **52** ($CDCl_3$)

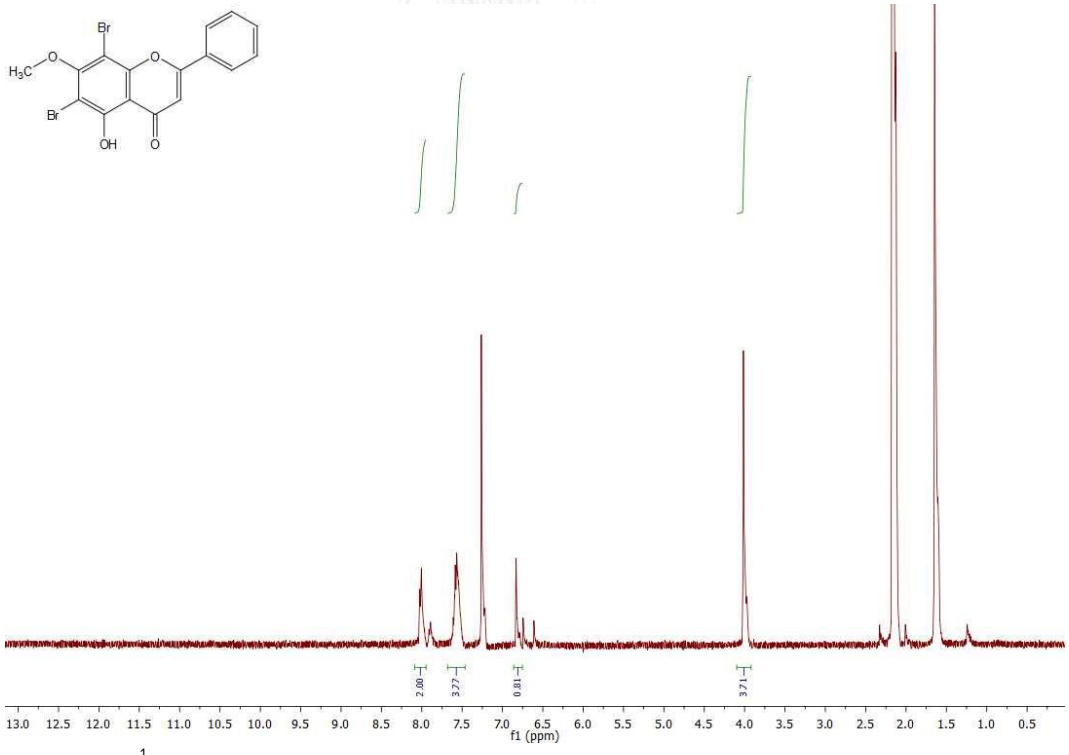


Figure A.16 ^{1}H-NMR (400 MHz) spectrum of compound **58** ($CDCl_3$)

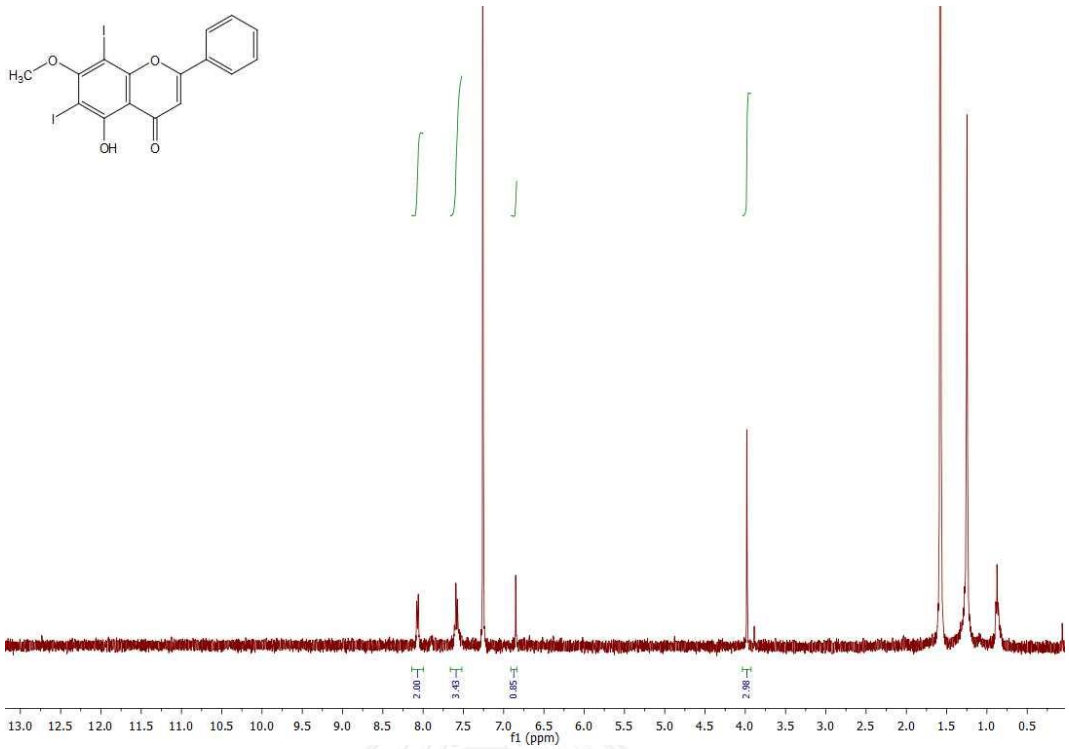


Figure A.17 ^{1}H-NMR (400 MHz) spectrum of compound **59** ($CDCl_3$)

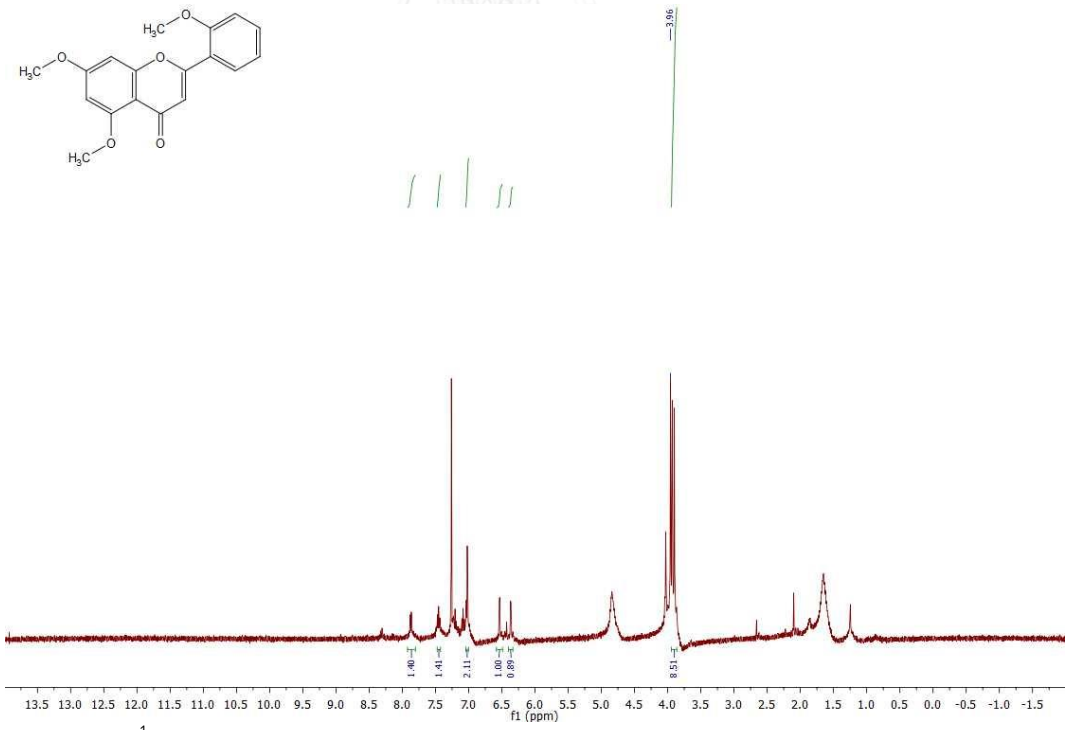


Figure A.18 ^{1}H-NMR (400 MHz) spectrum of compound **62** ($CDCl_3$)

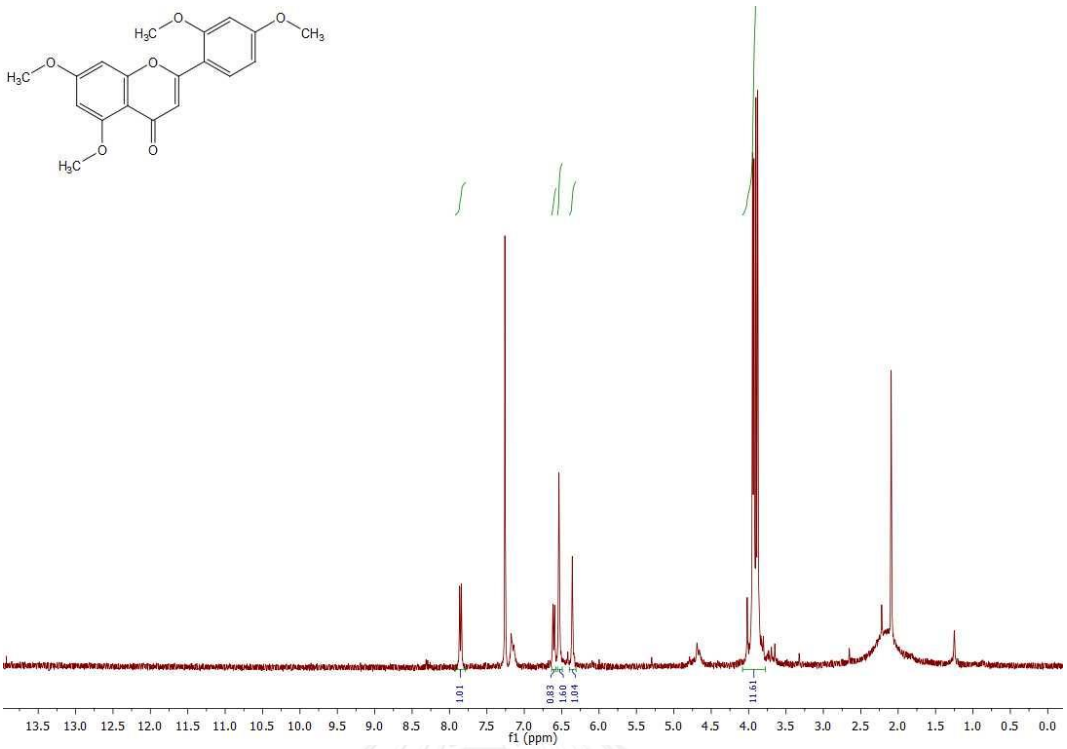


Figure A.19 ^{1}H-NMR (400 MHz) spectrum of compound **63** ($CDCl_3$)

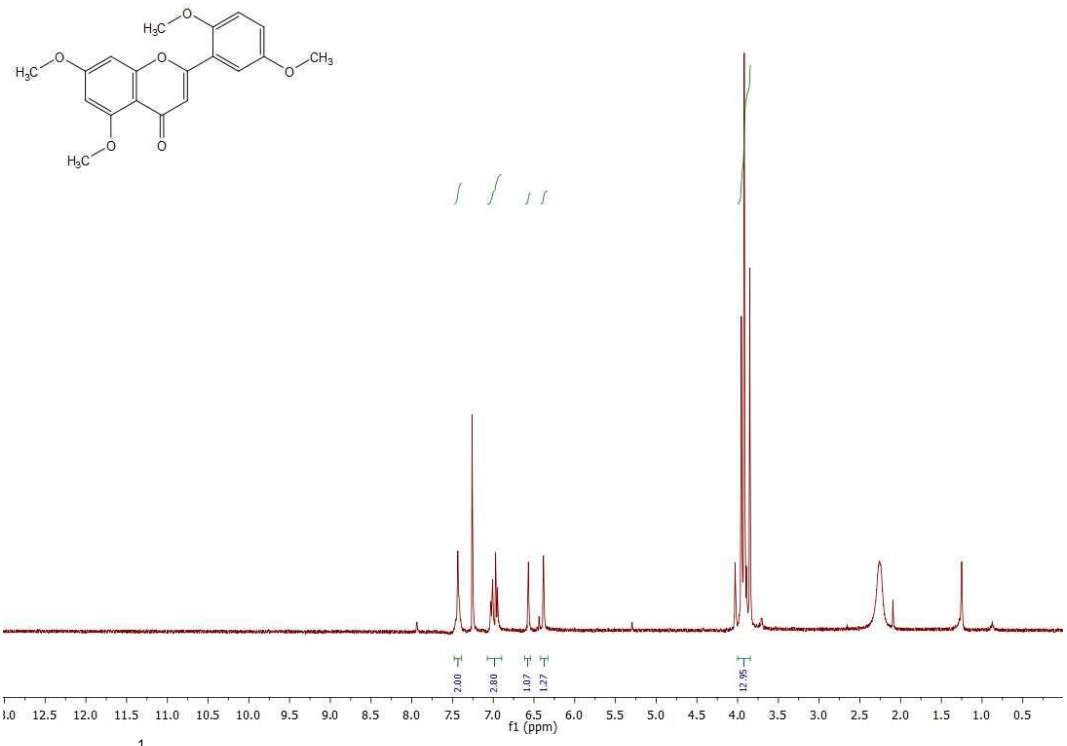


Figure A.20 ^{1}H-NMR (400 MHz) spectrum of compound **64** ($CDCl_3$)

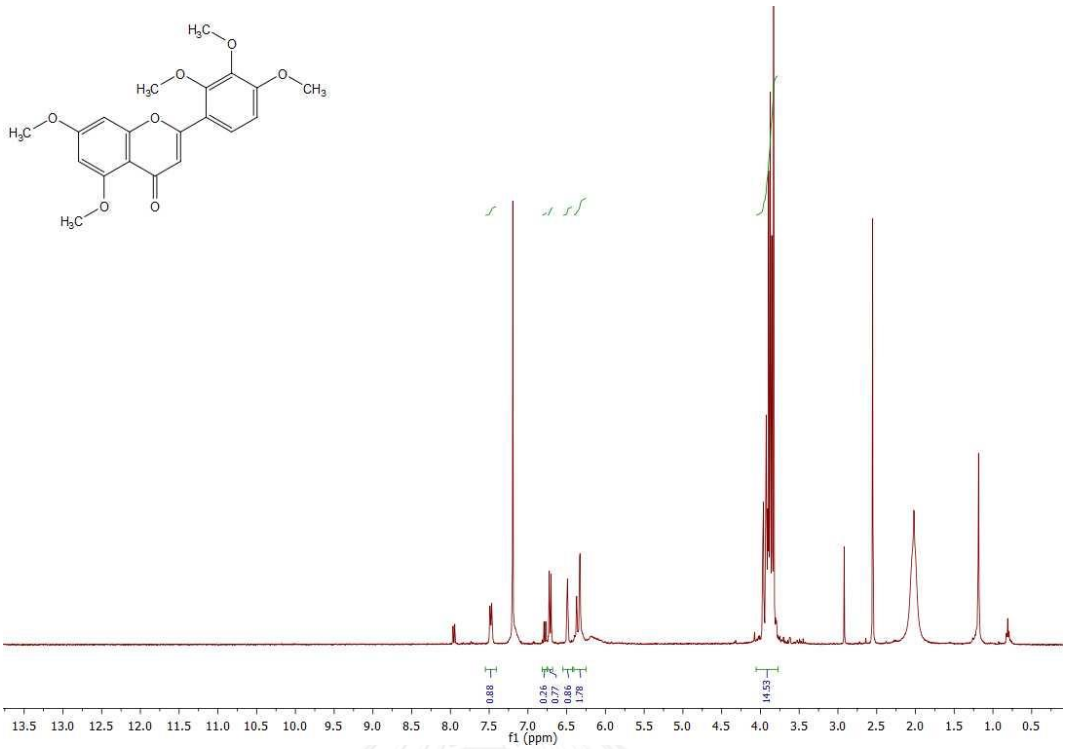


Figure A.21 ^{1}H-NMR (400 MHz) spectrum of compound **65** ($CDCl_3$)

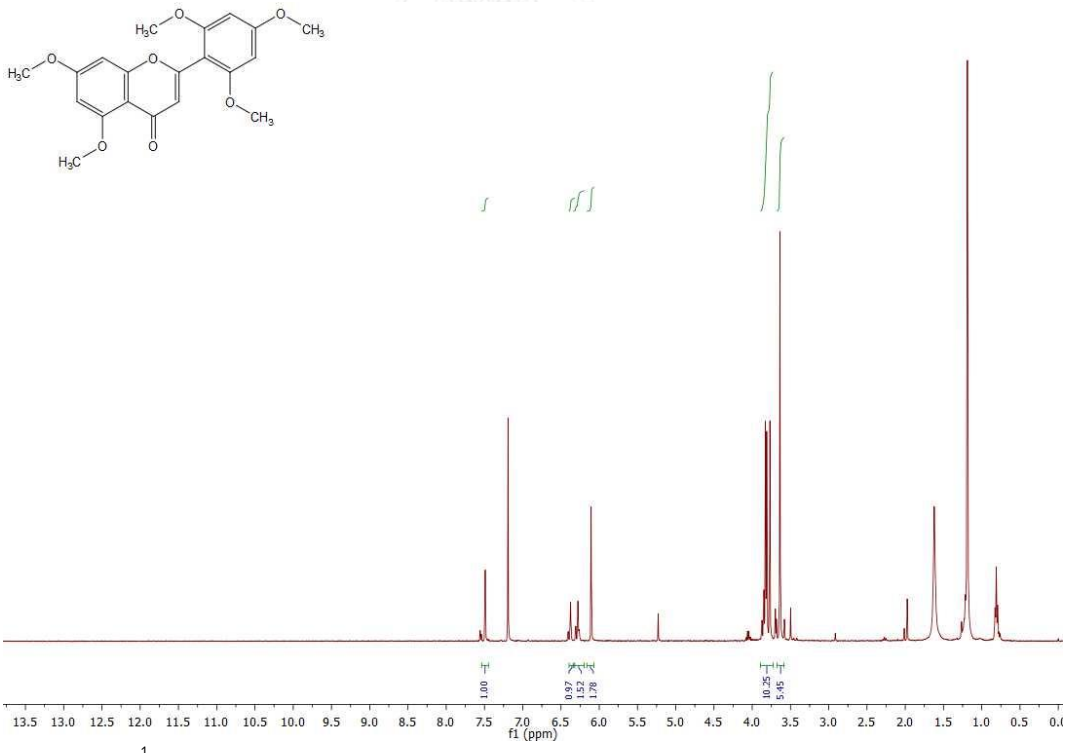


Figure A.22 ^{1}H-NMR (400 MHz) spectrum of compound **66** ($CDCl_3$)

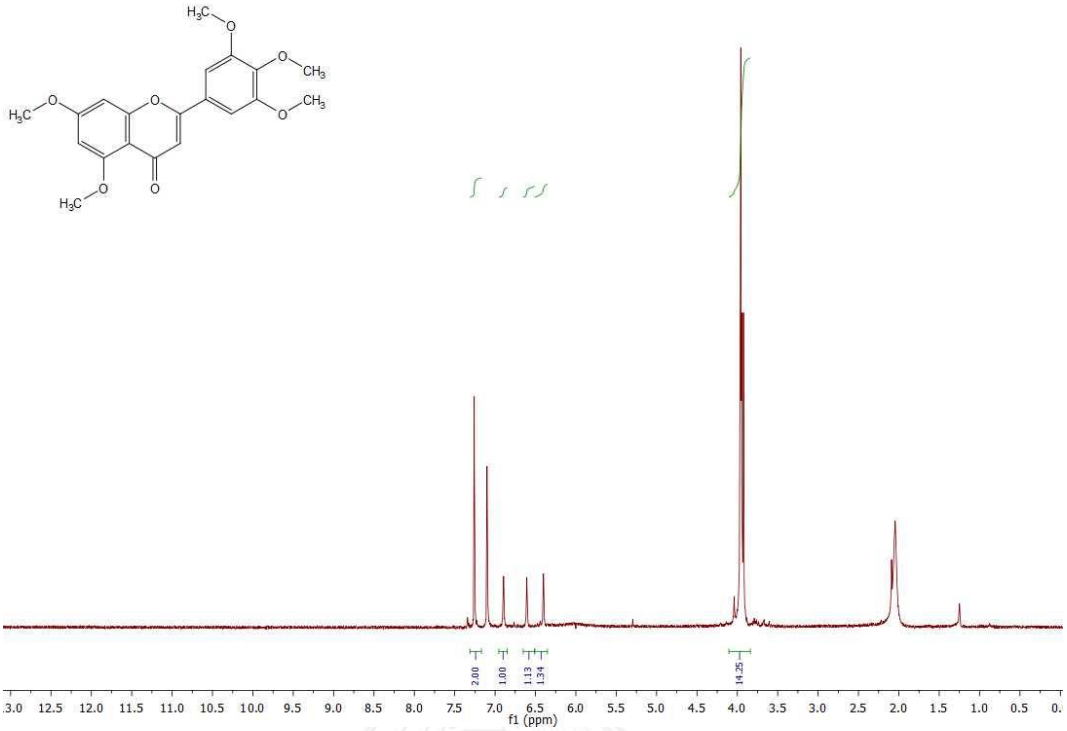


Figure A.23 ^{1}H-NMR (400 MHz) spectrum of compound **67** ($CDCl_3$)

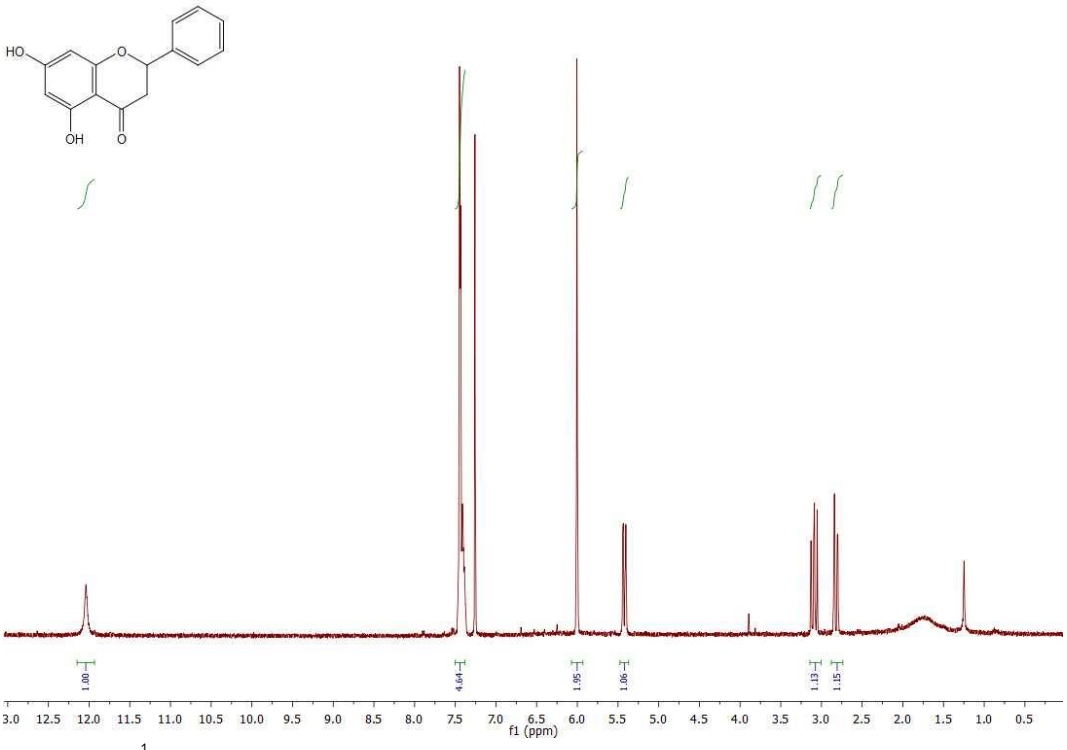


Figure A.24 ^{1}H-NMR (400 MHz) spectrum of compound **68** ($CDCl_3$)

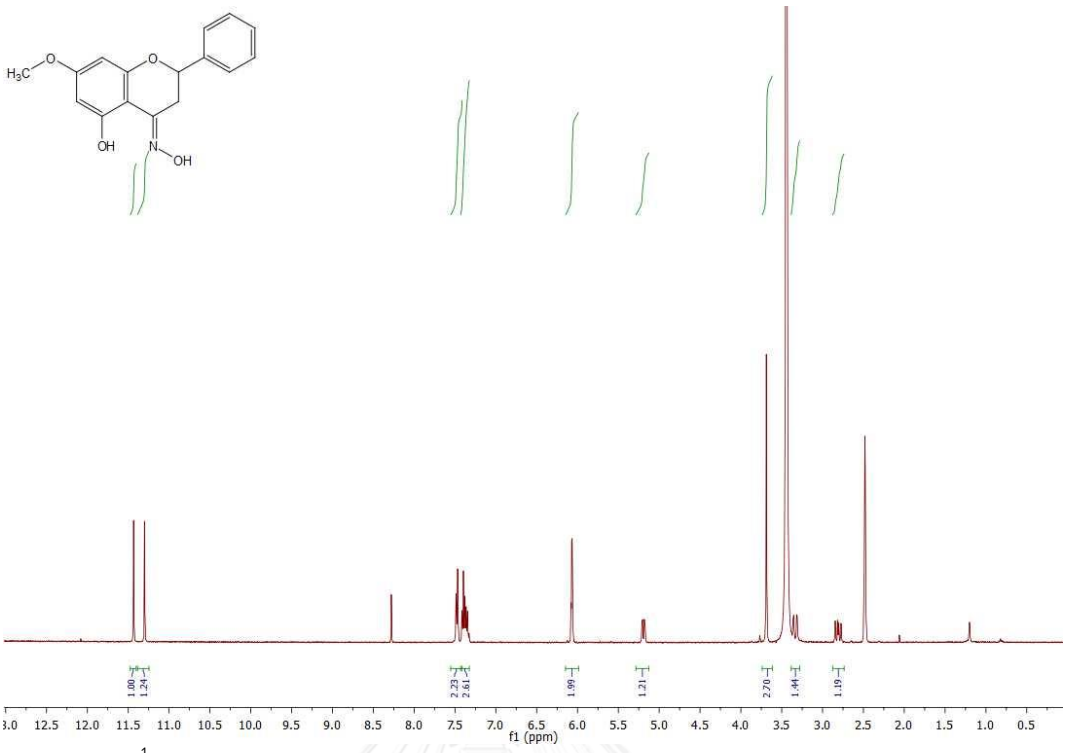


Figure A.25 ^{1}H-NMR (400 MHz) spectrum of compound **69** (DMSO-d_6)

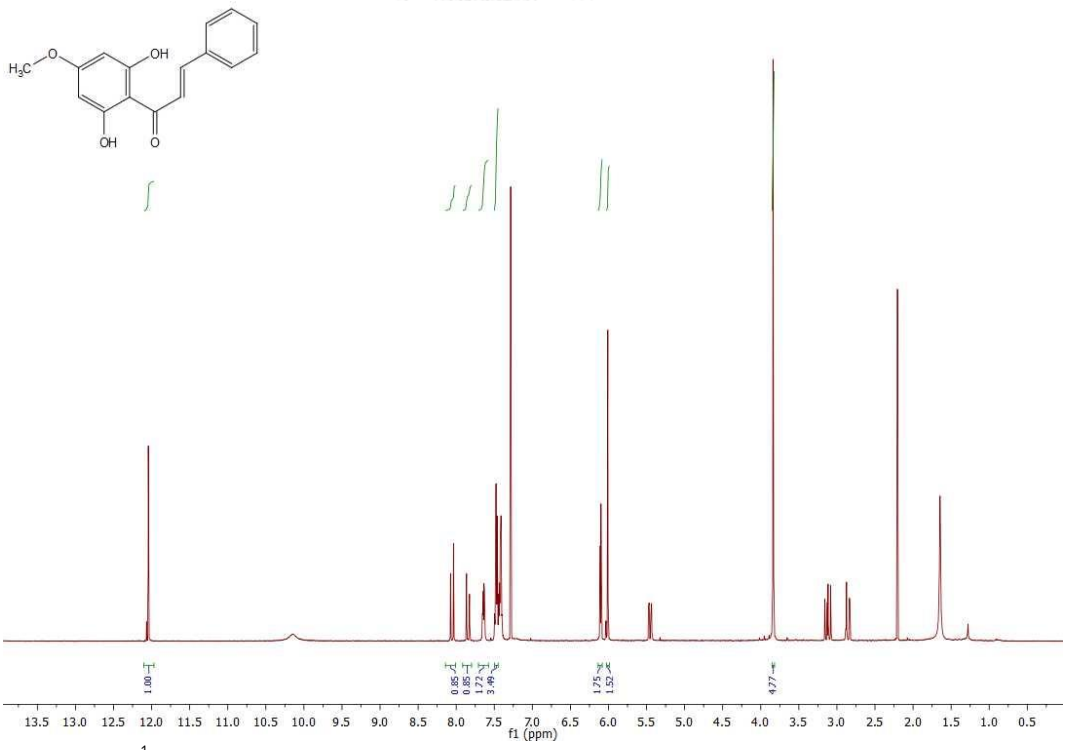


Figure A.26 ^{1}H-NMR (400 MHz) spectrum of compound **70** ($CDCl_3$)

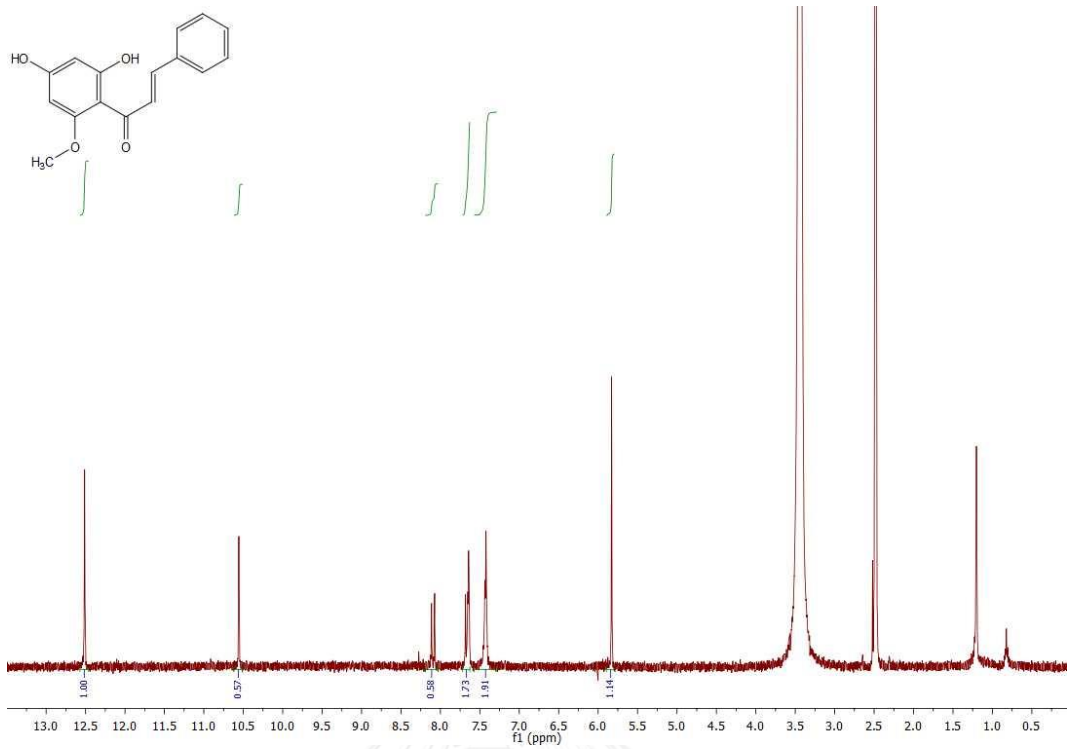


Figure A.27 ^{1}H-NMR (400 MHz) spectrum of compound **71** (DMSO-d_6)

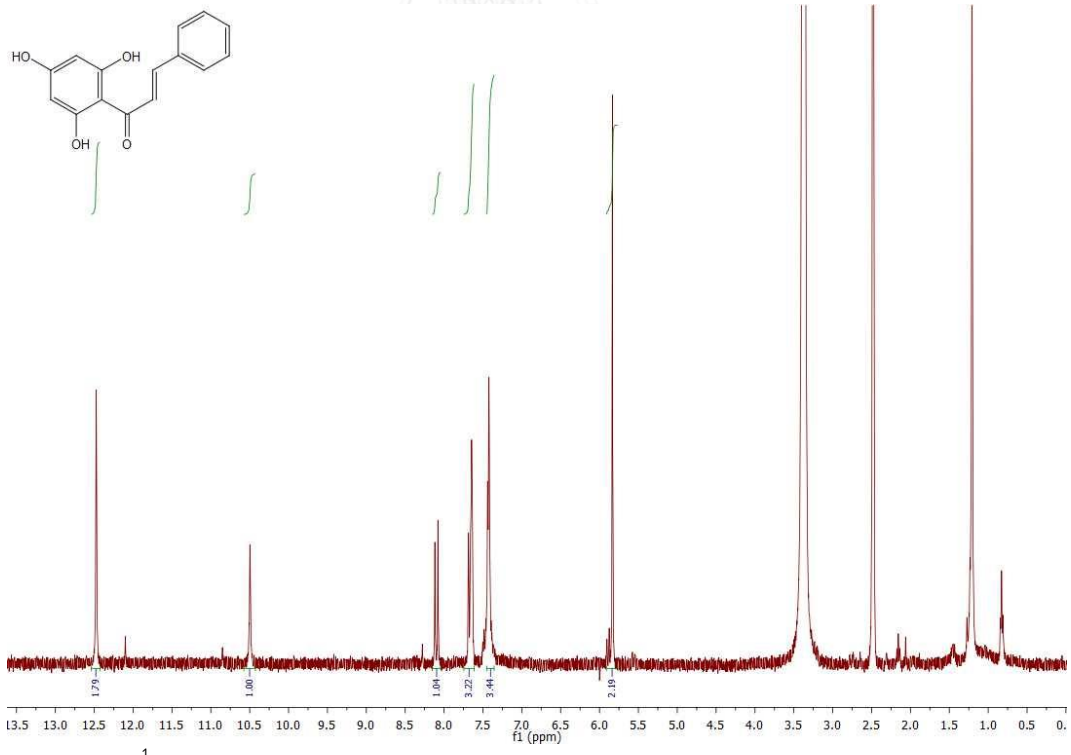


Figure A.28 ^{1}H-NMR (400 MHz) spectrum of compound **72** (DMSO-d_6)

VITA

Miss Krongkan Kingkaew was born on November 16th, 1990 in Ratchaburi province, Thailand. I was graduated with Bachelor's Degree of Science in Chemistry from Faculty of Science, Chulalongkorn University, in 2012. Then, she started as a Master Degree student with a major in Chemistry, Faculty of Science, Chulalongkorn University. My permanent address is 5/1 Angthong, Mueang, Ratchaburi, 70000, Thailand. E-mail: krongkan.kann@gmail.com