สารยับยั้งไทโรซิเนสจากละมุด *Manilkara zapota* L.

นางสาวสุทธิเดือน ชุณหกานต์

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรดุษฎีบัณฑิต
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TYROSINASE INHIBITORS FROM SAPODILLA PLUM *Manilkara zapota* L.

Miss Sutthiduean Chunhakant

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| Thesis Title | TYROSINASE INHIBITORS FROM SAPODILLA PLUM *Manilkara zapota* L. |
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| Field of Study | Biotechnology |
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สุทธิเดือน ชุณหกานต์ : สารยับยั้งไทโรซิเนสจากละมุด *Manilkara zapota* L. (TYROSINASE INHIBITORS FROM SAPODILLA PLUM *Manilkara zapota* L.) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. จรรยา ชัยเจริญพงศ์, 185 หน้า.

ส่วนต่าง ๆ ของละมุด 6 ส่วนประกอบด้วยเปลือก ดอก ผล ใบ ราก และเมล็ดถูกนำมาตรวจสอบหาปริมาณ สารประกอบฟีนอลิกทั้งหมด ปริมาณสารประกอบฟลาโวนอยด์ทั้งหมด ฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งไทโรซิเนส ส่วนสกัดหยาบเมทานอลของดอกแสดงปริมาณสารประกอบฟีนอลิกทั้งหมดสูงสุด (368.73 ± 0.65 mg GAE/g) ขณะที่ ส่วนสกัดหยาบเมทานอลของเมล็ดและรากแสดงปริมาณสารประกอบฟลาโวนอยด์ทั้งหมดสูง (90.21 ± 0.57 และ 89.03 ± 1.00 mg QE/g, ตามลำดับ) ส่วนสกัดหยาบเมทานอลของเมล็ดแสดงฤทธิ์ต้านอนุมูลอิสระ DPPH (IC$_{50}$ 282.05 ± 0.60 µg/mL) และ ABTS (IC$_{50}$ 205.11 ± 0.89 µg/mL) สูงสุดและแสดงความสามารถในการรีดิวซ์ FRAP สูงสุดด้วยค่า 296.46 ± 0.08 mg TEAC/mg ส่วนสกัดหยาบเมทานอลของรากแสดงฤทธิ์ยับยั้งไทโรซิเนสสูงสุดทั้งการยับยั้งมอนอฟีนอเลส (IC$_{50}$ 0.81 ± 0.92 mg/mL) และ ไดฟีนอเลส (IC$_{50}$ 0.55 ± 0.50 mg/mL) ปริมาณสารประกอบฟีนอลิกทั้งหมดและฤทธิ์ ต้านอนุมูลอิสระ แสดงความสัมพันธ์สูงโดยวิธี ABTS, DPPH และ FRAP ความสัมพันธ์ระหว่างปริมาณฟีนอลิกทั้งหมด กับฤทธิ์ยับยั้งไทโรซิเนสกับปริมาณสารประกอบฟลาโวนอยด์ทั้งหมดมีค่าต่ำมาก สารประกอบฟลาโวนอยด์ทั้งหมดกับ ฤทธิ์ต้านอนุมูลอิสระโดยวิธี DPPH, ABTS และ FRAP มีความสัมพันธ์ต่ำ สารประกอบฟาโวนอยด์ทั้งหมดกับฤทธิ์ยับยั้ง ไทโรซิเนส และฤทธิ์ต้านอนุมูลอิสระและฤทธิ์ยับยั้งไทโรซิเนสไม่มีความสัมพันธ์กัน นอกจากนี้การติดตามฤทธิ์ยับยั้งไท โรซิเนสถูกใช้ในการแยกสารยับยั้งไทโรซิเนสจากเปลือกต้นละมุด การแยกของส่วนสกัดหยาบนอร์มัลเฮกเซนและเอทิล แอซิเททของเปลือกต้นละมุดได้สาร 7 ชนิด คือ taraxerol methyl ether (I), 6-hydroxyflavanone (II), (+)-dihydrokaempferol (III), 3,4-dihydroxybenzoic acid (IV), taraxerol (V), taraxerone (VI) และ lupeol acetate (VII) (+)-dihydrokaempferol (III) (IC$_{50}$ 32.17 ± 0.32 µM) แสดงการยับยั้งไทโรซิเนสอย่างมีนัยสำคัญต่อฤทธิ์ยับยั้งมอนอ ฟีนอเลสได้ดีกว่า kojic acid (IC$_{50}$ 40.21 ± 0.63 µM) ยิ่งกว่านั้น (+)-dihydrokaempferol (III) (IC$_{50}$ 31.60 ± 0.73 µM) แสดงฤทธิ์ยับยั้งไทโรซิเนสแบบไดฟีนอเลสเทียบเท่ากับ kojic acid (IC$_{50}$ 30.07 ± 0.32 µM) ยิ่งกว่านั้นสารที่แยกได้ I-VII ถูกทดสอบหาฤทธิ์ต้านอนุมูลอิสระและความเป็นพิษต่อเซลล์มะเร็ง (+)-dihydrokaempferol (III) แสดงฤทธิ์ต้าน อนุมูลอิสระ DPPH (IC$_{50}$ 2.21 ± 0.77 µM) และ ABTS (IC$_{50}$ 214.83 ± 0.51 µM) สูงที่สุดและแสดงความสามารถในการ รีดิวซ์ FRAP สูงที่สุดด้วยค่า 6.23 ± 0.10 µM สารนี้แสดงความเป็นพิษต่อเซลล์มะเร็ง BT474, ChaGo-K-1, HepG$_2$, KATO-III และ SW620 ด้วยค่า IC$_{50}$ เท่ากับ 11.66 ± 0.42, 12.32 ± 0.73, 13.67 ± 0.38, 39.79 ± 0.38 และ 41.11 ± 1.08 µM, ตามลำดับ การศึกษานี้แสดงให้เห็นว่าละมุดอาจเป็นแหล่งที่มีปริมาณฟีนอลิกทั้งหมดสูงและแหล่งของสารต้านอนุมูล อิสระจากธรรมชาติ นอกจากนี้ (+)-dihydrokaempferol (III) สามารถพัฒนาเป็นสารยับยั้งไทโรซิเนสจากธรรมชาติได้

สาขาวิชา เทคโนโลยีชีวภาพ      ลายมือชื่อนิสิต _____

ปีการศึกษา 2560      ลายมือชื่อ อ.ที่ปรึกษาหลัก _____

# # 5472908523 : MAJOR BIOTECHNOLOGY

SUTTHIDUEAN CHUNHAKANT: TYROSINASE INHIBITORS FROM SAPODILLA PLUM *Manilkara zapota* L.. ADVISOR: ASST. PROF. CHANYA CHAICHAROENPONG, Ph.D., 185 pp.

Six different parts of *Manilkara zapota* which consisted of barks, flowers, fruits, leaves, roots and seeds were investigated for total phenolic content, total flavonoid content, antioxidant and antityrosinase activities. Methanol crude extract of flowers showed the highest total phenolic content ($368.73 \pm 0.65$ mg GAE/g), while methanol crude extracts of seeds and roots showed high total flavonoid content ($90.21 \pm 0.57$ and $89.03 \pm 1.00$ mg QE/g, respectively). Methanol crude extract of seeds showed the strongest DPPH ($IC_{50}$ $282.05 \pm 0.60$ μg/mL) and ABTS ($IC_{50}$ $205.11 \pm 0.89$ μg/mL) radical scavenging activities and showed the highest FRAP value of $296.46 \pm 0.08$ mg TEAC/mg. Methanol crude extract of roots showed the highest tyrosinase inhibitory activities on both monophenolase ($IC_{50}$ $0.81 \pm 0.92$ mg/mL) and diphenolase inhibitory activities ($IC_{50}$ $0.55 \pm 0.50$ mg/mL). Total phenolic content and antioxidant radical activities showed high correlation by ABTS, DPPH and FRAP assays. Correlations between total phenolic content with antityrosinase activities and with total flavonoid content were very low. A low correlation was found between total total flavonoid content and antioxidant activities by DPPH, ABTS and FRAP assays. There were no correlation between total flavonoid content with antityrosinase activities and no correlation between antioxidant and antityrosinase activities. Moreover, bioassay-guided fractionation on tyrosinase inhibitory activity was used to isolate tyrosinase inhibitors from *M. zapota* barks. Separation of *n*-hexane and ethyl acetate crude extracts of *M. zapota* barks afforded seven isolated compounds; taraxerol methyl ether (I), 6-hydroxyflavanone (II), (+)-dihydrokaempferol (III), 3,4-dihydroxybenzoic acid (IV), taraxerol (V), taraxerone (VI) and lupeol acetate (VII). (+)-Dihydrokaempferol (III) displayed significant tyrosinase inhibition ($IC_{50}$ $32.17 \pm 0.32$ μM) against monophenolase activity which was more potent than kojic acid ($IC_{50}$ $40.21 \pm 0.63$ μM). Furthermore, (+)-dihydrokaempferol (III) ($IC_{50}$ $31.60 \pm 0.73$ μM) showed similar activity on diphenolase inhibitory activity when compared with kojic acid ($IC_{50}$ $30.07 \pm 0.32$ μM). Furthermore, isolated compounds I-VII were evaluated antioxidant and cytotoxic activities. (+)-Dihydrokaempferol (III) exhibited the strongest scavenging activities on DPPH ($IC_{50}$ $2.21 \pm 0.77$ μM) and ABTS ($IC_{50}$ $214.83 \pm 0.51$ μM) and showed the highest FRAP value of $6.23 \pm 0.10$ μM. It displayed strong cytotoxic activity against human cancer cell lines; BT474, ChaGo-K-1, HepG$_2$, KATO-III and SW620 with $IC_{50}$ values of $11.66 \pm 0.42$, $12.32 \pm 0.73$, $13.67 \pm 0.38$, $39.79 \pm 0.38$ and $41.11 \pm 1.08$ μM, respectively. This study indicated that *M. zapota* might constitute a rich source of total phenolic contents and natural antioxidants. Moreover, (+)-dihydrokaempferol (III) could be developed as a natural tyrosinase inhibitor.

Field of Study:   Biotechnology          Student's Signature  _____

Academic Year:  2017                      Advisor's Signature  _____

# ACKNOWLEDGEMENTS

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# LIST OF ABBREVIATIONS

| | |
|---|---|
| $^1$H-NMR | Proton nuclear magnetic resonance spectroscopy |
| $^{13}$C-NMR | Carbon nuclear magnetic resonance spectroscopy |
| δ | Chemicals shift |
| ˚C | Celsius |
| % | Percentage |
| μg | Microgram |
| μL | Microliter |
| μm | Micrometre |
| μM | Micromolar |
| Abs | Absorbance |
| ABTS | 2,2-Azino-bis(3-ethylbenthiazoline-6-sulphonic acid) |
| ATCC | The American type culture collection |
| BT474 | Human breast carcinoma cell line |
| CC | Column chromatography |
| CDCl$_3$ | Deuterated chloroform |
| CD$_3$OD | Deuterated methanol |
| ChaGo-K-1 | Human lung bronchus carcinoma cell line |
| cm | Centimetre |
| cm$^{-1}$ | Reciprocal centimetre |
| COSY | Correlation spectroscopy |
| CC | Column chromatography |
| d | Doublet (for NMR spectra) |
| dd | Doublet of doublet (for NMR spectra) |
| DEPT | Distrotionless enhancement by polarization transfer |
| DHI | 5,6-Dihydroxyindole |
| DHICA | 5,6-Dihydroxyindole-2-carboxylic acid |
| DI water | Deionized water |
| DDI water | Distilled deionized water |
| DMSO | Dimethyl sulfoxide |
| DPPH | 2,2-Diphenyl-1-picryhydrazyl |
| e.g. | Exempli gratia |

| | |
|---|---|
| E | Enzyme |
| EAC | *Ehrlich ascites* carcinoma |
| EC | Enzyme commission |
| $EC_{50}$ | Half maximal effective concentration |
| ES | Enzyme-substrate complex |
| EI | Electron impact ionization |
| *et al.* | *et alii* |
| EtOH | Ethanol |
| $F_{254}$ | Fluorescent indicator 254 nm |
| Fast Blue BB | *N*-(4-amino-2,5-diethoxy phenyl) benzamide |
| FRAP | The ferric reducing antioxidant power |
| g | Gram |
| h | Hour |
| $HepG_2$ | Human liver carcinoma cell line |
| HL-60 | Human promyelocytic leukemia |
| HMBC | Heteronuclear multiple bond correlation |
| HMQC | Heteronuclear multiple quantum correlation |
| HPLC | High performance liquid chromatography |
| HREIMS | High resolution electron impact ionization mass spectrometry |
| HSQC | Heteronuclear single quantum correlation |
| HT-29 | Human colon adenocarcinoma cell line |
| Hz | Hertz |
| I | Inhibitor |
| $IC_{50}$ | Half maximal inhibitory concentration |
| In | Inch |
| $Inches^2$ | Square of inches |
| *J* | Coupling constant |
| KATO-III | Human gastric carcinoma cell line |
| kg | Kilogram |
| $K_{cat}$ | The turnover number |
| $K_i$ | Dissociation constant |
| $K_m$ | Michaelis constant |

| | |
|---|---|
| L | Litre |
| *L*-DOPA | *L*-3,4-Dihydroxyphenylalanine |
| m | Multiple (for NMR spectra) |
| M | Molar |
| m/z | Mass-to-charge ratio |
| $m^2$ | Square of metre |
| mg | Milligram |
| mg GAE | Milligram of gallic acid equivalent |
| mg QE | Milligram of quercetin equivqlent |
| mg TEAC | Milligram of trolox equivalent antioxidant capacity |
| min | Minute |
| mL | Millilitre |
| mm | Millimetre |
| mM | Millmolar |
| MIC | Minimum inhibitory concentrations |
| MMP | Matrix metalloproteinases |
| MTT | 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide |
| NA | No activity |
| ND | No detection |
| nm | Nanometre |
| NMR | Nuclear magnetic resonance spectroscopy |
| P | Product |
| pH | A logarithmic measure of hydrogen ion concentration |
| ppm | Part per million |
| PTLC | Preparative thin layer chromatography |
| ROS | Reactive oxygen |
| RPMI | Roswell park memorial institute |
| s | Siglet (for NMR spectra) |
| S | Substrate |
| SD | Standard deviation |
| SPSS | Statistical package for the social sciences |
| SW620 | Human colon carcinoma cell line |

| | |
|---|---|
| t | Triplet (for NMR spectra) |
| TLC | Thin layer chromatography |
| TMS | Tetramethylsilane |
| TYR | Tyrosine |
| TYRP1 | Tyrosinase-related protein 1 |
| TYRP2 | Tyrosinase-related protein 2 |
| UV | Ultraviolet |
| $\nu$ | The initial rate of reaction |
| v/v | Volume by volume |
| $V_{max}$ | The maximum rate |
| w | Weight |
| Wi-38 | Human diploid lung fibroblast |
| w/v | Weight by volume |
| w/w | Weight by weight |

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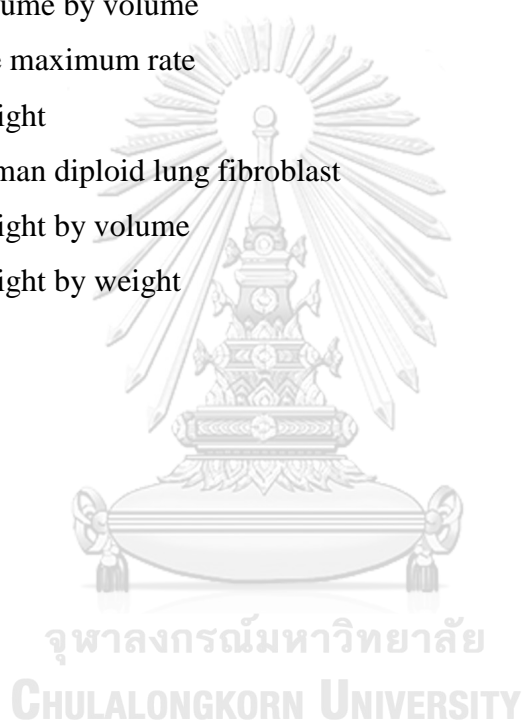
# CHAPTER I
# INTRODUCTION

Skin whitening assigns to the process of using chemical ingredients for lightening skin tone [1, 2]. One property of skin whitening cosmetics treats an overproduction of melanin. Melanin is the pigment that blocks ultraviolet (UV) radiation for protecting human skin [3]. The outer layer of skin is epidermis and consists of two cell types; keratinocytes and melanocytes. Melanocytes produce and keep melanin in melanosomes [4]. The two type of melanin calls eumelanin and pheomelanin. Both types are produced from biosynthesis pathway of melanin which tyrosinase is a key enzyme [5]. Tyrosinase (EC 1.14.18.1) contains two copper ions and catalyzes reaction in melanin synthesis. The reaction of tyrosinase involves in the initial step of the hydroxylation of $L$-tyrosine to $L$-3,4-dihydroxyphenylalanine ($L$-DOPA). Then, $L$-DOPA is oxidized to $L$-dopaquinone. Finally, a family of non-enzymatic reactions is converted to make either eumelanin or pheomelanin [6, 7]. Hyperpigmentation disorders produce abnormal melanin such as freckles, melasma, senile lentigo and spot on the body surface area [8, 9].

Tyrosinase inhibitors inhibit the increasing of skin color and reduce the activity of tyrosinase. Depigmentation mechanisms include pre-melanin synthesis, during melanin synthesis and after melanin synthesis [10]. Type of tyrosinase inhibitors is classified into three groups including competitive inhibitor, mixed type (competitive/uncompetitive) inhibitor and uncompetitive inhibitor [5]. Mechanism of depigmentation agents depends on the movement of melanin from melanocytes. Arbutin, kojic acid and vitamin C are commercially ingredients in cosmetic and pharmaceutical products and show slow-binding on diphenolase inhibitory activity [11].

Previously researches, tyrosinase inhibitors have been investigated from both natural and synthetic sources [12]. A small number of tyrosinase inhibitors have been used in cosmetic products because most of tyrosinase inhibitors showed harmful side effects on skin. Side effect of arbutin and kojic acid is a contact dermatitis and an allergic dermatitis, when a concentration of arbutin and kojic acid in cosmetic above

1% [13, 14]. From our preliminary study, ethanol and aqueous extracts of flowers of *Clitoria ternatea*, seeds of *Abrus precatorius*, barks of *Dalbergia oliveri*, barks of *Millettia brandisiana*, barks of *Smilax corbularia*, barks, twigs and roots of *Mimusops elengi*, barks, leaves and roots of *Manikara zapota* were evaluated for tyrosinase inhibitory activity using *L*-DOPA as a substrate. The results showed that ethanol extract of barks of *M. zapota* exhibited the highest antityrosinase activity with percentage inhibition value of 96.49 ± 0.10% at the concentration of 1.0 mg/mL.

Sapodilla plum (*M. zapota* L.) is a tree in family Sapotaceae. In Thailand, it is called Lamut or Lamutfarang. Its fruits are shape like eggs and ripe fruits are edible and sweet with rich fine flavor [15]. This plant has been shown many bioactivities such as antidiabetic, anti-inflammatory, antilipidemic, antimicrobial, antioxidant, anti-pyretic and antityrosinase activities [16-18]. However, the literatures were reported a few effective of the isolated compounds from *M. zapota* on tyrosinase inhibitory activity. Thus, the current study is undertaken to isolate chemical constituents of *M. zapota* barks and evaluate their tyrosinase inhibitory activity.

**Objectives**

To investigate the tyrosinase inhibitors from *M. zapota* L.

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# CHAPTER II
# THEORETICAL

## 2.1 Melanin

Skin is the site of various complex, helps to regulate temperature and protects structure from the external environment [19]. Skin is a living organism and consists of three main parts as epidermis, dermis and subcutaneous fat layers (Figure 1). The epidermal barrier prevents the skin from chemicals, microbes, physical injury and dehydration of epidermis. The epidermal barrier produces by differentiation of keratinocytes. They migrate from the basal cell layer into the stratum corneum. The keratinocytes of the epidermis are created and renewed by stem cells in the basal layer [20]. It produces every 28 days for replacement of epidermis. The basal layer takes 14 days to reach stratum corneum and scrubs for another 14 days. Keratinocytes build keratins. Keratins are structural proteins and make up the keratinocyte cytoskeleton. In stratum spinosum, keratin filaments emit from the nucleus and link with desmosomes. Then, they move into the stratum granulosum. Keratohyalin granules form keratin and profilaggrin. Profilaggrinis transforms into filaggrin. It flows and aligns keratin filaments into fully compressed parallel when the cell of stratum corneumare formed the matrix. The mutation of filaggrin genes connects with ichthyosis vulgaris and atopic dermatitis. Keratinocyte moves and develops a flat hexagon model into the stratum corneum [21].
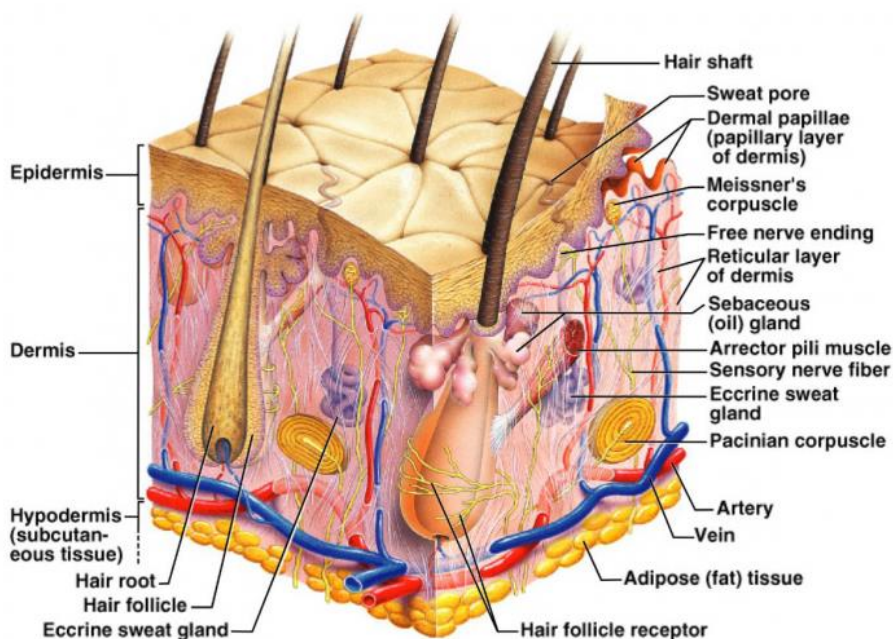
**Figure 1** Cross-section of skin structure

Melanins are widely disposed pigments in animals, plants, fungi and bacteria. Melanins are divided into two types as eumelanin and pheomelanin [22]. Melanin structure is a heterogeneous polymer with complex pigments. Melanin pigments are synthesized by melanocyte cells. Melanocyte cells contain a specific enzyme as tyrosinase in the stratum basale of dermis. Tyrosinase controls the produce of melanins. Synthesis of melanins occurs in melanosomes. It carries out from the melanocyte cells to keratinocyte cells. The function of melanin is protection of UV to damage the skin and discarded reactive oxygen species (ROS) in organisms [23-25]. The initial step of melanogenesis is the hydroxylation of *L*-tyrosine into *L*-DOPA. Then, *L*-DOPA is oxidized to *L*-dopaquinone. Both two steps are catalyzed by tyrosinase. After that, the reaction *L*-dopaquinone is cyclized into leukodopachrome at 1,4-addition position and rapidly oxidized to dopachrome. The dopachrome is brought to produce eumelanin into two difference reactions. The first reaction, dopachrome is decarboxylated into 5,6-dihydroxyindole (DHI). Then, it is oxidized to indole-5,6-quinone. Finally, indole-5,6-quinone is polymerized to eumelanin. The second reaction, dopachrome is transformed into 5,6-dihydroxyindole-2-carboxylic acid (DHICA) by tautomerase. Then, DHICA is oxidized into indole-5,6-quinone carboxylic acid. Then, indole-5,6-quinone carboxylic acid is polymerized to eumelanin. For synthesis of pheomelanin, *L*-dopaquinone is reacted with glutathione or cysteine to form glutathionyldopa or cysteinyldopa. The

glutathionyldopa or cysteinyldopa is then cyclized into 1,4-benzothiazinylalanine. Finally, 1,4-benzothiazinylalanine is polymerized to pheomelanin (Figure 2) [7, 26].
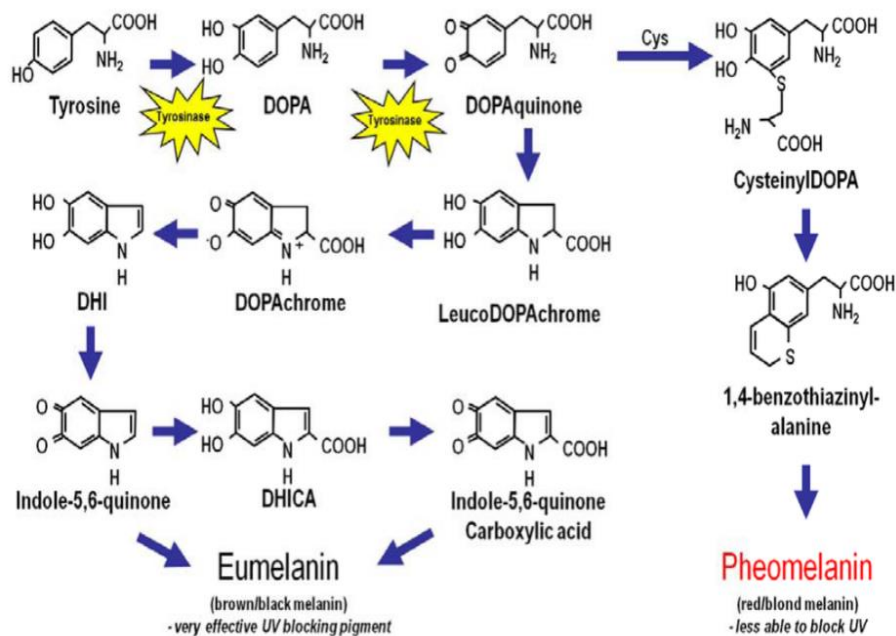


**Figure 2** Melanin biosynthesis pathway

Hyperpigmentation appears when melanin is overproduced on the skin. Many hyperpigmentation disorders such as acne scars, age spots, freckles, melisma and senile lentigo are attributed by the darkness of the human skin. The factors of hyperpigmentation are age, hormones, pregnancy, UV radiation [27] and others.

## 2.2 Tyrosinase enzyme

A family of tyrosinase as tyrosinase (TYR), tyrosinase-related protein 1 (TYRP1) and tyrosinase-related protein 2 (TYRP2) is related in human melanogenesis. Tyrosinase (EC 1.14.18.1) is a polyphenol oxidase. It contains binuclear copper on molecular oxygen (Figure 3). Tyrosinase catalyzes two initial steps in melanin biosynthesis pathway. The hydroxylation of *L*-tyrosine into *L*-DOPA is called monophenolase activity and the oxidation of *L*-DOPA into *L*-dopaquinone is called diphenolase activity [28]. TYRP1 and TYRP2 catalyze other step to control the type of melanin synthesis. However, if one reaction of melanin synthesis fails, melanins are not found on the skin [29].
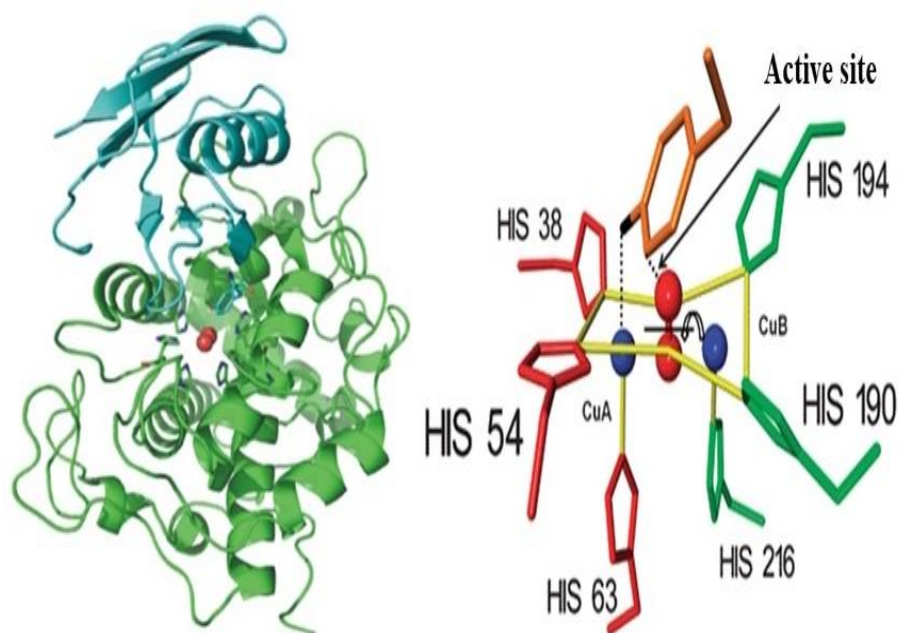
A= Tertiary structure of tyrosinase

1. B = Coordination sphere of binuclear copper center and location of tyrosinase substrate

**Figure 3** Structure of tyrosinase

## 2.3 Tyrosinase inhibitor

Various tyrosinase inhibitors were identified from both synthetic and natural sources. They were investigated using *L*-tyrosine or *L*-DOPA as substrates. The inhibitory activity of tyrosinase inhibitors are exhibited in the formation of dopachrome [6]. Monophenolase and diphenolase inhibitory activities of tyrosinase inhibitor can descript by one of the following:

(1) *L*-Dopaquinone is reduced by reducing agents. Reducing agents are used as melanogenesis inhibitors such as ascorbic acid.

(2) *L*-Dopaquinone is reacted with *L*-dopaquinone scavenger for the colorless of product. Then, the melagenesis process is slowed. The *L*-dopaquinone scavenger was used as thio-containing compounds and the reaction is overturn to original rates.

(3) Dopachrome formation is protected by alternative enzyme substrate such as phenolic compounds.

(4) Nonspecific enzyme in-activators denature the enzyme as acids or bases.

(5) Specific tyrosinase in-activators inhibit tyrosinase activity using suicide substrate such as kojic acid.

(6) Specific tyrosinase inhibitors reduce tyrosinase.

## 2.4 Mechanism of enzyme inhibition

Enzyme displays an essential role in biochemical processes. Enzyme is a specific function in few conditions. Enzyme (E) binds to the substrate (S) to form enzyme-substrate complex (ES). Then, it changes to enzyme and product (P). The reaction was described in equation 1 [30, 31].

$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E + P \qquad (1)$$
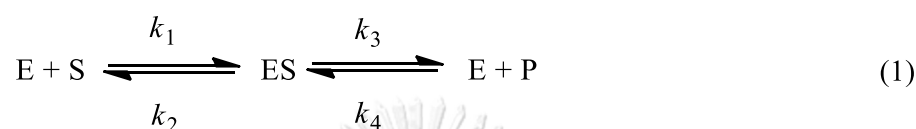
From theoretical, reactions are reversible reactions. Enzyme reactions are studied by using steady-state conditions. In steady-state conditions, ES is the rate constant, P is increasing and S is reducing. The conditions were described in equation 2.

$$k_1[E][S] = (k_2 + k_3)[ES] \qquad (2)$$

When equation 2 was rearrange to

$$[E][S]/[ES] = (k_2 + k_3) \quad /k_1 \qquad (3)$$

$$K_m = (k_2 + k_3)/k_1 \qquad (4)$$

Where $K_m$ is the Michaelis constant, it gives the enzyme-substrate binding constant under steady-state conditions and measures the rate in the initial milliseconds of the reaction.

$$[ES] = [E][S]/K_m \qquad (5)$$

$$[E] = [E_{total}] - [ES] \qquad (6)$$

$$[ES] = [E_{total}] - [E] \qquad (7)$$

Instead from equations 3 and 4 and solve for ES

$$[ES] = [E_{total}][S]/[S] + K_m \qquad (8)$$

The initial rate of reaction ($v$) is proportional to [ES] when [S] is small and the maximum rate ($V_{max}$) is proportional to [$E_{total}$] when [S] is saturating. The Michaelis-Menten equation can be described in term of the reaction rate.

$$v = V_{max}[S]/[S] + K_m \qquad (9)$$

$K_m$ is determined by measuring the reaction rate at various [S] and [$E_{total}$] is constant and $v$ is represented to [S] when [S] is low and $K_m = [S]$ when $v = V_{max}/2$

(Figure 4). Determination of kinetic parameters uses plotting of the reciprocals of the Michaelis-Menten equation (9). It is called a Lineweaver-Burk plot.

$1/v = 1/V_{max} + [K_m/V_{max}][1/[S]]$

(10)

At $V_{max}$, [ES] is equal to [$E_{total}$] and the rate of the reaction is proportional to $k_3$ which represented to the turnover number ($k_{cat}$). The equation can be determined, if [$E_{total}$] is known. $k_{cat}/K_m$ is called catalytic efficiency.

$k_3 = V_{max}/[E_{total}]$ (11)
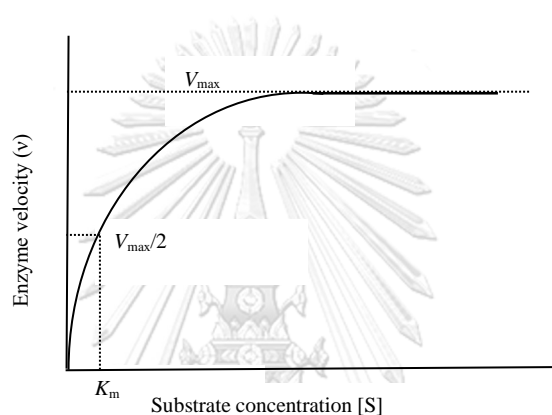


**Figure 4** Michaelis-Menten plot

2.4.1 Competitive inhibition

The enzyme inhibitor (I) binds to the enzyme at the active site as same as the substrate binds to the enzyme.



(12)
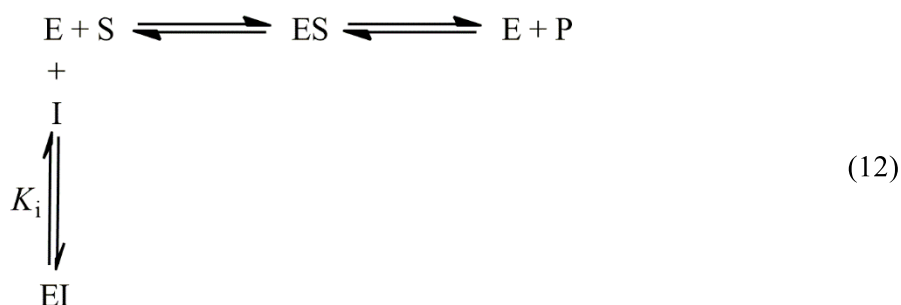
From Michaelis-Menten plot, $K_m$ is increased but $V_{max}$ is unchanged when the substrate concentration is increased (Figure 5). Dissociation constant ($K_i$) can be calculated from Michaelis-Menten equation or a reciprocal plot versus [I] in plotting

the slopes of the lines. In Lineweaver-Burk plot, the slopes of inhibitor and without inhibitor lines cross the y-axis at the same point (Figure 6).

$$K_i = [I]/[(K_{m, obs}/K_m)-1] \tag{13}$$
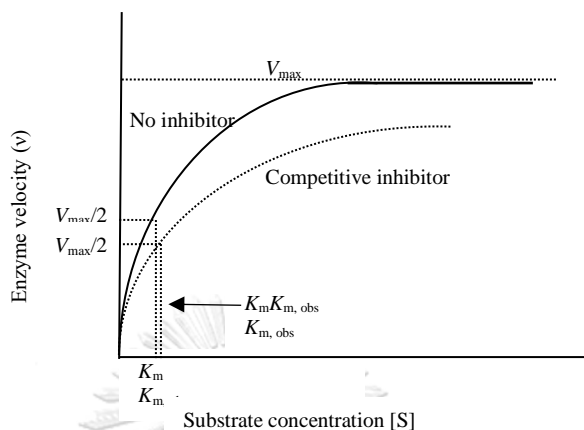


**Figure 5** Michaelis-Menten plot of competitive inhibition
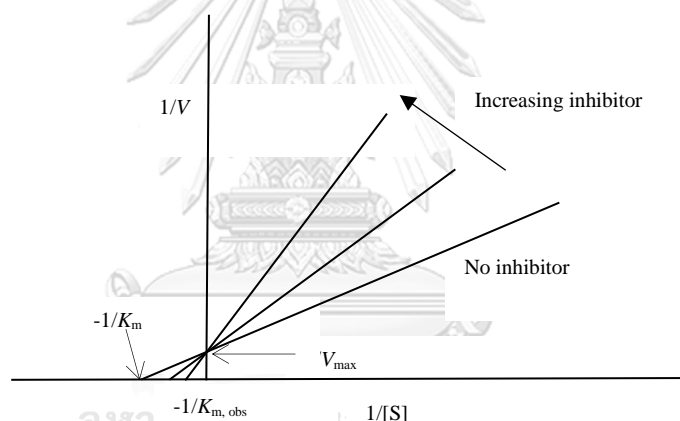


**Figure 6** Lineweaver-Burk plots of competitive inhibition

2.4.2 Mixed inhibition

The inhibitor binds to a site other than the active site of the enzyme to change form an inactive complex. Inhibitor binds to either the enzyme or the enzyme-substrate complex. From Michaelis-Menten equation, $K_m$ of inhibitor and without inhibitor is the same value. $V_{max}$ of inhibitor is lower than $V_{max}$ of without inhibitor (Figure 7) and $K_i$ can be calculated from Michaelis-Menten equation or plotting $1/v$ versus [I] in plotting the slopes of the lines. In a Lineweaver-Burk plot, the slopes of inhibitor and without inhibitor lines converge on the X-axis at $-1/K_m$ (Figure 8). It is called noncompetitive inhibition. In mixed inhibition, $K_i$ and $K_{si}$ are equivalent. The slopes of inhibitor and without inhibitor lines cross at the same point in the quadrant (Figure 9).

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

$$+ \qquad\qquad +$$

$$I \qquad\qquad I$$

$$K_i \qquad\qquad K_{si}$$

$$EI + S \rightleftharpoons ESI$$

(14)
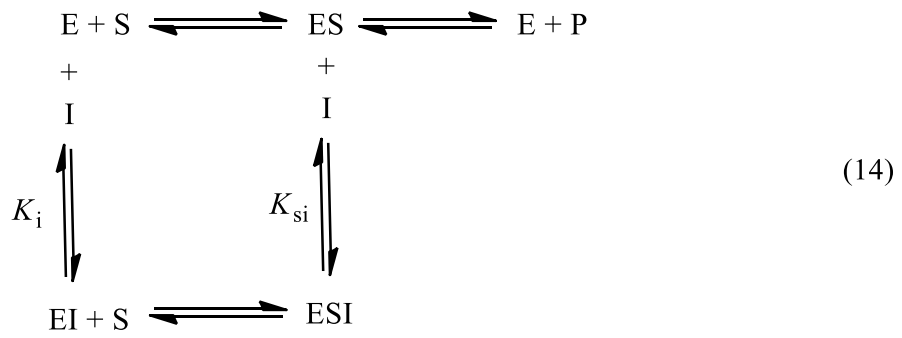
3
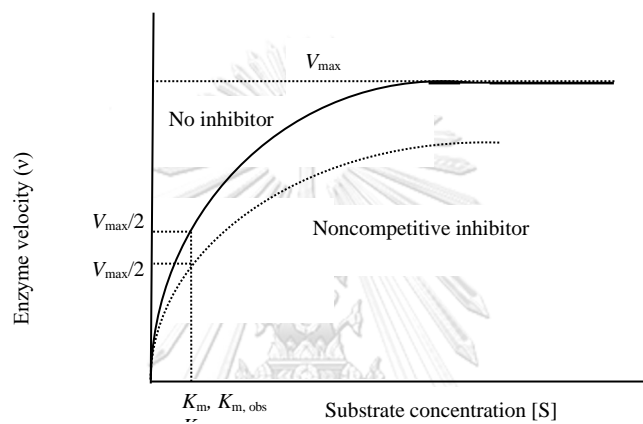


**Figure 7** Michaelis-Menten plot of noncompetitive inhibition
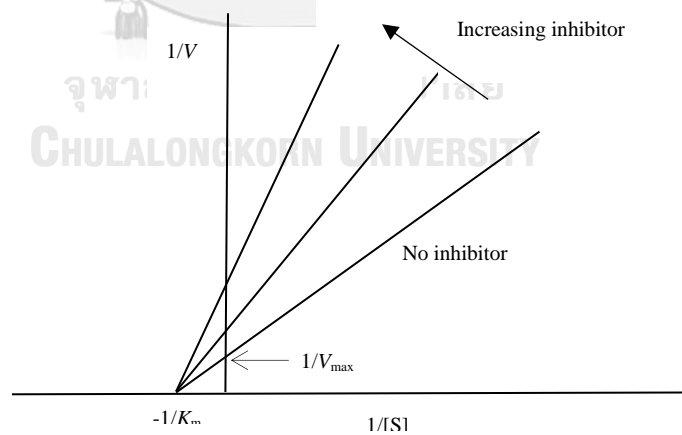


**Figure 8** Lineweaver-Burk plots of noncompetitive inhibition

$1/V$

Increasing inhibitor

No inhibitor

$1/V_{max}$

$-1/K_m$

$1/[S]$

**Figure 9** Lineweaver-Burk plots of mixed inhibition

2.4.3. Uncompetitive inhibition

The inhibitor binds to enzyme-substrate complex but it not binds enzyme. Both $K_m$ and $V_{max}$ reduce (Figure 10). In Lineweaver-Burk plot, both slopes of inhibitor line and without inhibitor line are parallel to each other lines. $K_i$ can be calculated from Michaelis-Menten equation (Figure 11).

$$E + S \rightleftharpoons ES \rightleftharpoons E + P$$

$$+$$

$$I$$

$$K_i$$

$$ESI$$

(15)



$V_{max}$

No inhibitor

Enzyme velocity (v)

$V_{max}/2$

$V_{max}/2$

Uncompetitive inhibitor

$K_{m, obs}$ $K_m$

Substrate concentration [S]

**Figure 10** Michaelis-Menten plot of uncompetitive inhibition

**Figure 11** Lineweaver-Burk plots of uncompetitive inhibition

## 2.5 Literature reviews

2.5.1 Tyrosinase inhibitory activity of medicinal plants

The various plants extracts and ethanol crude extract of medicine formulation of herbs were investigated for tyrosinase inhibitory activity. Antityrosinase assays were tested by either *in vitro* or *in vivo* methods on monophenolase and diphenolase inhibitory activities. For monophenolase inhibitory activity, ethyl acetate fraction of *Acanthopanax koreanum* leaves exhibited antityrosinase activity (IC$_{50}$ 186.20 ± 11.70 μg/mL) and triterpenoid compounds were isolated from this plant. 3$\alpha$,20$\alpha$,29-Trihydroxylupane-23,28-dioic acid (IC$_{50}$ 8.61 ± 1.09 μM) showed tyrosinase inhibitory activity and compared with kojic acid (IC$_{50}$ 36.36 ± 2.83 μM) [32]. (2*S*)-2′,4′-Dihydroxy-5′-(1′′′,1′′′-dimethylallyl)-8-prenyl pinocembrin which was isolated from *Dalea elegans* exhibited antityrosinase activity with IC$_{50}$ value of 2.32 ± 0.01 μM and compared with kojic acid with IC$_{50}$ value of 4.90 ± 0.01 μM [33]. Ethanol extract of fruit pericarps of *Dimocarpus longan* showed antityrosinase activity with percentage inhibition value of 23.60 ± 1.20 at a concentration of 100 μg/mL [34]. 2′,4′,6′-Trihydroxydihydrochalcone was isolated from *Greyia flanaganii* leaves and exhibited tyrosinase inhibitory activity with IC$_{50}$ value of 69.1 μM [35]. Dihydrokeampferol, naringenin and rhoiptelol which were isolated from *Juglans mandshurica* barks showed more potent antityrosinase activity than arbutin (IC$_{50}$ 22.40 ± 4.70 mM) [36]. Ethanol crude extracts of *Litchi sinensis* seeds showed antityrosinase activity in a concentration-dependent manner [37]. Methanol crude extract of *Mimusops elengi* flowers showed strong antityrosinase activity [38]. Essential oil of *Rubus pungens* var. *oldhamii* (IC$_{50}$ 0.92 mg/mL) showed strong tyrosinase inhibitory activity and compared with arbutin (IC$_{50}$ 0.66 mg/mL) [39]. Acetone and methanol crude extracts of

*Sideroxylon inerme* barks showed antityrosinase activity and compared with arbutin and kojic acid ($IC_{50}$ 63.00 ± 2.10, 82.10 ± 2.70, 149.00 ± 1.20 and 1.14 ± 0.05 μg/mL, respectively) [40].

For diphenolase inhibitory activity, dichloromethane fraction of *Acmella oleracea* showed antityrosinase activity ($IC_{50}$ 0.50 mM) and compared with kojic acid ($IC_{50}$ 0.13 mM) [41]. Artocaepin E, artocarpanone, liquiritigenin and steppogenin were isolated from *Artocarpus heterphyllous* woods showed more potent tyrosinase inhibitory activity than kojic acid ($IC_{50}$ 6.70 ± 0.80, 2.00 ± 0.10, 22.00 ± 2.5, 7.50 ± 0.5 and 44.60 ± 0.40 μM, respectively) [42]. Aqueous crude extract of *Asparagus officinalis* showed tyrosinase inhibitory activity with $IC_{50}$ value of 1.21 mg/mL and exhibited mixed-type of kinetic inhibition [43]. Essential oils which were extracted from *Cinnamomum zeylanicum*, *Citrus grandis* and *Citrus hystrix* showed potent tyrosinase inhibitory activity when compared with kojic acid ($IC_{50}$ 2.05 ± 0.07, 2.07 ± 0.15, 2.08 ± 0.25 and 44.60 ± 0.40 2.28 ± 0.05 μg/mL) [44]. Nobiletin which was isolated from *Citrus unshiu* peels showed stronger inhibition against tyrosinase than arbutin ($IC_{50}$ 1.49 and 27.31 mM, respectively) [45]. Ethanol extracts of root barks and twigs of *Morus alba* showed tyrosinase inhibitory activity with percentage inhibition values of 78.00 and 62.00 at a concentration of 60 μg/mL, respectively [46]. Moreover, methanol crude extract of *Podocarpus* species showed antityrosinase activity in a dose-dependent manner [47]. Purpurin which was isolated from roots of *Rubia cordifolia* showed antityrosinase activity and compared with kojic acid ($IC_{50}$ 0.29 ± 0.09 and 0.0019 mg/mL) [48]. Moracin M which was isolated from ethanol crude extract of woods of *Streblus ilicifolius* exhibited antityrosinase activity and and compared with kojic acid ($IC_{50}$ 67.69 and 38.67 μg/mL) [49]. In addition, herbal formulations were studied on tyrosinase inhibitory activity. Qian-wang-hong-bai-san composed of fruits of *Benincasa hispida*, pericarps of *Punica granatum*, tubers of *Bletilla striata* and tubers of *Typhonium giganteum* (1:1:1:1). Ethanol crude extract of Qian-wang-hong-bai-san showed mushroom tyrosinase inhibitory activity ($IC_{50}$ 1.21 mg/mL) and *in vivo* antityrosinase activity on mouse B16 cells ($IC_{50}$ 177.90 μg/mL). Qi-bai-gao consisted of rhizomes of *Atractylodes macrocephala*, rhizomes of *Smilax glabra*, roots of *Ampelopsis japonica*, roots of *Angelica dahurica*, root and rhizomes of *Asarum heterotropoides*, tubers of *B. striata* and tubers of *T. giganteum* (10:3:3:10:3:3:10).

Ethanol crude extract of Qi-bai-gao showed percentage of tyrosinase inhibition as 60.30 ± 0.90% at concentration of 1 mg/mL and percentage of tyrosinase inhibition on mouse B16 cells as 44.10 ± 8.60% at concentration of 1 mg/mL. Qiong-yu-gao consisted of root of *Rehmannia glutinosa*, roots and rhizomes of *Panax ginseng* and rhizomes of *S. glabra* (1:1:1). Qiong-yu-gao ($IC_{50} > 1000$ μg/mL) showed less *in vitro* tyrosinase inhibitory activity than *in vivo* assay on mouse B16 cells ($IC_{50}$ 531.20 μg/mL) [50]. The literature reviews of antityrosinase activity for crude extracts of plants are shown in Table 1. The literature reviews of antityrosinase activity for phytochemicals of plants are shown in Table 2 and Figure 12.

**Table 1** Antityrosinase activity of crude extracts of plants

| Scientific name | Plant part | Crude extract | Reference |
|---|---|---|---|
| *Acanthopanax koreanum* | Leaves | Ethyl acetate fraction of methanol extract ($IC_{50}$ = 186.20 ± 11.70 μg/mL on monophenolase inhibitory activity) | [32] |
| *Acmella oleracea* | Leaves Stems | Dichloromethane fraction of methanol extract ($IC_{50}$ = 0.50 mM on diphenolase inhibitory activity) | [41] |
| *Anacardium occidentale* | Fresh leaves | Methanol extract by blanching (% diphenolase inhibition = 44 ± 3.5% at 0.25 mg/mL) Methanol extract by maceration (% diphenolase inhibition = 40 ± 2.2% at 0.25 mg/mL) Methanol extract by microwave (% diphenolase inhibition = 49 ± 4.0% at 0.25 mg/mL) | [51] |
| *Ampelopsis japonica* | Roots | Ethanol extract (*in vitro*, $IC_{50}$ = 152.1 μg/mL on diphenolase inhibitory activity) (*in vivo*, $IC_{50}$ = 117.3 μg/mL on diphenolase inhibitory activity) | [50] |
| *Asparagus officinalis* | Whole plant | Aqueous extract (Mixed inhibitor, $IC_{50}$ = 1.21 mg/mL on diphenolase inhibitory activity) Aqueous extract inhibited melanin production (% diphenolase inhibition = 18.6 ± 1.7% at 0.05 mg/mL) (% diphenolase inhibition = 18.4 ± 2.2% at 0.20 mg/mL) (% diphenolase inhibition = 20.4 ± 2.3% at 1.00 mg/mL) | [43] |
| *Asphodelus microcarpus* | Flowers | Aqueous extract (% diphenolase inhibition = 6.55 ± 0.21% at 0.2 mg/mL) Ethanol extract (% diphenolase inhibition = 40.25 ± 4.4% at 0.2 mg/mL) Methanol extract (% diphenolase inhibition = 13.9 ± 2.4% at 0.2 mg/mL) | [52] |
| | Leaves | Aqueous extract (% diphenolase inhibition = 9.85 ± 0.21% at 0.2 mg/mL) Ethanol extract (% diphenolase inhibition = 29.9 ± 0.14% at 0.2 mg/mL) Methanol extract (% diphenolase inhibition = 20.4 ± 1.4% at 0.2 mg/mL) | |
| | Tubers | Aqueous extract (% diphenolase inhibition = 10.65 ± 1.34% at 0.2 mg/mL) Ethanol extract (% diphenolase inhibition = 8.4 ± 1.3% at 0.2 mg/mL) Methanol extract (% diphenolase inhibition = 2.25 ± 1.0% at 0.2 mg/mL) | |
| *Beilschmiedia pulverulenta* | Aerial parts | Essential oil (% diphenolase inhibition = 67.6 ± 0.40% at 1 mg/mL) | [53] |
| *Berberis aristata* | Stem barks and woods | Aqueous extract (% monophenolase inhibition = 97% at 110 μg/mL) Aqueous extract ($IC_{50}$ = 412.01 μg/mL on diphenolase inhibitory activity) Methanol extract (Mixed inhibitor, % monophenolase inhibition = 78% at 110 μg/mL) | [54] |

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**Table 1** Antityrosinase activity of crude extracts of plants (continue)

| Scientific name | Plant part | Crude extract | Reference |
|---|---|---|---|
| *Melaleuca quinquenervia* | Leaves | Essential oil<br>($IC_{50}$ = 150.28 ± 0.29 µg/mL on monophenolase inhibitory activity) | [44] |
| *Mentha pulegium* | Leaves | Ethanol extract<br>($IC_{50}$ = 286 ± 45 µg/mL on monophenolase inhibitory activity) | [60] |
| *Mentha rotundifolia* | Leaves | Ethanol extract<br>($IC_{50}$ = 108 ± 20 µg/mL on monophenolase inhibitory activity) | [60] |
| *Mentha spicata* | Leaves | Ethanol extract<br>($IC_{50}$ = 223 ± 41 µg/mL on monophenolase inhibitory activity) | [60] |
| *Mimusops elengi* | Flowers | Methanol extract<br>($IC_{50}$ = 401 µg/mL on monophenolase inhibitory activity) | [38] |
| *Morus alba* | Root barks | Ethanol extract<br>(% diphenolase inhibition = 0 - 62% at 0 - 60 µg/mL) | [46] |
| | Twigs | Ethanol extract<br>(% diphenolase inhibition = 0 - 78% at 0 - 60 µg/mL) | |
| *Peucedanum knappii* | Aerial parts | Ethyl acetate fraction of methanol extract<br>($IC_{50}$ = 517 µg/mL on diphenolase inhibitory activity) | [61] |
| *Pimenta dioica* | Leaves | Essential oil<br>($IC_{50}$ = 21.33 ± 0.127 µg/mL on monophenolase inhibitory activity) | [44] |
| *Piper betel* | Leaves | Essential oil<br>($IC_{50}$ = 87.97 ± 1.052 µg/mL on monophenolase inhibitory activity) | [44] |
| | Leaves | Methanol extract by maceration<br>(% diphenolase inhibition = -20 ± 5.5% at 0.25 mg/mL)<br>Methanol extract by blanching<br>(% diphenolase inhibition = -13 ± 3.8% at 0.25 mg/mL)<br>Methanol extract by microwave<br>(% diphenolase inhibition = -23 ± 7.3% at 0.25 mg/mL) | [51] |
| *Piper magnibaccum* | Leaves | Essential oil<br>(% diphenolase inhibition = 49.50 ± 0.6%) | [62] |
| | Stems | Essential oil<br>(% diphenolase inhibition = 57.01 ± 0.8%)<br>Fraction IV of ethanol extract<br>(Uncompetitive inhibitor, $IC_{50}$ = 5.81 ± 0.08 µg/mL on diphenolase inhibitory activity) | |
| *Podocarpus elonggatus* | Leaves | 50% Methanol extract<br>($EC_{50}$ = 0.47 ± 0.001 mg/mL) | [47] |
| | Stems | 50% Methanol extract<br>($E_{50}$ = 0.14 ± 0.001 mg/mL) | |
| *Podocarpus falcatus* | Leaves | 50% Methanol extract<br>($EC_{50}$ = 0.29 ± 0.002 mg/mL on diphenolase inhibitory activity) | [47] |
| | Stems | 50% Methanol extract<br>($EC_{50}$ = 0.35 ± 0.002 mg/mL on diphenolase inhibitory activity) | |
| *Podocarpus henkelii* | Leaves | 50% Methanol extract<br>($EC_{50}$ = 0.37 ± 0.003 mg/mL on diphenolase inhibitory activity) | [47] |
| | Stems | 50% Methanol extract<br>($EC_{50}$ = 0.40 ± 0.003 mg/mL on diphenolase inhibitory activity) | |
| *Podocarpus latifolius* | Leaves | 50% Methanol extract<br>($EC_{50}$ = 0.41 ± 0.005 mg/mL on diphenolase inhibitory activity) | [47] |
| | Stems | 50% Methanol extract<br>($EC_{50}$ = 0.36 ± 0.001 mg/mL on diphenolase inhibitory activity) | |
| *Polygonatum odoratum* | Rhizomes | 30% Ethanol extract<br>(*in vitro*, $IC_{50}$ = 98.4 µg/mL on diphenolase inhibitory activity)<br>(*in vivo*, $IC_{50}$ = 830.1 µg/mL on diphenolase inhibitory activity) | [50] |
| *Polygonum cuspidatum* | Fruits | Extract by supercritical carbon dioxide fluid<br>(% monophenolase inhibition = 14.8 ± 1.23% at 100 µg/mL)<br>(% monophenolase inhibition = 22.6 ± 1.64% at 250 µg/mL) | [26] |
| *Populus nigra* | Buds | Ethanol extract<br>(Competitive inhibitor, *in vitro*, $IC_{50}$ = 77 ± 8 ppm on monophenolase inhibitory activity)<br>(*in vivo*, $IC_{50}$ = 27 ± 1 ppm on monophenolase inhibitory activity)<br>(Reducing melanin content in B16 cells, $IC_{50}$ = 39 ± 9 µg/mL) | [63] |
| *Prunus davidiana* | Seeds | 30% Ethanol extract<br>(*in vitro*, $IC_{50}$ > 1,000 µg/mL on diphenolase inhibitory activity)<br>(*in vivo*, $IC_{50}$ > 1,000 µg/mL on diphenolase inhibitory activity) | [50] |

**Table 1** Antityrosinase activity of crude extracts of plants (continue)

| Scientific name | Plant part | Crude extract | Reference |
|---|---|---|---|
| *Psiadia arguta* | Leaves | Essential oil<br>(IC$_{50}$ = 20.03 ± 1.542 μg/mL on monophenolase inhibitory activity) | [44] |
| *Psiadia terebinthina* | Leaves | Essential oil<br>(IC$_{50}$ = 135.13 ± 0.117 μg/mL on monophenolase inhibitory activity) | [44] |
| *Psidium guajava* | Leaves | Essential oil<br>(IC$_{50}$ = 76.34 ± 0.854 μg/mL on monophenolase inhibitory activity) | [44] |
| *Rhodiola rosea* | Roots | Ethanol extract<br>(% monophenolase inhibition = 53.3 ± 1.5% at 4 mg/mL)<br>Ethyl acetate extract<br>(% monophenolase inhibition = 77.1 ± 0.5% at 4 mg/mL) | [64] |
| *Rosa damascena* | Flowers | Rose oil distillation water<br>(Uncompetitive inhibitor, IC$_{50}$= 0.41 ± 0.03 μg/mL on monophenolase inhibitory activity)<br>Fraction III of rose oil distillation water<br>(Noncompetitive inhibitor, IC$_{50}$ = 89.09 ± 0.16 μg/mL on monophenolase inhibitory activity) | [65] |
| *Rubus pungens* | Leaves | Essential oil<br>(IC$_{50}$ = 0.92 mg/mL on diphenolase inhibitory activity) | [39] |
| *Salvia officinalis* | Leaves | Essential oil<br>(IC$_{50}$ = 99.76 ± 1.750 μg/mL on monophenolase inhibitory activity) | [44] |
| *Schinus terebinthifolius* | Leaves | Essential oil<br>(IC$_{50}$ = 105.03 ± 2.485 μg/mL on monophenolase inhibitory activity) | [44] |
| *Sideroxylon inerme* | Stem barks | Acetone extract<br>(IC$_{50}$ = 63 ± 2.1 μg/mL on monophenolase inhibitory activity)<br>Acetone extract<br>(IC$_{50}$ > 400 μg/mL on diphenolase inhibitory activity)<br>Dichloromethane extract<br>(IC$_{50}$ > 400 μg/mL on monophenolase inhibitory activity | [40] |
| *Sideroxylon inerme* | Stem barks | Methanol extract<br>(IC$_{50}$ = 82.1 ± 2.7 μg/mL on monophenolase inhibitory activity) | [40] |
| *Sideroxylon inerme* | Stem barks | Methanol extract<br>(IC$_{50}$ = 82.1 ± 2.7 μg/mL on monophenolase inhibitory activity)<br>Methanol extract<br>(IC$_{50}$ > 400 μg/mL on diphenolase inhibitory activity)<br>Ethyl acetate fraction<br>(% diphenolase inhibition = 40% at 25 μg/mL)<br>Ethyl acetate fraction inhibited melanin production in melanocyte at 50 μg/mL | [40] |
| *Solanum ovalifolium* | Fruits | Methanol extract<br>(% monophenolase inhibition = 93.6 ± 0.5%) | [66] |
| *Sophora japonica* | Flowers | 50% Ethanol extract<br>(IC$_{50}$ = 0.56 μg/mL on monophenolase inhibitory activity) | [67] |
| *Streblus ilicifolius* | Woods | Aqueous extract<br>(% diphenolase inhibition = 3.17 ± 5.24% at 20 μg/mL)<br>Ethanol extract<br>(% diphenolase inhibition = 75.52 ± 5.42% at 20 μg/mL)<br>Ethyl acetate extract<br>(% diphenolase inhibition = 24.38 ± 1.04% at 20 μg/mL)<br>Petroleum ether extract<br>(% diphenolase inhibition = 2.45 ± 5.90% at 20 μg/mL) | [49] |
| *Tamarindus indica* | | Ethyl acetate extract<br>(IC$_{50}$ = 96.15 ± 0.62 μg/mL on diphenolase inhibitory activity) | [59] |
| *Triphasia trifolia* | Leaves | Essential oil<br>(IC$_{50}$ = 19.87 ± 0.892 μg/mL on monophenolase inhibitory activity) | [44] |

**Table 2** Antityrosinase activity of phytochemicals of plants

| Scientific name | Plant part | Phytochemical | Reference |
|---|---|---|---|
| *Acanthopanax koreanum* | Leaves | 3$\alpha$-Hydroxy-lup-20(29)-en-23-al-28-oic acid (**1**) ($IC_{50}$ = 25.81 ± 1.40 $\mu$M on monophenolase inhibitory activity) (4*S*)-$\alpha$-Terpinyl 8-*O*-$\beta$-*D*-glucopyranoside (**2**) ($IC_{50}$ = 16.90 ± 0.62 $\mu$M on monophenolase inhibitory activity) 3$\alpha$,11$\alpha$,30-Trihydroxylup-23-al-20(29)-en-28-oic acid (**3**) ($IC_{50}$ = 63.50 ± 3.43 $\mu$M on monophenolase inhibitory activity) 3$\alpha$,20$\alpha$,29-Trihydroxylupane-23,28-dioic acid (**4**) ($IC_{50}$ = 8.61 ± 1.09 $\mu$M on monophenolase inhibitory activity) | [32] |
| *Artocarpus heterophyllus* | Heart woods | Artocarpin (**5**) ($IC_{50}$ = 0.90 ± 1.63 $\mu$g/mL on diphenolase inhibitory activity) Brosimon I (**6**) ($IC_{50}$ = 1.78 ± 0.94 $\mu$g/mL on diphenolase inhibitory activity) Cudraflavon B (**7**) ($IC_{50}$ = 1.03 ± 0.65 $\mu$g/mL on diphenolase inhibitory activity) Morachalcon A (**8**) ($IC_{50}$ = 0.18 ± 0.10 $\mu$g/mL on diphenolase inhibitory activity) | [42] |
| | Roots | (*E*)-4-[(1*E*)-3-methyl-1-buten-1-yl]-3,5,2′,4′-tetra hydroxylstilbene (**9**) ($IC_{50}$ = 0.20 $\mu$g/mL on diphenolase inhibitory activity) | [68] |
| | Woods | Artocarpanone (**10**) ($IC_{50}$ = 2.0 ± 0.1 $\mu$M on diphenolase inhibitory activity) Artocaepin E (**11**) ($IC_{50}$ = 6.7 ± 0.8 $\mu$M on diphenolase inhibitory activity) Artocaepin F (**12**) ($IC_{50}$ > 50 $\mu$M on diphenolase inhibitory activity) Dihydromorin (**13**) ($IC_{50}$ > 50 $\mu$M on diphenolase inhibitory activity) Liquiritigenin (**14**) ($IC_{50}$ = 22.0 ± 2.5 $\mu$M on diphenolase inhibitory activity) Norartocarpetin (**15**) ($IC_{50}$ > 50 $\mu$M on diphenolase inhibitory activity) Steppogenin (**16**) ($IC_{50}$ = 7.5 ± 2.5 $\mu$M on diphenolase inhibitory activity) | [42] |
| *Artocarpus integer* | Roots | Artocarpanone (**10**) ($IC_{50}$ = 44.56 $\mu$g/mL on diphenolase inhibitory activity) Artocarpin (**5**) ($IC_{50}$ > 200 $\mu$g/mL on diphenolase inhibitory activity) Cudraflavone C (**17**) ($IC_{50}$ > 200 $\mu$g/mL on diphenolase inhibitory activity) | [25] |
| *Blumea balsamifera* | Leaves | Blumeatin (**18**) ($IC_{50}$ = 0.624 ± 0.029 mM on diphenolase inhibitory activity) Dihydroquercetin-7,4′-dimethyl ether (**19**) (Competitive inhibitor, $IC_{50}$ = 0.162 ± 0.042 mM on diphenolase inhibitory activity) Dihydroquercetin-4′-methyl ether (**20**) (Competitive inhibitor, $IC_{50}$ = 0.115 ± 0.013 mM on diphenolase inhibitory activity) Luteolin (**21**) (Noncompetitive inhibitor, $IC_{50}$ = 0.258 ± 0.015 mM on diphenolase inhibitory activity) Luteolin-7-methyl ether (**22**) (Noncompetitive inhibitor, $IC_{50}$ = 0.350 ± 0.002 mM on diphenolase inhibitory activity) Quercetin (**23**) (Competitive inhibitor, $IC_{50}$ = 0.096 ± 0.004 mM on diphenolase inhibitory activity) Rhamnetin (**24**) (Competitive inhibitor, $IC_{50}$ = 0.107 ± 0.017 mM on diphenolase inhibitory activity) Tamarixetin (**25**) ($IC_{50}$ = 0.144 ± 0.004 mM on diphenolase inhibitory activity) 5,7,3′,5′-Tetrahydroxyflavanone (**26**) (Competitive inhibitor, $IC_{50}$ = 0.423 ± 0.049 mM on diphenolase inhibitory activity) | [69] |
| *Cinnamomum cassia* | Stem barks | Cinnamaldehyde (**27**) (Mixed inhibitor, $IC_{50}$ = 4.04 ± 0.08 mM on diphenolase inhibitory activity) | [70] |

**Table 2** Antityrosinase activity of phytochemicals of plants (continue)

| Scientific name | Plant part | Phytochemical | Reference |
|---|---|---|---|
| *Citrus unshiu* | Peels | Hesperidin (**28**)<br>(Noncompetitive inhibitor, $IC_{50}$ = 16.08 mM on diphenolase inhibitory activity)<br>Nobiletin (**29**)<br>(Competitive inhibitor, $IC_{50}$= 1.49 mM on diphenolase inhibitory activity) | [45] |
| *Cleyera japonica* | Branches | Aviculin (**30**)<br>($IC_{50}$ > 200 µg/mL on diphenolase inhibitory activity)<br>3,3′-Di-*O*-methylellagic acid (**31**)<br>($IC_{50}$ > 200 µg/mL on diphenolase inhibitory activity)<br>3,3′-Di-*O*-methylellagic acid 4′-*O*-β-D-xylofuranoside (**32**)<br>($IC_{50}$ = 0.078 mM on diphenolase inhibitory activity)<br>3,5,7-Trihydroxychromone 3-*O*-α-*L*-arabinofuranoside (**33**)<br>($IC_{50}$ > 200 µg/mL on diphenolase inhibitory activity) | [71] |
| *Dalea elegans* | Aerial parts | Comptonin (**34**)<br>(% monophenolase inhibition = 22.7 ± 0.6% at 100 µM)<br>Demethoxymatteucinol (**35**)<br>($IC_{50}$ = 97.60 ± 0.30 µM on monophenolase inhibitory activity)<br>(2*S*)-2′,4′-Dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-prenyl pinocembrin) (**36**)<br>(Competitive inhibitor, $IC_{50}$ = 2.32 ± 0.01 µM on monophenolase inhibitory activity)<br>7-Hydroxy-5-methoxy-6,8-dimethylflavanone (**37**)<br>(% monophenolase inhibition = 6.97 ± 0.6% at 100 µM)<br>(2*S*)-8-Prenylpinocembrin (**38**)<br>($IC_{50}$ = 80.60 ± 0.30 µM on monophenolase inhibitory activity)<br>Triangularin (**39**)<br>(Uncompetitive inhibitor, $IC_{50}$ = 33.30 ± 0.10 µM on monophenolase inhibitory activity) | [33] |
| *Greyia flanaganii* | Leaves | 5,7-Dihydroxyflavanone [(2*S*)-pinocembrin] (**40**)<br>($IC_{50}$ > 200 µg/mL on monophenolase inhibitory activity)<br>2′,6′-Dihydroxy-4-methoxydihydrochalcone (**41**)<br>($IC_{50}$ > 200 µg/mL on monophenolase inhibitory activity)<br>(3*S*)-4-Hydroxyphenethyl 3-hydroxy-5-phenylpentanoate (**42**)<br>($IC_{50}$ > 200 µg/mL on monophenolase inhibitory activity)<br>2′,4′,6′-Trihydroxydihydrochalcone (**43**)<br>($IC_{50}$ = 17.86 µg/mL on monophenolase inhibitory activity)<br>(2*R*,3*R*)-3,5,7-Trihydroxy-3-*O*-acetylflavanone (**44**)<br>($IC_{50}$ > 200 µg/mL on monophenolase inhibitory activity)<br>2′,6′,4′-Trihydroxy-4-methoxydihydrochalcone (**45**)<br>($IC_{50}$ > 200 µg/mL on monophenolase inhibitory activity) | [35] |
| *Herotheca inuloides* | Flowers | Quercetin (**23**)<br>(Competitive inhibitor, $IC_{50}$ = 0.13 mM on diphenolase inhibitory activity) | [72] |
| *Intsia palembanica* | | 4′-Dehydroxyrobidanol (**46**)<br>(Competitive inhibitor, $IC_{50}$ = 15.2 µM on monophenolase inhibitory activity)<br>(Uncompetitive inhibitor, $IC_{50}$ = 50.0 µM on diphenolase inhibitory activity)<br>(+)-Epirobidanol (**47**)<br>(Mixed inhibitor, $IC_{50}$ = 20.2 µM on monophenolase inhibitory activity)<br>(Uncompetitive inhibitor, $IC_{50}$ = 178.5 µM on diphenolase inhibitory activity)<br>(-)-Robidanol (**48**)<br>(Competitive inhibitor, $IC_{50}$ = 8.7 µM on monophenolase inhibitory activity<br>(Noncompetitive inhibitor, $IC_{50}$ = 26.6 µM on diphenolase inhibitory activity) | [58] |
| *Juglans mandshurica* | Barks | Dihydroquercetin (**49**)<br>($IC_{50}$ = 98.5 ± 17.8 mM on diphenolase inhibitory activity)<br>Dihydrokaempferol (**50**)<br>($IC_{50}$ = 2.7 ± 0.7 mM on diphenolase inhibitory activity)<br>Kaempferol (**51**)<br>($IC_{50}$ = 40.8 ± 9.7 mM on diphenolase inhibitory activity)<br>Kaempferol-3-*O*-α-*L*-rhamnoside (**52**)<br>($IC_{50}$ = 117.7 ± 21.6 mM on diphenolase inhibitory activity) | [61] |

**Table 2** Antityrosinase activity of phytochemicals of plants (continue)

| Scientific name | Plant part | Phytochemical | Reference |
|---|---|---|---|
| | | Quercetin-3-$O$-$α$-$L$-rhamnoside (**53**) | |
| | | (IC$_{50}$ = 124.9 ± 20.1 mM on diphenolase inhibitory activity) | |
| | | Rhamnetin-3-$O$-$β$-$D$-glucopyranoside (**54**) | |
| | | (IC$_{50}$ = 27.95 µg/mL on diphenolase inhibitory activity) | |
| | | Soybean isoflavone (**55**) | |
| | | (IC$_{50}$ = 75.3 ± 10.1 mM on diphenolase inhibitory activity) | |
| *Juniperus communis* | Fruits | Amentoflavone (**56**) | [73] |
| | | (% diphenolase inhibition = 91.10 ± 2.78% at 25 µM) | |
| | | (% diphenolase inhibition = 94.85 ± 1.80% at 50 µM) | |
| | | Apigenin (**57**) | |
| | | (% diphenolase inhibition = 82.96 ± 1.80% at 25 µM) | |
| | | (% diphenolase inhibition = 90.06 ± 6.60% at 50 µM) | |
| | | Cupressuflavone (**58**) | |
| | | (% diphenolase inhibition = 83.87 ± 5.27% at 25 µM) | |
| | | (% diphenolase inhibition = 88.73 ± 4.04% at 50 µM) | |
| | | Hinokiflavone (**59**) | |
| | | (% diphenolase inhibition = 77.12 ± 2.25% at 25 µM) | |
| | | (% diphenolase inhibition = 98.47 ± 2.09% at 50 µM) | |
| | | Hypolaetin 7-$O$-$β$-xylopyranoside (**60**) | |
| | | (% diphenolase inhibition = 39.01 ± 3.36% at 25 µM) | |
| | | (% diphenolase inhibition = 54.52 ± 0.67% at 50 µM) | |
| | | Isoscutellarein 7-$O$-$β$-xyloparanoside (**61**) | |
| | | (% diphenolase inhibition = 88.11 ± 3.55% at 25 µM) | |
| | | (% diphenolase inhibition = 93.67 ± 6.04% at 50 µM) | |
| | | Podocarpusflavone A (**62**) | |
| | | (% diphenolase inhibition = 91.38 ± 1.30% at 25 µM) | |
| | | (% diphenolase inhibition = 95.76 ± 2.17% at 50 µM) | |
| | | Robustaflavone (**63**) | |
| | | (% diphenolase inhibition = 91.59 ± 2.92% at 25 µM) | |
| | | (% diphenolase inhibition = 92.35 ± 2.69% at 50 µM) | |
| *Morus notabilis* | Stems | (2′$R$)-2′,3′-Dihydro-2′-(1-hydroxy-1-methylethyl)-2,6′-bibenzofuran-6,4′-diol (**64**) | [74] |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | 3,4-Dihydro-7-(6-hydroxy-2-benzofuranyl)-2,2-dimethyl-2$H$-1-benzopyran-3,5-doil (**65**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | 5,6-Dimethoxy-2-(3-hydroxy-5-methoxyphenyl)benzofuran (**66**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | 2-(3-Hydroxy-5-methoxyphenyl)benzofuran-6-ol (**67**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | Moracin B (**68**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | Moracin C (**69**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | Moracin D (**70**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| | | Moracin O (**71**) | |
| | | (IC$_{50}$ = 6.29 ± 1.31 µM on diphenolase inhibitory activity) | |
| | | Moracin P (**72**) | |
| | | (IC$_{50}$ = 2.90 ± 0.17 µM on diphenolase inhibitory activity) | |
| | | Procyanidin B1 (**73**) | |
| | | (IC$_{50}$ = 200 ± 2.2 µg/mL, 346 µM on monophenolase inhibitory activity) | |
| | | (IC$_{50}$ > 200 µg/mL on diphenolase inhibitory activity) | |
| | | Sanggenfuran B (**74**) | |
| | | (Low inhibition, < 50 % diphenolase inhibition at 30 µM) | |
| *Peucedanum knappii* | Aerial part | 4′-Dihydrophaseic acid (**75**) | [36] |
| | | (IC$_{50}$ = 278.5 ± 20.4 mM on diphenolase inhibitory activity) | |
| | | 3,3′-Dimethoxyellagic acid (**76**) | |
| | | (IC$_{50}$ = 627.5 ± 23.7 mM on diphenolase inhibitory activity) | |
| | | 3,3′-Dimethoxyellagic acid xylopyranoside (**77**) | |
| | | (IC$_{50}$ = 350.2 ± 28.9 mM on diphenolase inhibitory activity) | |
| | | 4,4′-Dimethoxyellagic acid (**78**) | |
| | | (IC$_{50}$ = 310.4 ± 18.5 mM on diphenolase inhibitory activity) | |
| | | Ellagic acid (**79**) | |
| | | (IC$_{50}$ = 92.0 ± 13.4 mM on diphenolase inhibitory activity) | |

**Table 2** Antityrosinase activity of phytochemicals of plants (continue)

| Scientific name | Plant part | Phytochemical | Reference |
|---|---|---|---|
| | | ($3R$)-3′,4″-Epoxy-1-(4-hydroxyphenyl)-7-(3-methoxy phenyl) heptan-3-ol (**80**)<br>($IC_{50}$ = 59.6 ± 17.2 mM on diphenolase inhibitory activity)<br>Ethyl gallate (**81**)<br>($IC_{50}$ = 190.7 ± 18.2 mM on diphenolase inhibitory activity)<br>Gallic acid (**82**)<br>($IC_{50}$ = 89.1 ± 12.3 mM on diphenolase inhibitory activity)<br>Juglanthrthracenoside A (**83**)<br>($IC_{50}$ = 39.1 ± 11.1 mM on diphenolase inhibitory activity)<br>Methyl gallate (**84**)<br>($IC_{50}$ = 187.2 ± 22.1 mM on diphenolase inhibitory activity)<br>Naringenin (**85**)<br>($IC_{50}$ = 5.1 ± 1.5 mM on diphenolase inhibitory activity)<br>Rhoiptelol (**86**)<br>($IC_{50}$ = 83.3 ± 19.7 mM on diphenolase inhibitory activity)<br>Rhoiptelol C (**87**)<br>($IC_{50}$ = 1.5 ± 0.4 mM on diphenolase inhibitory activity)<br>Regiolone (**88**)<br>($IC_{50}$ = 78.2 ± 15.6 mM on diphenolase inhibitory activity)<br>$\beta$-Sitosterol (**89**)<br>($IC_{50}$ = 395.5 ± 21.2 mM on diphenolase inhibitory activity) | |
| *Polyalthia longifolia* | Leaves | Proanthocynidins (**90**)<br>($IC_{50}$= 773.09 ± 1.47 µg/mL on diphenolase inhibitory activity) | [75] |
| *Rhamnus nakaharai* | Heart woods | Alaternin (**91**)<br>($IC_{50}$ = 327.3 µM on diphenolase inhibitory activity)<br>Emodin (**92**)<br>($IC_{50}$ = 187.5 µM on diphenolase inhibitory activity)<br>6-Methoxysorigenin- 8-*O*-$\beta$-glucopyranoside (**93**)<br>($IC_{50}$ = 42.2 µM on diphenolase inhibitory activity)<br>6-Methoxysorigenin (**94**)<br>($IC_{50}$ = 97.4 µM on diphenolase inhibitory activity) | [76] |
| *Rhus verniciflua* | Woods | Butin (**95**)<br>(Competitive inhibitor, $IC_{50}$ = 16.00 µM on diphenolase inhibitory activity)<br>Sulfuretin (**96**)<br>(Competitive inhibitor, $IC_{50}$ = 13.64 µM on diphenolase inhibitory activity) | [77] |
| *Rosa damascena* | Flowers | Ellagic acid (**79**)<br>(Mixed inhibitor, $IC_{50}$ = 1.58 ± 0.09 µg/mL on diphenolase inhibitory activity)<br>Kaempferol (**51**)<br>(Competitive inhibitor, $IC_{50}$ = 1.58 ± 0.18 µg/mL on diphenolase inhibitory activity)<br>Quercetin (**23**)<br>(Competitive inhibitor, $IC_{50}$ = 1.27 ± 0.06 µg/mL on diphenolase inhibitory activity) | [73] |
| *Rubia cordifolia* | Roots | Purpurin (**97**)<br>(Competitive inhibitor, $IC_{50}$ = 0.11 ± 0.02 mg/mL on monolase activity)<br>(Competitive inhibitor, $IC_{50}$ = 0.29 ± 0.09 mg/mL on diphenolase activity) | [48] |
| *Sideroxylon inerme* | Stem barks | Epigallocatechin gallate (**98**)<br>($IC_{50}$ = 30±1.9 µg/mL, 65.5 µM on monophenolase inhibitory activity) | [40] |
| *Stewartia pseudocamellia* | Twigs | Stewartianol (**99**)<br>($IC_{50}$ = 49.33 ± 3.11 µM on melanin production by α-MSH)<br>Stewartianol-3-*O*-glucoside (**100**)<br>($IC_{50}$ = 39.23 ± 6.04 µM on melanin production by α-MSH) | [78] |
| *Streblus ilicifolius* | Woods | ($E$)-2,4-Dihydroxy-3-(3,7-dimethyl -2,6-octadienyl) benzaldehyde (**101**)<br>($IC_{50}$ > 200 µg/mL on diphenolase inhibitory activity)<br>Flavokawain C (**102**)<br>(Competitive inhibitor, $IC_{50}$ = 60.2 µM on monophenolase inhibitory activity)<br>(Competitive inhibitor, $IC_{50}$ = 106.7 µM on diphenolase inhibitory activity) | [49] |

**Table 2** Antityrosinase activity of phytochemicals of plants (continue)

| Scientific name | Plant part | Phytochemical | Reference |
|---|---|---|---|
| | | *p*-Hydroxybenzoic acid methyl ester (**103**) (IC$_{50}$ > 200 µg/mL on diphenolase inhibitory activity) Isoxanthohumol (**104**) (Mixed inhibitor, IC$_{50}$ = 77.4 µM on monophenolase inhibitory activity) Methylxanthohumol (**105**) (Competitive inhibitor, IC$_{50}$ = 34.3 µM on monophenolase inhibitory activity) (Competitive inhibitor, IC$_{50}$ = 70.5 µM on diphenolase inhibitory activity) Moracin M (**106**) (IC$_{50}$ = 67.69 µg/mL on monophenolase inhibitory activity) (Noncompetitive inhibitor, IC$_{50}$ = 77.2 µM on diphenolase inhibitory activity) Xanthohumol (**107**) (Competitive inhibitor, IC$_{50}$ = 15.4 µM on monophenolase inhibitory activity) (Competitive inhibitor, IC$_{50}$ = 31.1 µM on diphenolase inhibitory activity) Xanthoumol B (**108**) (Competitive inhibitor, IC50 = 22.1 µM on monophenolase inhibitory activity) (Competitive inhibitor, IC50 = 46.7 µM on diphenolase inhibitory activity) Xanthohumol C (**109**) (Competitive inhibitor, IC$_{50}$ = 20.6 µM on monophenolase inhibitory activity) (Competitive inhibitor, IC$_{50}$ = 41.3 µM on diphenolase inhibitory activity) | |
| *Zanthoxylum piperitum* | Leaves | Quercetin (**23**) (Competitive inhibitor, IC$_{50}$ = 3.8 µg/mL on monophenolase inhibitory activity( | [79] |



3α-Hydroxy-lup-20(29)-en-23-al-28-en-28-oic acid (**1**)

(4*S*)-α-Terpinyl 8-*O*-β-*D*-glucopyranoside (**2**)

3α,11α,30-Trihydroxylup-23-al-20(29) oic acid (**3**)

3α,20α,29-Trihydroxylupane-23,28-dioic acid (**4**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors

Artocarpin (**5**)

Brosimon I (**6**)

Cudraflavon B (**7**)

Morachacon (**8**)

(*E*)-4-[(1*E*)-3-methyl-1-buten-1-yl-3,5,2′,4′
-tetrahydroxystilbene (**9**)

Artocarpanone (**10**)

Artocaepin E (**11**)

Artocaepin F (**12**)

Dihydromorin (**13**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Liquiritigenin (**14**)

Norartocarpetin (**15**)

Steppogenin (**16**)

Cudraflavone C (**17**)

Blumeatin (**18**)

Dihydroquercetin-7,4′-dimethyl ether (**19**)

Dihydroquercetin-4′-methyl ether (**20**)

Luteolin (**21**)

Luteolin-7-methyl ether (**22**)

Quercetin (**23**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Rhamnetin (**24**)

Tamarixetin (**25**)

5,7,3′,5′-Tetrahydroxyflavanone (**26**)

Cinnamaldehyde (**27**)

Hesperidin (**28**)

Nobiletin (**29**)

Aviculin (**30**)

3,3′-Di-*O*-methylellagic acid (**31**)

3,3′-Di-*O*-methylellagic acid 4′-*O*-β-
*D*-xylofuranoside (**32**)

3,5,7-Trihydroxychromone
3-*O*-α-*L*-arabinofuranoside (**33**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Comptonin (**34**)

Demethoxymatteucinol (**35**)

(2*S*)-2′,4′-Dihydroxy-5′-(1‴,1‴-dimethylallyl)-8-
prenylpinocembrin) (**36**)

7-Hydroxy-5-methoxy-6,8-
dimethylflavanone (**37**)

(2*S*)-8-Prenylpinocembrin (**38**)

Triangularin (**39**)

5,7-Dihydroxyflavanone
[(2*S*)-pinocembrin] (**40**)

2′,6′-Dihydroxy-4-methoxy
dihydrochalcone (**41**)

(3*S*)-4-Hydroxyphenethyl 3-hydroxy-5-
phenylpentanoate (**42**)

2′,4′,6′-Trihydroxydihydrochalcone (**43**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

(2*R*,3*R*)-3,5,7-Trihydroxy-3-*O*-acetyl flavanone (**44**)

2′,6′,4′-Trihydroxy-4-methoxy dihydrochalcone (**45**)

4′-Dehydroxyrobidanol (**46**)

(+)-Epirobidanol (**47**)

(-)-Robidanol (**48**)

Dihydroquercetin (**49**)

Dihydrokaempferol (**50**)

Kaempferol (**51**)

Kaempferol-3-*O*-α-*L*-rhamnoside (**52**)

Quercetin-3-*O*-α-*L*-rhamnoside (**53**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Rhamnetin-3-*O*-β-*D*-glucopyranoside (**54**)

Soybean isoflavone (**55**)

Apigenin (**57**)

Amentoflavone (**56**)

Cupressuflavone (**58**)

Hinokiflavone (**59**)

Hypolaetin 7-*O*-*β*-xylopyranoside (**60**)

Isoscutellarein 7-*O*-*β*-xyloparanoside (**61**)

Podocarpusflavone A (**62**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Robustaflavone (**63**)

(2′*R*)-2′,3′-Dihydro-2′-(1-hydroxy-1-methyl
ethyl)-2,6′-bibenzofuran-6,4′-diol (**64**)

3,4-Dihydro-7-(6-hydroxy-2-benzo
furan-2,2-dimethyl-2*H*-1benzopyran-3,5-doil
(**65**)

5,6-Dimethoxy-2-(3-hydroxy-
5-methoxy phenyl) (**66**)

2-(3-Hydroxy-5-ethoxyphenyl)benzofuran-6-
ol (**67**)

Moracin B (**68**)

Moracin C (**69**)

Moracin D (**70**)

Moracin O (**71**)

Moracin P (**72**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Procyanidin B1 (**73**)
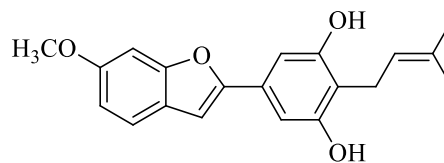
Sanggenfuran B (**74**)
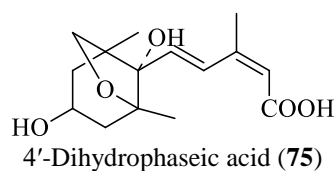
4′-Dihydrophaseic acid (**75**)
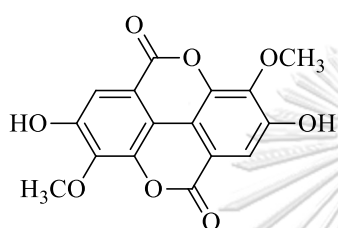
3,3′-Dimethoxyellagic acid (**76**)
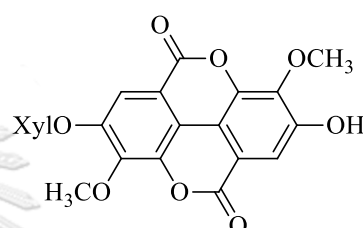
3,3′-Dimethoxyellagic acid xylopyranoside (**77**)
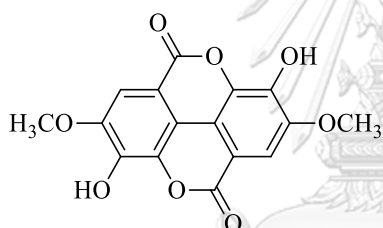
4,4′-Dimethoxyellagic acid (**78**)
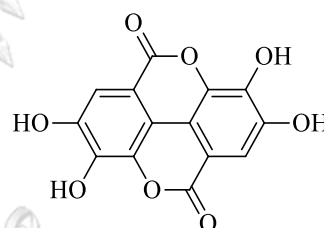
Ellagic acid (**79**)
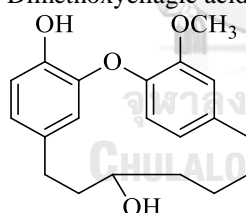
(*3R*)-3′,4″-epoxy-1-(4-hydroxyphenyl)-7(3-methoxyphenyl) heptan-3-ol (**80**)
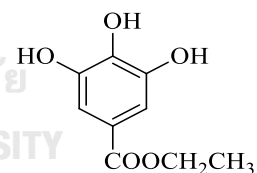
Ethyl gallate (**81**)
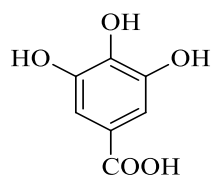
Gallic acid (**82**)
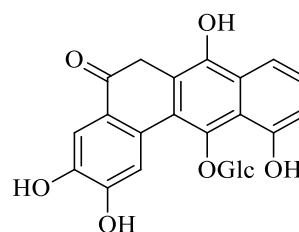
Juglanthrthracenoside A (**83**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)
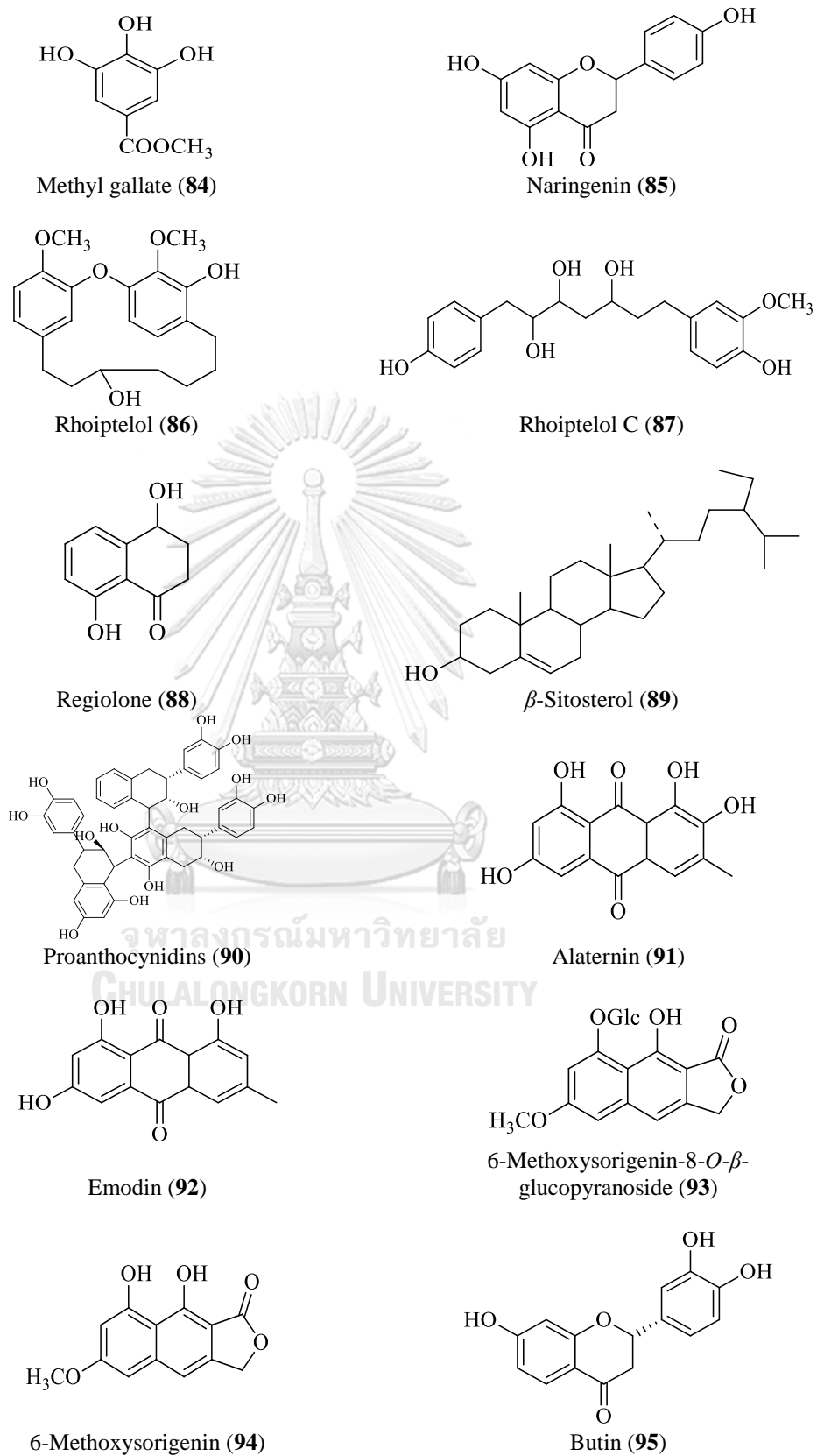
Methyl gallate (**84**)

Naringenin (**85**)

Rhoiptelol (**86**)

Rhoiptelol C (**87**)

Regiolone (**88**)

$\beta$-Sitosterol (**89**)

Proanthocynidins (**90**)

Alaternin (**91**)

Emodin (**92**)

6-Methoxysorigenin-8-*O*-$\beta$-glucopyranoside (**93**)

6-Methoxysorigenin (**94**)

Butin (**95**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Sulfuretin (**96**)

Purpurin (**97**)

Epigallocatechin gallate (**98**)

Stewartianol (**99**)

Stewartianol-3-*O*-glucoside (**100**)

(*E*)-2,4-Dihydroxy-3-(3,7-dimethyl- octadienyl) benzaldehyde (**101**)

Flavokawain C (**102**)

*p*-Hydroxybenzoic acid methyl ester (**103**)

Methylxanthohumol (**105**)

Isoxanthohumol (**104**)

**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

Moracin M (**106**)

Xanthohumol (**107**)

Xanthoumol B (**108**)

Xanthohumol C (**109**)
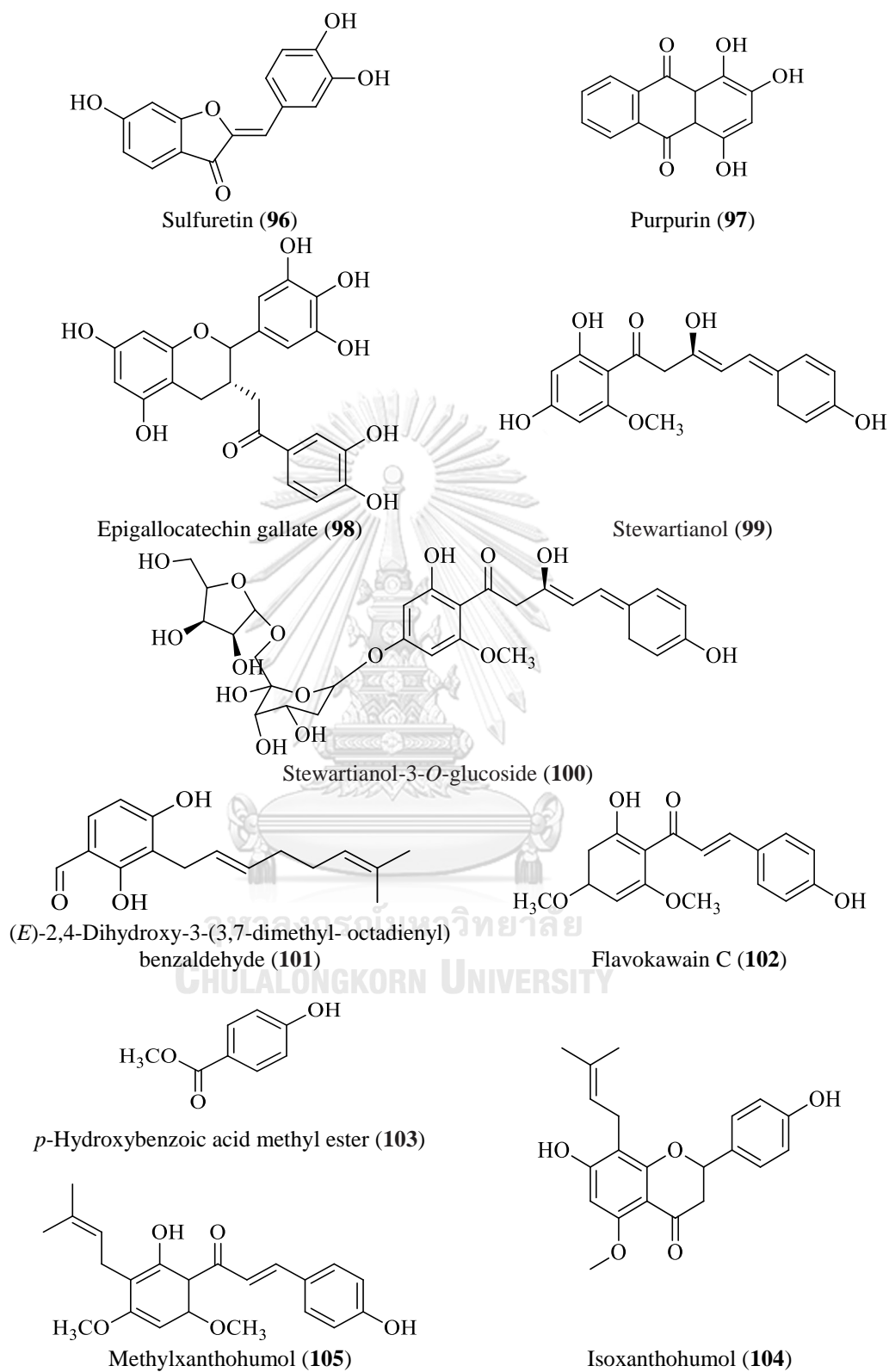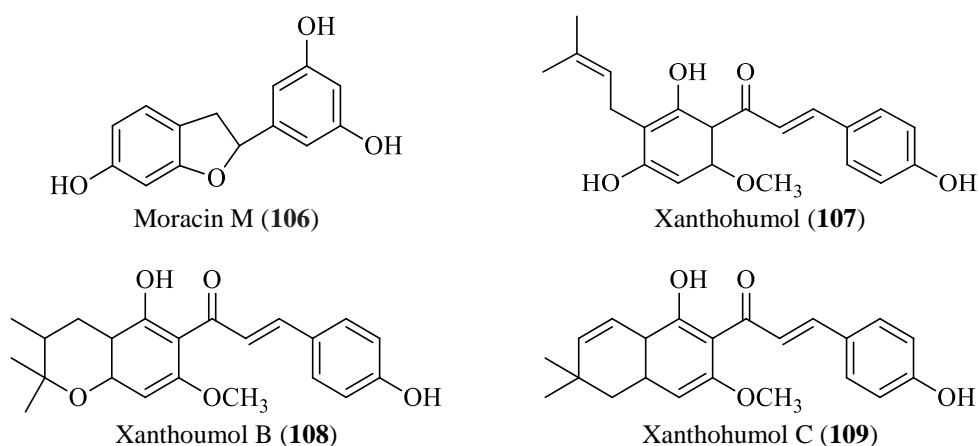
**Figure 12** Phytochemical structures of tyrosinase inhibitors (continue)

2.5.2 Tyrosinase inhibitory activity of synthetic chemicals

Many synthetic tyrosinase inhibitors were developed and designed. They were changed action of altered chemical structure but their chemicals cause unacceptable side effect in the body. While, natural tyrosinase inhibitors were able to interact with biological molecules in the body. Natural tyrosinase inhibitors could be less sustainable. Antityrosinase activity depends on classification of synthetic compounds such as the 'O' position-1 in pyranone ring was changed to synthesize a conjugate of hydroxypyridinone-amono acid and hydroxypyridinone-dipeptide [80]. Coumarin and flavonoid derivatives were synthesized and evaluated for their tyrosinase inhibitory activity [81]. The literature reviews of antityrosinase activity for synthetic compounds are shown in Table 3 and their structures are shown in Figure 13.

**Table 3** Antityrosinase activity of synthetic compounds

| Compound | Tyrosinase inhibitory activity | Reference |
|---|---|---|
| Amoxicillin (**110**) | Mixed inhibitor | [82] |
|  | IC$_{50}$ = 9.0 ± 1.8 mM on diphenolase inhibitory activity |  |
| Anisic acid (**111**) | Noncompetitive inhibitor | [80] |
|  | IC$_{50}$ = 0.60 mM on diphenolase inhibitory activity |  |
| Azaresveratrol analogs A-F |  | [83] |
| Azaresveratrol analog A (**112**) | IC$_{50}$ = 44.89 μg/mL on monophenolase inhibitory activity |  |
| Azaresveratrol analog B (**113**) | IC$_{50}$ = 72.58 μg/mL on monophenolase inhibitory activity |  |
| Azaresveratrol analog C (**114**) | IC$_{50}$ = 160.1 μg/mL on monophenolase inhibitory activity |  |
| Azaresveratrol analog D (**115**) | IC$_{50}$ = 28.66 μg/mL on monophenolase inhibitory activity |  |
| Azaresveratrol analog E (**116**) | IC$_{50}$ = 49.47 μg/mL on monophenolase inhibitory activity |  |
| Azaresveratrol analog F (**117**) | IC$_{50}$ = 147.96μg/mL on monophenolase inhibitory activity |  |

**Table 3** Antityrosinase activity of synthetic compounds (continue)

| Compound | Tyrosinase inhibitory activity | Reference |
|---|---|---|
| Caffeic acid *N*-nonyl ester (**118**) | Mixed inhibitor at lower concentration than 50 μM Irreversible inhibitor at higher concentration than 50 μM $IC_{50} = 37.5$ μM on diphenolase inhibitory activity | [84] |
| Coumarin-resveratrol hybrids 1-8 | | [81] |
| Coumarin-resveratrol hybrid 1 (**119**) | $IC_{50} > 0.1$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 2 (**120**) | $IC_{50} > 0.1$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 3 (**121**) | $IC_{50} > 0.1$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 4 (**122**) | $IC_{50} = 1.56$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 5 (**123**) | $IC_{50} > 0.1$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 6 (**124**) | $IC_{50} = 3.68$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 7 (**125**) | $IC_{50} > 0.1$ mM on diphenolase inhibitory activity | |
| Coumarin-resveratrol hybrid 8 (**126**) | $IC_{50} = 0.27$ mM on diphenolase inhibitory activity | |
| Curcumin-diaryl pentanoid analogues | | [85] |
| Curcumin-diaryl pentanoid analogue 4 (**127**) | $IC_{50} = 79.05 \pm 3.92$ μM on diphenolase inhibitory activity | |
| Curcumin-diaryl pentanoid analogue 10 (**128**) | $IC_{50} > 250$ μM on diphenolase inhibitory activity | |
| Curcumin-diaryl pentanoid analogue 15 (**129**) | $IC_{50} > 250$ μM on diphenolase inhibitory activity | |
| Curcumin-diaryl pentanoid analogue 16 (**130**) | $IC_{50} > 250$ μM on diphenolase inhibitory activity | |
| Curcumin-diaryl pentanoid analogue 22 (**131**) | $IC_{50} > 250$ μM on diphenolase inhibitory activity | |
| (2RS,4R)-2-(2,4-Dihydroxyphenyl)thiazolidine-4-carboxylic acid (**132**) | $IC_{50} = 1.81 \pm 0.90$ μM on diphenolase inhibitory activity | [86] |
| Furfural (**133**) | $IC_{50} = 3.38$ mM on diphenolase inhibitory activity | [87] |
| Furfuryl alcohol (**134**) | $IC_{50} = 17.75$ mM on diphenolase inhibitory activity | [87] |
| Furoic acid (**135**) | Uncompetitive inhibitor $IC_{50} = 10.00$ mM on diphenolase inhibitory activity | [87] |
| Glabridin (**136**) | Noncompetitive inhibitor $IC_{50} = 0.43$ μM on diphenolase inhibitory activity | [88] |
| Hydroxypyridinone derivatives | | [89] |
| Hydroxypyridinone derivative 6a (**137**) | $IC_{50} = 11.76$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 6b (**138**) | $IC_{50} = 28.71$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 6c (**139**) | $IC_{50} = 15.62$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 6d (**140**) | $IC_{50} = 12.48$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 6e (**141**) | $IC_{50} = 1.95$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 12a (**142**) | $IC_{50} = 2.79$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 12b (**143**) | $IC_{50} = 6.93$ μM on diphenolase inhibitory activity | |
| Hydroxypyridinone derivative 12c (**144**) | $IC_{50} = 14.26$ μM on diphenolase inhibitory activity | |
| 5-Hydroxy-4-acetyl-2,3-dihydronaphtho[1,2-b]furans derivatives: | | [90] |
| 1-(2-Ethoxy-5-hydroxy-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**145**) | $IC_{50} = 83.17 \pm 0.12$ μg/mL on diphenolase inhibitory activity Competitive inhibitor | |
| 1-(5-Hydroxy-2-propoxy-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**146**) | $IC_{50} = 8.91 \pm 0.24$ μg/mL on diphenolase inhibitory activity | |
| 1-(5-Hydroxy-2-isobutoxy-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**147**) | $IC_{50} = 59.56 \pm 0.95$ μg/mL on diphenolase inhibitory activity | |
| 1-(2-Butoxy-5-hydroxy-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**148**) | $IC_{50} = 69.82 \pm 0.72$ μg/mL on diphenolase inhibitory activity | |
| 1-(5-Hydroxy-2-methoxy-2-methyl-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**149**) | $IC_{50} > 100$ μg/mL on diphenolase inhibitory activity | |
| 1-(*trans*-2-Ethoxy-5-hydroxy-3-methyl-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**150**) | $IC_{50} = 30.61 \pm 0.91$ μg/mL on diphenolase inhibitory activity | |
| *cis*-1-(5-Hydroxy-6b,7,8,9a-tetrahydrofuro[2,3-b]naphtho[2,1-d]furan-6-yl)ethanone (**151**) | $IC_{50} > 100$ μg/mL on diphenolase inhibitory activity | |
| *cis*-1-(5-Hydroxy-7,8,9,10a-tetrahydro-6bH-naphtho[2',1':4,5]furo[2,3-b]pyran-6-yl)ethanone (**152**) | $IC_{50} = 50.00 \pm 0.82$ μg/mL on diphenolase inhibitory activity | |
| 1-(5-Hydroxy-2-methyl-2-phenyl-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**153**) | $IC_{50} > 100$ μg/mL on diphenolase inhibitory activity | |
| 1-(5-Hydroxy-2-methyl-2-phenyl-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**154**) | $IC_{50} > 100$ μg/mL on diphenolase inhibitory activity | |
| 1-(5-Hydroxy-2-(4-methoxyphenyl)-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**155**) | $IC_{50} > 100$ μg/mL on diphenolase inhibitory activity | |
| 2-Phenylacetic acid (**156**) | Uncompetitive inhibitor $IC_{50} = 2.38$ mM on diphenolase inhibitory activity | [91] |

**Table 3** Antityrosinase activity of synthetic compounds (continue)

| Compound | Tyrosinase inhibitory activity | Reference |
|---|---|---|
| 2-Phenylacetaldehyde (157) | Uncompetitive inhibitor IC50 = 0.39 mM on diphenolase inhibitory activity | [91] |
| 2-Phenylethanol (158) | Mixed inhibitor IC50 = 3.04 mM on diphenolase inhibitory activity | [91] |
| Rhododendrol derivatives: | | [92] |
| Rhododendrol derivative 3 (159) | IC50 = 4.72 ± 0.58 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 4 (160) | IC50 = 2.30 ± 0.15 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 5 (161) | IC50 = 2.17 ± 0.20 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 6 (162) | IC50 = 1.78 ± 0.10 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 7 (163) | IC50 = 4.56 ± 0.48 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 8 (164) | IC50 = 1.72 ± 0.17 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 9 (165) | IC50 = 3.83 ± 0.45 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 10 (166) | IC50 = 1.51 ± 0.10 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 11 (167) | IC50 = 4.13 ± 0.69 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 12 (168) | IC50 = 1.98 ± 0.17 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 20 (169) | IC50 = 9.15 ± 0.71 μM on diphenolase inhibitory activity | |
| Rhododendrol derivative 21 (170) | IC50 = 0.56 ± 0.02 μM on diphenolase inhibitory activity | |

Amoxicillin (**110**)

Anisic acid (**111**)

Azaresveratrol analog A (**112**)

Azaresveratrol analog B (**113**)

Azaresveratrol analog C (**114**)

Azaresveratrol analog D (**115**)

**Figure 13** Structure of synthetic compounds of tyrosinase inhibitors

Azaresveratrol analog E (**116**)

Azaresveratrol analog F (**117**)

Caffeic acid *N*-nonyl ester (**118**)

Coumarin-resveratrol hybrid 1 (**119**)

Coumarin-resveratrol hybrid 2 (**120**)

Coumarin-resveratrol hybrid 3 (**121**)

Coumarin-resveratrol hybrid 4 (**122**)

Coumarin-resveratrol hybrid 5 (**123**)

Coumarin-resveratrol hybrid 6 (**124**)

Coumarin-resveratrol hybrid 7 (**125**)

Coumarin-resveratrol hybrids 8 (**126**)

(2*RS*,4*R*)-2-(2,4-Dihydroxyphenyl)
thiazolidine-4-carboxylic acid (**132**)

**Figure 13** Structure of synthetic compounds of tyrosinase inhibitors (continue)

Curcumin-diaryl pentanoid analogue 4 (**127**): R= 4-OH
Curcumin-diaryl pentanoid analogue 10 (**128**): R= 4-F
Curcumin-diaryl pentanoid analogue 15 (**129**): R= 3-OCH₃, 4-OH
Curcumin-diaryl pentanoid analogue 16 (**130**): R= 2-F, 4-OCH₃
Curcumin-diaryl pentanoid analogue 22 (**131**): R = 4-OH
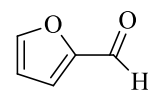
Furfural (**133**)
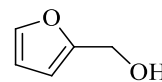
Furfuryl alcohol (**134**)
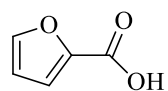
Furoic acid (**135**)
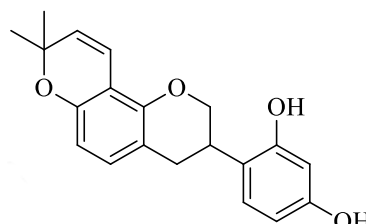
Glabridin (**136**)
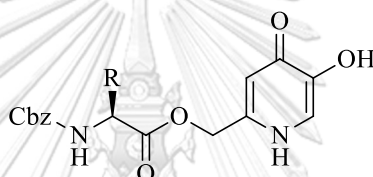
Hydroxypyridinone derivative 6a (**137**): R = CH₃
Hydroxypyridinone derivative 6b (**138**): R = CH₃CH(CH₃)₂
Hydroxypyridinone derivative 6c (**139**): R = (CH₃)CHCH₂CH₃
Hydroxypyridinone derivative 6d (**140**): R = CH(CH₃)₂

Hydroxypyridinone derivative 6e (**141**): R =

Hydroxypyridinone derivative 12a (**142**): R =
Hydroxypyridinone derivative 12b (**143**): R = CH₂CH(CH₃)₂
Hydroxypyridinone derivative 12c (**144**): R = CH(CH₃)₂
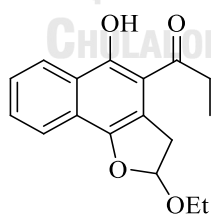
1-(2-Ethoxy-5-hydroxy-2,3
dihydronaphtho[1,2-b]furan-4-yl)ethanone (**145**)
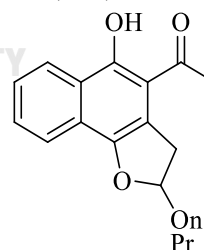
1-(5-Hydroxy-2-propoxy-2,3-dihydro
naphtho[1,2-b]furan-4-yl)ethanone (**146**)

**Figure 13** Structure of synthetic compounds of tyrosinase inhibitors (continue)

1-(5-Hydroxy-2-isobutoxy-2,3-dihydro naphtha[1,2-b]furan-4-yl)ethanone (**147**)

1-(2-Butoxy-5-hydroxy-2,3-dihydro naphtho[1,2-b]furan-4-yl)ethanone (**148**)

1-(5-Hydroxy-2-methoxy-2-methyl-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**149**)

1-(*trans*-2-Ethoxy-5-hydroxy-3-methyl-2,3-dihydronaphtho[1,2-b]furan-4-yl) ethanone (**150**)

*cis*-1-(5-Hydroxy-6b,7,8,9a-tetrahydrofuro[2,3-b] naphtho[2,1-d]furan-6-yl)ethanone (**151**)

*cis*-1-(5-Hydroxy-7,8,9,10a-tetrahydro-6bH-naphtho[2',1':4,5]furo[2,3-b] pyran-6-yl)ethanone (**152**)

1-(5-Hydroxy-2-methyl-2-phenyl-2,3-dihydronaphtho[1,2-b]furan-4-yl)ethanone (**153**)

1-(5-Hydroxy-2-methyl-2-phenyl-2,3-dihydronaphtho[1,2-b]furan-4-yl) ethanone (**154**)

**Figure 13** Structure of synthetic compounds of tyrosinase inhibitors (continue)

1-(5-Hydroxy-2-(4-methoxyphenyl)-2,3-
dihydronaphtho[1,2-b]furan-4-yl)ethanone (**155**)

2-Phenylacetic acid (**156**)
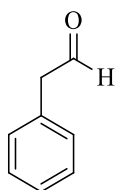
2-Phenylacetaldehyde (**157**)
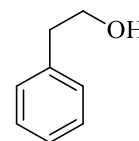
2-Phenylethanol (**158**)
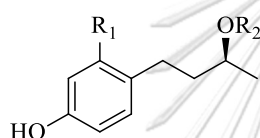
Rhododendrol derivative 3 (**159**): $R_1$ = OH $R_2$ = Glc
Rhododendrol derivative 5 (**161**): $R_1$ = OH $R_2$ = H
Rhododendrol derivative 7 (**163**): $R_1$ = OH $R_2$ = Xyl
Rhododendrol derivative 9 (**165**): $R_1$ = OH $R_2$ = Cel
Rhododendrol derivative 11 (**167**):$R_1$ = OH $R_2$ = Mal
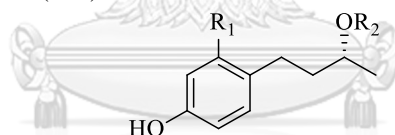
Rhododendrol derivative 4 (**160**): $R_1$ = OH $R_2$ = Glc
Rhododendrol derivative 6 (**162**): $R_1$ = OH $R_2$ = H
Rhododendrol derivative 8 (**164**): $R_1$ = OH $R_2$ = Xyl
Rhododendrol derivative 10 (**166**): $R_1$ = OH $R_2$ = Cel
Rhododendrol derivative 12 (**168**): $R_1$ = OH $R_2$ = Mal
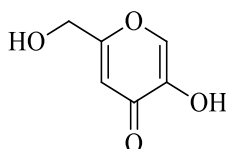
Rhododendrol derivative 20 (**169**)
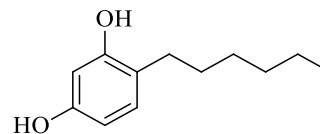
Rhododendrol derivative 21 (**170**)

**Figure 13** Structure of synthetic compounds of tyrosinase inhibitors (continue)

## 2.6 *Manilkara zapota* Linn.

### 2.6.1 Botanical

*M. zapota* belongs to the Sapotaceae family. The genus *Manilkara* are found only four species in Thailand; *M. hexandra*, *M. littoralis*, *M. kauki* and *M. zapota*. Thai

name of *M. zapota* is called Lamut, Cha-wa-ni-lo (Pattani) and general name of *M. zapota* is called Lamut farang. It is a native of South America and is widely cultivated in tropical zones. This tree grows up to 6 m tall. Branches are reddish brown and leaves are elliptic oblong, 1.3-2.6 cm long. Solitary flower is 2-2.5 cm long and reddish brown tomentose. Ellipsoid fruits are 5-6 cm long. Ripe fruits are edible, soft and sweet [15].

2.6.2   Phytochemical and biological activities

*M. zapota* contains many phytochemical compounds like triterpenoid such as 3-acetyltaraxer-14-en-12-one, 3-acetyltaraxerol, beturinic acid and lupeol. These compounds were isolated from *M. zapota* barks and showed cytotoxicity against the human Caucasian prostate adencarcinoma cell line PC-3 [93]. Antioxidant compound, methyl-4-*O*-galloylchlorogenate was isolated from *M. zapota* fruits and displayed antioxidant activity on DPPH radical scavenging with $IC_{50}$ value of 12.9 μM [94]. Moreover, ethanol extract of *M. zapota* fruits showed DPPH and ABTS radical scavenging with $IC_{50}$ values of $37.63 \pm 1.18$ and $73.14 \pm 2.84$ μg/mL, respectively. Ethanol extract of *M. zapota* fruits inhibited matrix metalloproteinases types MMP-1, MMP-2 and elastase activities with $IC_{50}$ values of $89.61 \pm 0.96$, $86.47 \pm 3.04$ and $35.73 \pm 0.61$ μg/mL, respectively [95]. Myricetin-3-*O*-α-*L*-rhamnoside was isolated from *M. zapota* leaves and showed antityrosinase activity (% diphenolase inhibition = 30% at 100 μg/mL) and DPPH radical scavenging activity (% radical scavenging = 94% at 40 μg/mL). *D*-Quercitol and saccharose were isolated from *M. zapota* seeds [96]. Acetone extract of *M. zapota* leaves showed antimicrobial activity against *Klebsiella pneumoniae* with zone of inhibition value of $15 \pm 0.29$ mm [18]. Aqueous extract of *M. zapota* fruit pulps and leaves showed *in vivo* antidiabetic and antipidemic acitivities on metabolic parameter of Wistar rats [16]. Ethyl acetate extract of stem barks showed antitumor activity against *Ehrlich ascites* carcinoma cell bearing mice that the weight gain was reduced with 200 mg/kg dose extract and showed antimicrobial activity against gram positive and negative bacteria with the average value of 7-13.5 mm zone of inhibition at the concentration of 400 µg/disc [97]. Manilkoraside, a pentacyclic triterpenoid saponin was isolated from ethanol extract. It showed anticancer activity with the half maximal effective concentration values ($EC_{50}$) of 20 and 48 μg/mL against human promyelocytic leukemia (HL-60 and HL-29), respectively [98]. Aqueous extract

of *M. zapota* leaves showed acaricidal activity against *Rhipicephalus microplus* with $IC_{50}$ value of 16.72 mg/L [99]. Acetone extract of *M. zapota* seeds showed antibacterial activity againsts *Vibrio cholerae* with an $IC_{50}$ value 93 µg/mL [100]. Acetone extract of *M. zapota* seeds showed DPPH free radical scavenging activity with $IC_{50}$ value of 400 µg/mL [101]. Ethyl acetate extract of *M. zapota* seed coats exhibited tyrosinase inhibitory activity with $IC_{50}$ value of 138 µg/mL [102]. Phytochemicals and biological activities of *Manilkara* genus are summarized in Table 4. Structure of phytochemicals of genus *Manilkara* is shown in Figure 14.

**Table 4** Phytochemical and biological activities of genus *Manilkara*

| Scientific name | Plant part | Crude extract/phytochemical | Reference |
|---|---|---|---|
| *Manilkara bidentata* | Resin | 3*β*-*O*-Acetyl-α-amyrin (**171**) | [103] |
| | | 3*β*-*O*-trans-Cinnamyl-α-amyrin (**172**) | |
| | | 3*β*-*O*-trans-Cinnamyl Lupeol (**173**) | |
| *Manilkara caffra* | Leaves | Ursolic acid (**174**) | [104] |
| | | Antiplasmodial activity | |
| | | ($IC_{50}$ = 6.8 µg/mL) | |
| *Manilkara obtusfolia* | Stem barks | Taraxerol (**175**) | [104] |
| | | Antiplasmodial activity | |
| | | ($IC_{50}$ > 100 µg/mL) | |
| | | Taraxerol methyl ether (**176**) | |
| | | Antiplasmodial activity | |
| | | ($IC_{50}$ > 100 µg/mL) | |
| *Manilkara subsericea* | Fruits | α-Amyrin acetate (**177**) | [105] |
| | | *β*-Amyrin acetate (**178**) | |
| | | α-Amyrin caproate (**179**) | |
| | | *β*-Amyrin caproate (**180**) | |
| | | α-Amyrin caprylate (**181**) | |
| | | *β*-Amyrin caprylate (**182**) | |
| | | Hexadecanoic acid (**183**) | |
| | | Hexadecanoic acid ethyl ester (**184**) | |
| | | Octadecanoic acid ethyl ester (**185**) | |
| | | (*E*)-9-Octadecanoic acid ethyl ester (**186**) | |
| *Manilkara zapota* | Barks | 3-Acetyloleanolic acid (**187**) | [93] |
| | | ($IC_{50}$ = 16.2 ± 0.7 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | 3-Acetyltaraxer-14-en-12-one (**188**) | |
| | | ($IC_{50}$ = 27.8 ± 0.5 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | α-Amyrin (**189**) | |
| | | *β*-Amyrin (**190**) | |
| | | Betulinic acid (**191**) | |
| | | ($IC_{50}$ = 19.8 ± 0.6 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | Lupeol (**192**) | |
| | | ($IC_{50}$ = 30.6 ± 0.9 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | Lupeol acetate (**193**) | |
| | | ($IC_{50}$ = 61.2 ± 0.7 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | Lupeone (**194**) | |
| | | Oleanolic acid (**195**) | |
| | | 7-Oxobetulinic acid (**196**) | |
| | | ($IC_{50}$ = 14.1 ± 0.5 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | 3-Oxolup-20(29)-en-28-oic acid (**197**) | |
| | | ($IC_{50}$ = 24.8 ± 0.8 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |

**Table 4** Phytochemical and biological activities of genus *Manilkara* (continue)

| Scientific name | Plant part | Crude extract/phytochemical | Reference |
|---|---|---|---|
| *Manilkara zapota* | Fruits | Taraxerone (**198**) | |
| | | ($IC_{50}$ = 62.5 ± 0.6 µg/mL on cytotoxic activity against the human Caucasian prostate adenocarcinoma cell line PC-3) | |
| | | Ethanol extract | [106] |
| | | DPPH radical scavenging | |
| | | ($IC_{50}$ = 37.63 ± 1.18 µg/mL) | |
| | | ABTS radical scavenging | |
| | | ($IC_{50}$ = 73.14 ± 2.84 µg/mL) | |
| | | Collagenase inhibition on MMP-1 | |
| | | ($IC_{50}$ = 89.61 ± 0.96 µg/mL) | |
| | | Collagenase inhibition on MMP-1 | |
| | | ($IC_{50}$ = 86.47 ± 3.04 µg/mL) | |
| | | Elastase inhibition | |
| | | ($IC_{50}$ = 35.73 ± 0.61 µg/mL) | |
| | | 4-Caffeoylquinic acid (**199**) | [94] |
| | | Methyl-4-*O*-Galloylchlorogenate (**200**) | |
| | | DPPH radical scavenging | |
| | | ($IC_{50}$ = 12.9 µM) | |
| | | Cytotoxicity against the HCT-116 and SW-480 human colon cancer cell lines | |
| | | ($IC_{50}$ = 154 and 134 µM) | |
| | | 4-*O*-Galloylchlorogenicacid (**201**) | |
| | | Methyl chlorogenate (**202**) | |
| | | Dihydromyricetin (**203**) | |
| | | Quercitrin (**204**) | |
| | Fruit pulps, leaves | Aqueous extract | [16] |
| | | *In vivo* antidiabetic and antipidemic activities | |
| | Leaves | Aqueous extract | [99] |
| | | Acaricidal activity against *Rhipicephalus microplus* | |
| | | ($IC_{50}$ = 16.72 mg/L) | |
| | | Acetone extract | [18] |
| | | Antimicrobial activity against *Klebsiella pneumoniae* | |
| | | (zone of inhibition = 15 ± 0.29 mm) | |
| | | Myricetin-3-*O*-α-L- rhamnoside (**205**) | [96] |
| | | (% diphenolase inhibition = 30% at 100 µg/mL) | |
| | | (% elastase inhibition = 34% at 40 µg/mL) | |
| | | (% radical scavenging = 94% at 40 µg/mL) | |
| | | (+)-Catechin (**206**) | |
| | | (-)-Epicatechin (**207**) | |
| | | (+)-Gallocatechin (**208**) | |
| | | Gallic acid (**82**) | |
| | Seeds | Acetone extract | [100] |
| | | Antibacterial activity againsts *Vibrio cholerae* | |
| | | ($IC_{50}$ = 93 µg/mL) | |
| | | Acetone extract | [101] |
| | | DPPH radical scavenging | |
| | | ($IC_{50}$ = 400 µg/mL) | |
| | | D-quercitol (**209**) | [96] |
| | | Saccharose (**210**) | [96] |
| | | 2-(4-Hydroxyphenethyl) tetratriacontanoate (**211**) | [107] |
| | | ($IC_{50}$ = 8.50 ± 0.55 µg/mL on DPPH radical scavenging) | |
| | | Mixture of phenylethanoyls | [107] |
| | | ($IC_{50}$ = 62.52 ± 1.25 µg/mL on DPPH radical scavenging) | |
| | | 2-(4-Hydroxyphenethyl) tetracosanoate (212) | |
| | | 2-(4-Hydroxyphenethyl) docosanoate (213) | |
| | | 2-(4-Hydroxyphenethyl) eicosanoate (214) | |
| | | 2-(4-Hydroxyphenethyl) octadecanoate (215) | |
| | | 2-(4-Hydroxyphenethyl) hexadecanoate (216) | |
| | | β-Amyrin (190) | [107] |
| | | ($IC50$ = 201.14 ± 4.53 µg/mL on DPPH radical scavenging) | |
| | | Oleanolic acid (195) | [107] |
| | | ($IC50$ = 151.80 ± 2.00 µg/mL on DPPH radical scavenging) | |

**Table 4** Phytochemical and biological activities of genus *Manilkara* (continue)

| Scientific name | Plant part | Crude extract/phytochemical | Reference |
|---|---|---|---|
| | | Lupeol (192) | [107] |
| | | (IC50 = 223.11 ± 5.64 µg/mL on DPPH radical scavenging) | |
| | | Betulinic acid (191) | [107] |
| | | DPPH radical scavenging | |
| | | (IC50 = 105.20 ± 0.41 µg/mL) | |
| | | Stigmasterol-3-O-β-D-glucopyranoside (217) | [107] |
| | | Stigmasterol (218) | [107] |
| | | β-Sitosterol (89) | [107] |
| | Seed coats | Ethyl acetate extract | [102] |
| | | Tyrosinase inhibitory activity | |
| | | (IC50 = 138 µg/ml) | |
| | Stem barks | Ethyl acetate extract | [97] |
| | | Antitumor activity against EAC | |
| | | Antimicrobial activity against gram positive and negative bacteria | |
| | | (zone of inhibition range 7-13.5 mm) | |
| | | Manilkoraside (219) | [98] |
| | | Anticancer activity against HL-60 | |
| | | (EC50 = 20 µg/mL) | |
| | | Anticancer activity against HL-29 | |
| | | (EC50 = 48 µg/mL) | |



3β-O-Acetyl-α-amyrin (**171**)          3β-O-*trans*-Cinnamyl-α-amyrin (**172**)

3β-*O*-trans-Cinnamyl lupeol (**173**)          Ursolic acid (**174**)

Taraxerol (**175**)          Taraxerol methyl ether (**176**)

**Figure 14** Phytochemical structure of genus *Manilkara*

α-Amyrin acetate (**177**)

β-Amyrin acetate (**178**)

α-Amyrin caproate (**179**)

β-Amyrin caproate (**180**)

α-Amyrin caprylate (**181**)

β-Amyrin caprylate (**182**)

Hexadecanoic acid (**183**)

Hexadecanoic acid ethyl ester (**184**)

3-Acetyloleanolic acid (**187**)

Octadecanoic acid ethyl ester (**185**)

(*E*)-9-Octadecanoic acid ethyl ester (**186**)

3-Acetyltaraxer-14-en-12-one (**188**)

**Figure 14** Phytochemical structure of genus *Manilkara* (continue)

α-Amyrin (**189**)

β-Amyrin (**190**)

Betulinic acid (**191**)

Lupeol (**192**)

Lupeol acetate (**193**)

Lupeone (**194**)

Oleanolic acid (**195**)

7-Oxobetulinic acid (**196**)

3-Oxolup-20(29)-en-28-oic acid (**197**)

Taraxerone (**198**)

**Figure 14** Phytochemical structure of genus *Manilkara* (continue)

4-Caffeoylquinic acid (**199**)

Methyl-4-*O*-Galloylchlorogenate (**200**)

4-*O*-Galloylchlorogenic acid (**201**)

Methyl chlorogenate(**202**)

Dihydromyricetin (**203**)

Quercitrin (**204**)

Myricetin-3-*O*-α-*L*-rhamnoside (**205**)

(+)-Catechin (**206**)

(-)-Epicatechin (**207**)

(+)-Gallocatechin (**208**)

D-quercitol (**209**)

Saccharose (**210**)

**Figure 14** Phytochemical structure of genus *Manilkara* (continue)

2-(4-Hydroxyphenethyl) tetracosanoate (**211**)

2-(4-Hydroxyphenethyl) docosanoate (**212**)

2-(4-Hydroxyphenethyl) eicosanoate (**213**)

2-(4-Hydroxyphenethyl) octadecanoate (**214**)

Stigmasterol-3-O-β-D-glucopyranoside (**215**)

Stigmasterol (**216**)

Manilkoraside (**217**)

**Figure 14** Phytochemical structure of genus *Manilkara* (continue)

# CHAPTER III

# MATERIALS AND METHODS

## 3.1 Materials

### 3.1.1 Plant material of *M. zapota*

The barks, flowers, fruits, leaves, roots and seeds of *M. zapota* were collected from Saraburi Province, Thailand in May 2013. A voucher specimen (BKF No. 187749) was deposited at the Forest Herbarium, Department of National Parks, Wildlife and Plant Conservation, Bangkok, Thailand.

### 3.1.2 Chemicals and reagents

3.1.2.1 All commercial grade solvents were distilled prior to use such as hexane, dichloromethane, ethyl acetate, acetone and methanol.

3.1.2.2 All HPLC grade solvents were used for HPLC analysis such as acetonitrile, methanol and DDI water (Sigma, Germany).

3.1.2.3 All analytical grade chemicals, monosodium phosphate, disodium phosphate, dimethyl sulfoxide (DMSO), absolute ethanol, sulfuric acid, vanillin reagent, *L*-tyrosine, *L*-DOPA, kojic acid, chloroform-*d*, acetone-*d*$_6$ were purchased from Merck (Germany). Mushroom tyrosinase was purchased from Sigma-Aldrich (USA).

## 3.2 General techniques and procedures

### 3.2.1 Thin layer chromatography (TLC)

| | |
|---|---|
| Techniques: | One dimension |
| Stationary phase: | Silica gel 60 F$_{254}$ (Merck, Germany) pre-coated plate |
| Layer thickness: | 0.2 mm |
| Distance of mobile phase: | 4 cm |
| Mobile phase: | Various solvent systems |
| Detection: | a. UV light at 254 and 365 nm |
| | b. dipping in 10% sulfuric acid in aqueous ethanol and heating on hot plate |

3.2.2 Preparative thin layer chromatography (PTLC)

| | |
|---|---|
| Techniques: | One dimension |
| Stationary phase: | Silica gel 60 F$_{254}$ (Merck, Germany) glass-coated plate |
| Layer thickness: | 1 mm |
| Distance of mobile phase: | 18 cm |
| Mobile phase: | Various solvent systems |
| Detection: | a. UV light at 254 and 365 nm |
| | b. dipping in 10% sulfuric acid in aqueous ethanol and heating on hot plate |

3.2.3 Column chromatography (CC)

| | |
|---|---|
| Stationary phase: | Silica gel 60, 70-230 mesh ASTM (Merck, Germany) and Sephadex LH-20, 8-111 μm (Bioscience, USA) |
| Mobile phase: | Various solvent systems |
| Packing method: | Dry and wet packing |
| Detection: | Eluted fractions were monitored by TLC |

3.2.4 High performance liquid chromatography (HPLC)

| | |
|---|---|
| Stationary phase: | Hypersyl ODS Cyano column (4.6 mm × 25 cm, 5 μm, Thermo Hypersil-Keystone, Germany) |
| Mobile phase: | Acetonitrile, methanol and DDI water |
| Injection value: | 10 μL |
| Detection: | UV-Visible spectrophotometer, range of wavelength was 200-600 nm |

3.2.5 UV-Visible spectrophotometer

UV spectra were recorded with a Microplate reader Multiscan GO (Thermo Fisher Scientific, USA).

3.2.6 Nuclear magnetic resonance spectrophotometer (NMR)

The one dimensional spectra ($^1$H-NMR, $^{13}$C-NMR, DEPT90 and DEPT135) and two dimensional spectra (correlation spectrophotometer (COSY), heteronuclear

multiple quantum coherence (HMQC), heteronuclear single quantum correlation (HSQC) and heteronuclear multiple bond correlation (HMBC)) were determined on a Bruker model Fourier 300 spectrometer instrument (Bruker Daltonics Inc, Bremen, Germany). The chemical shifts were recorded in ppm with the reference solvent signals. Tetramethylsilane (TMS) was used as an internal standard.

3.2.7 High resolution electron impact mass spectrophotometer

The high resolution mass spectra were obtained on Bruker model MICROTOF (Bruker Daltonics Inc, Bremen, Germany). The coupled mass spectrometer was carried on electron impact ionization (EI) mode. Range of scan was 25-3000 m/z.

**3.3 Methods**

3.3.1 Extraction

3.3.1.1 Extraction of different parts of *M. zapota*

The six different parts of *M. zapota*; barks, flowers, fruits, leaves, roots and seeds were chopped and dried in a hot air oven at 60 °C. Each dried sample (500 g) was extracted with methanol ($2 \times 0.5$ L) and water ($2 \times 0.5$ L) by maceration for 24 h at 30 °C. Then, the filtrate was evaporated *in vacuo* to give methanol and aqueous crude extracts.

Barks, flowers, fruits, leaves, roots and seeds of *M. zapota*

Dry 60 °C

Dried plants

Maceration, filtration and evaporation

Methanol crude extracts          Aqueous crude extracts
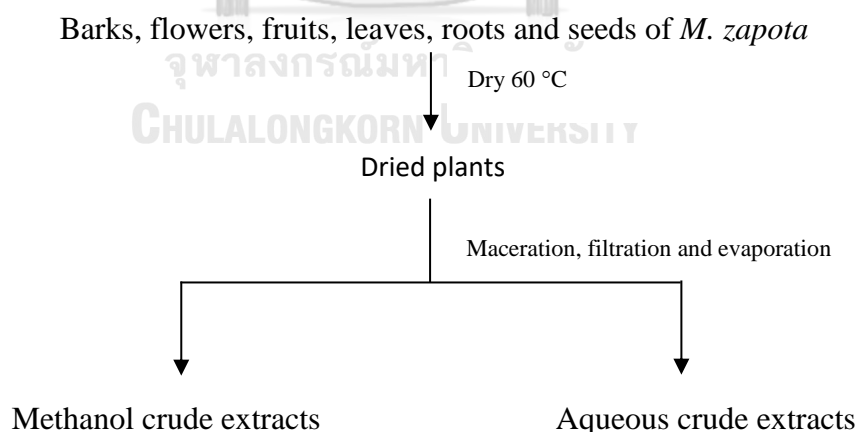
**Figure 15** Extraction procedure of six different parts of *M. zapota*

3.3.1.2 Extraction of *M. zapota* barks

Fresh barks of *M. zapota* (175 kg) were collected and dried in an oven at 60 °C. The dried barks of *M. zapota* (7 kg) were then powdered and extracted by maceration with *n*-hexane ($3 \times 5.0$ L), ethyl acetate ($3 \times 5.5$ L), methanol ($3 \times 5.0$ L) and water (3

$\times$ 3.5 L), respectively. Each the extract was filtrated and evaporated under *in vacuo* evaporator to get *n*-hexane crude extract (140 g), ethyl acetate crude extract (138 g), methanol crude extract (820 g) and aqueous crude extract (97 g). All crude extracts of *M. zapota* barks were evaluated for tyrosinase inhibitory activity.

Fresh barks of *M. zapota*

Dry 60 °C

Dried barks of *M. zapota*

Hexane

Residue | Hexane crude extract

Ethyl acetate

Residue | Ethyl acetate crude extract

Methanol

*Residue* | *Methanol crude extract*

Water

*Residue* | *Aqueous crude extract*
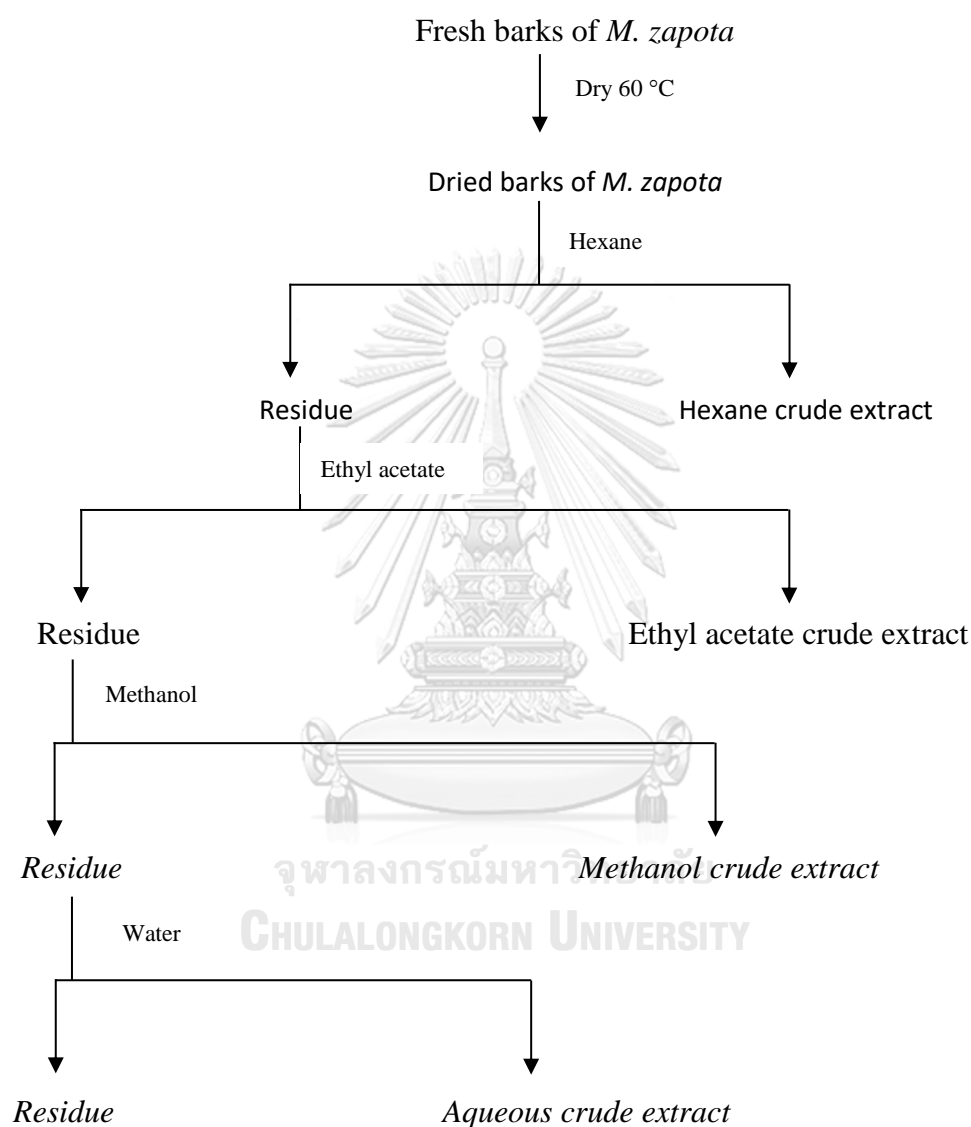
**Figure 16** Extraction procedure of *M. zapota* barks

3.3.2 Total phenolic content

Total phenolic content was determined using the Folin-Ciocalteu assay according to the modified method of Dorman *et al* [22]. The 100 µL of sample (1 mg/mL) was added to 6 mL of water. After that, 500 µL of undiluted Folin–Ciocalteu reagent was added to the solution and incubated for 1 min at 30 °C. Then, 1.5 mL of 20% (w/v) $Na_2CO_3$ was added and the reaction mixture was made up to 10 mL of

volumetric flask with water. The absorbance was measured at 760 nm after 2 h incubation using the spectrophotometer. The total phenolic content was expressed as mg gallic acid equivalents (mg GAE)/g dry sample. The concentrations of standard curve were 50, 100, 200, 400 and 800 μM, respectively.

### 3.3.3 Total flavonoid content

Total flavonoid content was determined by a modified method of Tohidi *et al* [23]. The 125 μL of sample (1 mg/mL) was added to 75 μL of 5% (w/v) $NaNO_2$. The mixture solution was incubated for 6 min at 30 °C and 150 μL of 10% (w/v) $AlCl_3$ was then added. After 5 min of incubation, 750 μL of 1 M NaOH was added and the final volume of the reaction mixture was made up to 2500 μL with water. After incubation for 15 min at 30 °C, the absorbance was measured at 510 nm using the spectrophotometer. The total flavonoid content was expressed as mg quercetin equivalents (mg QE)/g dry sample using quercetin calibration curve. Standard curve was prepared at concentration of 50, 100, 200, 400 and 800 μM, respectively.

### 3.3.4 Isolation of crude extracts of *M. zapota* barks

### 3.3.4.1 Bio-assay guided isolation of *n*-hexane crude extract

*n*-Hexane crude extract (135 g) was chromatographed on a silica gel quick column chromatography and eluted with *n*-hexane, a mixture of *n*-hexane:ethyl acetate (1:1 v/v), ethyl acetate and a mixture of ethyl acetate:methanol (19:1 v/v) to yield four fractions [108]. Fractions A-D were tested for tyrosinase inhibitory activity using *L*-DOPA as a substrate.

The fraction A (60 g) was applied for a silica gel column chromatography and eluted with *n*-hexane:dichloromethane (100:0, 19:1, 1:1 and 0:100 v/v) to obtain four subfractions (A1-A4). These subfractions were investigated for tyrosinase inhibitory activity. Fraction A1 (900.0 mg) was subjected to silica gel column chromatography using *n*-heptane, a mixture gradient of *n*-heptane:*n*-hexane (9:1 and 2:3 v/v) and *n*-hexane to afford four subfractions (A11-A14). Each subfractions was evaluated for tyrosinase inhibitory activity using *L*-DOPA as a substrate. Subfraction A14 was recrystallized in a mixture of dichloromethane and methanol (9:1 v/v) to give colorless crystal (60.8 mg). Moreover, fraction A2 was afforded as colorless crystal (26.2 mg).

Isolated crystal from subfractions A14 and A2 exhibited the same $R_f$ value on TLC. Thus, subfractions A14 and A2 were obtained as compound **I** (87.0 mg).

The fraction C (1.3 g) was chromatographed by column chromatography on silica gel with eluents as *n*-heptane:*n*-hexane:dichloromethane (1:4:0, 0:100:0 and 0:9:1 v/v) to yield three subfractions (C1-C3). Each subfraction was investigated for tyrosinase inhibitory activity. Subfraction C3 (140.0 mg) was subjected to silica gel column chromatography and eluted with petroleum ether:dichloromethane (2:3 and 1:4 v/v) and dichloromethane:ethyl acetate (100:0 and 9:1 v/v) to give four subfractions (C31-C34). Subfractions C31-C34 were tested for antityrosinase activity.

### 3.3.4.2 Bio-assay guided isolation of ethyl acetate crude extract

Ethyl acetate crude extract (130 g) was chromatographed by a silica gel quick column chromatography and eluted with a mixture gradient of *n*-hexane:ethyl acetate (100:0, 1:1 and 0:100 v/v) and a mixture gradient of ethyl acetate:methanol (19:1 v/v) to yield four fractions (E-H). Fractions E-H were evaluated for tyrosinase inhibitory activity using *L*-DOPA as a substrate.

Fraction E (1.0 g) was chromatographed over silica gel column chromatography and eluted with *n*-hexane in dichloromethane (100:0, 9:1, 7:3, 3:2, 1:1, 4:5, 3:7 and 1:9 v/v) to give nine subfractions (E1-E9). Subfractions E2, E5, E6 and E8 were evaluated against tyrosinase activity. Subfraction E2 (25.0 mg) was separated on silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:dichloromethane (9:1, 4:1, 7:3, 3:2, 3:7, 1:4, 1:9 and 0:100 v/v) to obtain eight subfractions (E21-E28). Only subfraction E22 was determined for tyrosinase inhibitory activity. Subfraction E5 (75.0 mg) was rechromatographed using preparative TLC with petroleum ether:dichloromethane (3:2 v/v) as eluent to give three subfractions (E51-E53).

Fraction F (19.0 g) was subjected to column chromatography over silica gel and eluted with a mixture gradient of *n*-hexane:ethyl acetate (4:1, 7:3, 3:2, 3:7, 1:4 and 0:100 v/v) to yield six subfractions (F1-F6). All subfractions F1-F6 were evaluated for tyrosinase inhibitory activity. Subfraction F1 (1.0 g) was fractionated by silica gel column chromatography and eluted with *n*-hexane:dichloromethane (9:1, 4:1, 7:3, 3:2, 3:7 and 1:9 v/v) to give six subfractions (F11-F16). From tyrosinase inhibitory activity,

subfraction F11 (120.0 mg) was chromatographed by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:dichloromethane (100:0, 9:1, 4:1, 3:2, 1:1 and 0:100 v/v) to afford six subfractions (F111-F116). Subfraction F113 (60.0 mg) was purified by silica gel column chromatography which eluted with dichloromethane:ethyl acetate (4:1, 7:3, 3:2, 1:1 and 1:4 v/v) to yield five subfractions (F1131-F1135). Subfractions F1131 and F1132 were tested for antityrosinase activity. Subfractions F1131 (25.0 mg) was chromatographed by silica gel column chromatography and eluted with petroleum ether:*n*-hexane (3:2 and 1:9 v/v), *n*-hexane: dichloromethane (100:0, 4:1, 7:3, 3:2, 1:9 and 0:100 v/v) to provide eight subfractions (F11311-F11318). Subfraction F2 (100 mg) was chromatographed by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:dichloromethane (9:1, 7:3 and 1:9 v/v) to obtain three subfractions (F21-F23). Subfraction F21 displayed as same $R_f$ value on TLC as compound **I**. Subfraction F3 (9.6 g) was applied to silica gel column chromatography and eluted with a mixture solvent of *n*-hexane:ethyl acetate (4:1, 7:3, 3:7 and 0:100 v/v) to obtain four subfractions (F31-F34). TLC analysis of subfractions F31 and compound **I** showed the same $R_f$ value. Thus subfractions F31 was compound **I**. Subfraction F4 (1.0 g) was subjected to silica gel column chromatography using a mixture gradient of *n*-hexane:ethyl acetate (100:0, 9:1, 7:3, 1:1, 1:4 and 0:100 v/v) as eluent to yield six subfractions (F41-F46). Subfraction F43 (100.0 mg) was further subjected to silica gel column chromatography eluting with *n*-hexane:ethyl acetate (4:1, 7:3, 1:1, 1:4 and 0:100 v/v) to yield five subfractions (F431-F435). Subfraction F433 (65.0 mg) was separated by silica gel column chromatography and eluted with gradient solvent of *n*-hexane:ethyl acetate (100:0, 4:1, 7:3, 1:1, 1:4 and 0:100 v/v) to obtain six subfractions (F4331-F4336). Subfraction F4332 (72.9 mg) was separated by preparative TLC and eluted with a mixture of *n*-hexane:ethyl acetate (7:3 v/v) to obtain two subfractions (F43321-F43322). Subfraction F4334 (10.0 mg) was fractionated on an analytical $C_{18}$ column using an isocratic system of methanol and aqueous (3:2 v/v, 1 mL/min). The retention time of subfraction F4334 is 3.5 min to yield compound **II** (8.0 mg). Subfraction F4335 (5.0 mg) was submitted to Sephadex LH-20 column chromatography and used methanol as an eluent to afford three subfractions (F53351-F43353). Subfraction F43353 (2.4 mg) was purified by an analytical HPLC using an isocratic system of methanol and water (7:3 v/v, 0.5 mL/min)

to yield compound **III** (2.4 mg). Retention time of compound **III** was recorded at 5.9 min. Subfraction F5 (3.8 g) was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:ethyl acetate (9:1, 4:1, 7:3, 3:2, 3:7, 1:4, 1:9 and 0:100 v/v) to afford nine subfractions (F51-F59). Subfraction F53 (70.0 mg) was separated by preparative TLC and eluted with a mixture gradient of *n*-hexane and ethyl acetate (9:1 v/v) to afford two subfractions (F531-F532). Subfraction F54 (17.9 mg) was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:ethyl acetate (9:1, 7:3, 3:2, 1:4, 1:9 and 0:100 v/v) to afford six subfractions (F541-F546). Subfraction F59 (150.0 mg) was separated by silica gel column chromatography using a mixture gradient of *n*-hexane:acetone (9:1, 4:1, 7:3, 3:2, 1:1, 2:3, 3:7, 1:4 and 0:100 v/v) to afford ten subfractions (F591- F5910). Subfraction F596 was obtained as brown powder (60.0 mg, compound **IV**). Subfraction F6 (1.0 g) was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:ethyl acetate (100:0, 4:1 and 7:3 v/v) to give three subfractions (F61-F63). Subfraction F62 (20.0 mg) was separated by silica gel column chromatography and eluted with a mixture of *n*-hexane:dichloromethane (4:1, 7:3, 1:4, 1:9 and 0:100 v/v) to give six subfractions (F621-F626).

Subfraction G (4.0 g) was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:dichloromethane:ethyl acetate (100:0:0, 1:4:0 and 0:7:3 v/v) to give five subfractions (G1-G5). Subfraction G3 (150 mg) was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:ethyl acetate (100:0, 4:1 and 2:3 v/v) and ethyl acetate:methanol (9.5:0.5 v/v) to give four subfractions (G31-G34). Subfraction G33 (45.0 mg) was separated using silica gel column chromatography and eluted with *n*-heptane:dichloromethane (9:1, 7:3 and 1:9 v/v) to give three subfractions (G331-G333). Subfraction G332 (24.0 mg) was further purified by preparative TLC and eluted with a mixture of petroleum ether and dichloromethane (7:3 v/v) to obtain two subfractions (G3321-G3322). Subfraction G3322 was obtained as white crystal (10.5 mg, compound **V**). Subfraction G333 was obtained as white crystal (15.4 mg, compound **VI**). Subfraction G5 (180.0 mg) was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:ethyl acetate (3:7 and 2:8 v/v) to obtain two subfractions (G51-G52). Subfraction G51 (80.0 mg) was further separated by silica gel column chromatography

and eluted with a mixture of dichloromethane:ethyl acetate (3:7 and 1:9 v/v) to obtain two subfractions (G511-G512). Subfrction G511 was obtained as white powder (20.0 mg, compound **VII**).

3.3.5 Tyrosinase inhibitory activity

Antityrosinase activity was determined using a modified protocol of Dej-adisai *et al*. [26]. *L*-Tyrosine and *L*-DOPA were used as substrates for monophenolase and diphenolase inhibitory activities, respectively. Briefly, test sample (1 mg/mL) was dissolved in 20% dimethyl sulfoxide (DMSO) in ethanol. The reaction mixture of control without test sample (A) consisted of 50 µL of tyrosinase solution (200 U/mL), 150 µL of 0.2 M sodium phosphate buffer (pH 6.8) and 50 µL of 20% DMSO in ethanol. The reaction mixture of blank of control (B) consisted of 200 µL of 0.2 M sodium phosphate buffer and 50 µL of 20% DMSO in ethanol. The reaction mixture of test sample and positive control (C) consisted of 50 µL of tyrosinase solution, 150 µL of 0.2 M sodium phosphate buffer (pH 6.8) and 50 µL of test sample. The reaction mixture of blank of test sample and positive control (D) consisted of 200 µL of 0.2 M sodium phosphate buffer (pH 6.8) and 50 µL of test sample. The reaction was mixed and incubated for 10 min at 30 °C. After that, 50 µL of substrate solution (500 µM for *L*-tyrosine or *L*-DOPA) was added and absorbance was immediately measured (t = 0 min) at 490 nm. The assay mixture was then incubated for 20 min at 30 °C and the absorbance was measured at 490 nm (t = 20 min). The percentage of inhibition was calculated using the following equation:

$$\% \text{ inhibition} = \left[\frac{(A\text{-}B)\text{-}(C\text{-}D)}{(A\text{-}B)}\right] \times 100$$

Where A is the difference of the absorbance of control without test sample at t = 0 min and t = 20 min, B is the difference of the absorbance of blank of control at t = 0 min and t = 20 min, C is the difference of the absorbance of test sample and positive control at t = 0 min and t = 20 min and D is the difference of the absorbance of blank of test sample and positive control at t = 0 min and t = 20 min.

3.3.6 Antioxidant activities

3.3.6.1 2,2-Diphenyl-1-picryhydrazyl (DPPH) radical scavenging activity

The scavenging activity on DPPH radical of test sample was determined as previously reported [109]. Briefly, the reaction mixture consisted of 50 µL of test sample (100 mg/mL) and 150 µL of 0.05 M DPPH solution in methanol. Then, the reaction mixture was mixed and incubated for 30 min in the dark at 30 °C. The absorbance was measured at 517 nm using spectrophotometer. Trolox was used as a positive control. DPPH scavenging effect was calculated using the following equation:

$$\text{DPPH radical scavenging activity (\%)} = \left[1 - \frac{A_s - A_b}{A_d}\right] \times 100$$

Where $A_s$ is the absorbance of the isolated compound mixed with DPPH solution, $A_b$ is the absorbance of the sample without DPPH solution and $A_d$ is the absorbance of DPPH solution without sample.

3.3.6.2 ABTS radical scavenging activity

The ABTS scavenging capacity was determined in accordance with a reported method [110]. The stock solution consisted of 100 mL of 7.0 mM ABTS solution and 100 mL of 2.4 mM potassium persulfate solution. Then, the stock solution was left in the dark for 14 h at 30 °C. The solution was diluted by 1 mL of ABTS solution with 60 mL of absolute ethanol to determine an absorbance of $0.700 \pm 0.001$ absorbance units at 734 nm using the spectrophotometer. Five hundred µL of test sample (100 mg/mL) were reacted with 500 µL of the ABTS solution and measured at 734 nm after incubation for 7 min using the spectrophotometer. The results were compared with a trolox standard and percentage scavenging was calculated with equation below:

$$\text{ABTS radical scavenging activity (\%)} = \left[\left(\frac{A_c - A_s}{A_c}\right) \times 100\right]$$

Where $A_c$ is the absorbance of ABTS radical with absolute ethanol and $A_s$ is the absorbance of ABTS radical with test sample or positive control.

3.3.6.3 The ferric reducing antioxidant power (FRAP) assay

Reducing power was evaluated using a modification of the FRAP assay as followed protocol [110]. The FRAP reagent consisted of 25 mL of 0.3 M acetate buffer

(pH 3.6), 2.5 mL of 20 mM ferric chloride solution, 2.5 mL of 10 mM 4,6-tripryridyls-triazine and made up to a last volume of 50 mL using 40 mM HCl solution. Then, the FRAP reagent put on water bath for about half an hour at 50 °C. Then, 600 μL of FRAP reagent was added to 25 μL of test sample (100 mg/mL). The absorbance was measured at 595 nm after incubation for 4 min at room temperature using the spectrophotometer. Trolox was used as a positive control. The ferric reducing capacity was expressed as ferrous sulphate equivalent.

### 3.3.7 *In vitro* cytotoxicity assay

Cytotoxic activity of isolated compounds was tested using the MTT assay which following descripted [111]. In this study, five human cancer cell lines were evaluated including breast carcinoma (BT474, ATCC® HTB20™), lung bronchus carcinoma (ChaGo-K-1, National Cancer Institute, Thailand), liver carcinoma (HepG₂, ATCC® HB8065™), gastric carcinoma (KATO-III, ATCC®HTB103™) and colon carcinoma (SW620, ATCC® CCL227™). Human diploid lung fibroblasts (Wi-38, ATCC®CCL75™) were used as the comparison. Culturing of these cell lines were derived in complete medium including RPMI-1640 medium, 5% v/v of fetal bovine serum, 25 mM HEPES, 0.25% w/v of sodium bicarbonate and kanamycin (100 μg/mL). Doxorubicin and DMSO were used as reference control. The culture medium of cell lines (198 μL) were added into each well of 96 plate and incubated with 5% (v/v) $CO_2$ for 24 h at 37 °C. Then the culture cells were treated with 2 μL/well of the test sample and further incubated for 72 h at 37 °C. Two μL of MTT solution (5 mg/mL) in normal saline were added in each well of plate and incubated for 4 h. The medium solution was discarded and the mixture of 25 μL of 0.1 M glycine and 150 μL of DMSO were added. The plates were mixed for dissolving the purple-blue crystal before its absorbance was determined using the spectrophotometer at 540 nm. Relative of the cell survival and a percentage of the DMSO as only control which set 100% were calculated using the formula:

$$\text{The percentage of cell survival} = \left[ \frac{A_S}{A_C} \times 100 \right]$$

Where $A_s$ is the absorbance of test sample and $A_c$ is the absorbance of control.

3.3.8   Statistics

All expressed data are the mean ± standard deviation (SD) of experiment. All statistical of the three experiments carried out in triplicate and used GraphPad Prism 6 and SPSS 24. Difference were evaluated statistically significant at $p < 0.05$.

# CHAPTER IV
# RESULTS AND DISCUSSIONS

*M. zapota* contained many phytochemical constituents such as phenolic compound, triterpenoid, saponin and steroid. The reviews were reported on biological of crude extracts and phytochemical of *M. zapota* such as antibacterial, anticancer, antidiabetic, antioxidant and antityrosinase activities. However, little research on tyrosinase inhibitory activity of *M. zapota* had been previously reported. In the present study, the quantity of total phenolic content was evaluated for a correlation with total flavonoid content, antioxidant activities with DPPH, ABTS radical scavenging, FRAP assays and antityrosinase activities of crude extracts of six different parts. Consequently, tyrosinase inhibitors and mechanism of tyrosinase inhibition of isolated compounds from *M. zapota* barks.

## 4.1 Extraction of different parts of *M. zapota*

The barks, flowers, fruits, leaves, roots and seeds of *M. zapota* were macerated with methanol and water. The yield of methanol crude extracts of six different parts of *M. zapota* was ranged between 6.71-39.67% and the yield of aqueous crude extracts was ranged between 5.38-34.00% (Table 5).

**Table 5** Extraction yield of crude extracts of different parts of *M. zapota*

| Plant part | Solvent extract | Appearance | Extraction yield (% w/w dry weight) |
|---|---|---|---|
| Barks | Aqueous | Brown gum | 12.52 |
| | Methanol | Brown gum | 14.45 |
| Flowers | Aqueous | Yellow gum | 9.40 |
| | Methanol | Yellow gum | 6.71 |
| Fruits | Aqueous | Brown gum | 34.00 |
| | Methanol | Brown gum | 39.67 |
| Leaves | Aqueous | Brown gum | 20.67 |
| | Methanol | Brown gum | 20.11 |
| Roots | Aqueous | Brown gum | 11.55 |
| | Methanol | Brown gum | 11.01 |
| Seeds | Aqueous | Brown gum | 5.38 |
| | Methanol | Brown gum | 7.25 |

**4.2 Determination of total phenolic and total flavonoid contents of different parts of *M. zapota***

The results of total phenolic content of methanol and aqueous crude extracts of six different parts of *M. zapota* are shown in Table 6. Total phenolic content was estimated using a standard calibration curve of gallic acid (Figure 31) and expressed as mg gallic acid equivalents (mg GAE)/g dry sample. Methanol crude extract of flowers showed the highest amount of total phenolic content of $368.73 \pm 0.65$ mg GAE/g, followed by methanol crude extract of barks with total phenolic value of $343.44 \pm 0.50$ mg GAE/g and methanol crude extract of seeds with total phenolic value of $341.68 \pm 0.60$ mg GAE/g. Whereas, aqueous crude extract of flowers showed the lowest amount of total phenolic content of $114.03 \pm 0.94$ mg GAE/g.

4.2.2 Total flavonoid content

The results of total flavonoid content of methanol and aqueous crude extracts of six different parts of *M. zapota* are shown in Table 6. Total flavonoid content was determined using calibration curve of quercetin (Figure 32) and expressed as mg quercetin equivalents (mg QE)/g dry sample. Methanol crude extract of seeds displayed the highest amount of total flavonoid content of $90.21 \pm 0.57$ mg QE/g which followed by methanol crude extract of roots with total flavonoid value of $89.03 \pm 1.00$ mg QE/g and aqueous crude extract of seeds with total flavonoid value of $82.56 \pm 1.34$ mg QE/g. While, aqueous crude extract of roots showed the lowest amount of total flavonoid content of $24.32 \pm 0.82$ mg QE/g.

**Table 6** Total phenolic and total flavonoid contents of crude extracts of different parts
of *M. zapota*

| Plant part | Solvent extract | Total phenolic content (mg GAE/g) | Total flavonoid content (mg QE/g) |
|---|---|---|---|
| Barks | Aqueous | 286.97 ± 0.28 | 38.15 ± 0.54 |
| | Methanol | 343.44 ± 0.50 | 49.62 ±1.68 |
| Flowers | Aqueous | 114.03 ± 0.94 | 43.15 ± 0.54 |
| | Methanol | 368.73 ± 0.65 | 54.32 ± 0.48 |
| Fruits | Aqueous | 246.97 ± 0.11 | 59.32 ±1.01 |
| | Methanol | 141.97 ± 0.36 | 67.56 ±1.08 |
| Leaves | Aqueous | 209.91 ± 0.60 | 32.26 ± 0.97 |
| | Methanol | 271.67 ± 0.72 | 71.68 ± 0.53 |
| Roots | Aqueous | 305.21 ± 0.76 | 24.32 ± 0.82 |
| | Methanol | 145.50 ± 0.65 | 89.03 ±1.00 |
| Seeds | Aqueous | 299.62 ± 0.53 | 82.56 ±1.34 |
| | Methanol | 341.68 ± 0.60 | 90.21 ± 0.57 |

These results showed that total phenolic and total flavonoid contents were differences in crude extracts of six different parts of *M. zapota*. Total phenolic and total flavonoid contents are the two role factors for representation the antioxidant activity. Previous research of this plant has reported that the ethanol crude extract of fruit pulps showed total phenolic value of 38.56 ± 1.98 mg GAE/g [106]. While, acetone, aqueous, ethyl acetate and toluene crude extracts of leaves showed total phenolic contents of 241.06 ± 0.81, 106.19 ± 1.99, 137.63 ± 1.12 and 4.45 ± 0.09 mg GAE/g, respectively [18]. Amount of total flavonoid content of acetone, aqueous, ethyl acetate and toluene crude extracts of leaves was 166.84 ± 0.31, 37.04 ± 0.37, 127.63 ± 0.20 and 59.84 ± 0.59 mg QE/g, respectively [18]. Total phenolic content of acetone, ethyl acetate and aqueous crude extracts of *M. zapota* seeds was 2.26 ± 0.01, 2.54 ± 0.02 and 1.21 ± 0.01 mg GAE/g, respectively [101]. Moreover, total flavonoid content of acetone, ethyl acetate and aqueous crude extracts of *M. zapota* seeds was 2.19 ± 0.01, 1.01 ± 0.02 and 0.74 ± 0.01 mg QE/g, respectively [101]. In this study, crude extracts of six different parts of *M. zapota* showed high total phenolic and total flavonoid contents than the previous reported. The results conducted that the different parts of plant and polarity solvents can affect the total phenolic and total flavonoid contents of crude extract of *M. zapota*.

4.3 Determination of antioxidant and antityrosinase activities of different parts of *M. zapota*

4.3.1 Antioxidant activities

4.3.1.1 DPPH radical scavenging activity

The results of DPPH radical scavenging activity of methanol and aqueous crude extracts of six different parts of *M. zapota* are shown in Table 7. Methanol crude extracts of six different parts of *M. zapota* showed higher DPPH radical scavenging activity than aqueous crude extracts of six different parts of *M. zapota*. Methanol crude extracts of seeds, flowers and barks showed strong DPPH radical scavenging capacity with $IC_{50}$ values of $282.05 \pm 0.60$, $297.18 \pm 0.49$ and $299.53 \pm 0.31$ µg/mL, respectively. Whereas, aqueous crude extract of seeds exhibited the weakest DPPH radical scavenging capacity with $IC_{50}$ value of $670.31 \pm 0.24$ µg/mL. Trolox was used as a positive control and exhibited stronger DPPH radical scavenging activity than all crude extracts of six different parts of *M. zapota* with $IC_{50}$ value of $124.84 \pm 0.78$ µg/mL.

4.3.1.2 ABTS radical scavenging activity

The results of ABTS radical scavenging activity of crude extracts of six different parts of *M. zapota* are shown in Table 7. Methanol crude extracts of *M. zapota* displayed stronger ABTS radical scavenging activity than aqueous crude extracts of *M. zapota*. Methanol crude extracts of seeds, roots and flowers exhibited potent scavenger of ABTS radical with $IC_{50}$ values of $205.11 \pm 0.89$, $262.80 \pm 0.51$ and $308.51 \pm 0.57$ µg/mL, respectively. While, aqueous crude extract of barks showed the lowest scavenger of ABTS radical with $IC_{50}$ value of $942.47 \pm 0.26$ µg/mL. Trolox exhibited a significant strong ABTS radical scavenging capacity with $IC_{50}$ value of $164.09 \pm 0.21$ µg/mL.

4.3.1.3 FRAP activity

The results of reducing capacity of crude extracts of six different parts of *M. zapota* are shown in Table 7. FRAP values were calculated using calibration curve of ferrous sulphate (Figure 33). Methanol crude extract of seeds displayed the highest reducing capacity with FRAP value of $296.46 \pm 0.08$ mg TEAC/g, followed by methanol crude extracts of flowers and roots with FRAP values of $292.27 \pm 0.09$ and

278.46 ± 0.02 mg TEAC/g, respectively. Trolox exhibited strong reducing capacity with FRAP value of 349.18 ± 0.06 mg TEAC/g. While, aqueous and methanol crude extracts of fruits showed the lowest reducing capacity with FRAP values of 155.18 ± 0.08 and 150.27 ± 0.06 mg TEAC/g, respectively.

**Table 7** Antioxidant activities of crude extracts of different parts of *M. zapota*

| Plant part | Solvent extract | $IC_{50}$ (μg/mL) | | FRAP (mg TEAC/g) |
|---|---|---|---|---|
| | | DPPH | ABTS | |
| Barks | Aqueous | 453.23 ± 0.72 | 942.47 ± 0.26 | 222.27 ± 0.06 |
| | Methanol | 299.53 ± 0.31 | 649.13 ± 0.40 | 252.27 ± 0.02 |
| Flowers | Aqueous | 369.05 ± 0.10 | 447.91 ± 0.67 | 168.64 ± 0.06 |
| | Methanol | 297.18 ± 0.49 | 308.51 ± 0.57 | 292.27 ± 0.09 |
| Fruits | Aqueous | 424.10 ± 0.21 | 564.25 ± 0.25 | 155.18 ± 0.08 |
| | Methanol | 444.59 ± 0.56 | 514.35 ± 0.24 | 150.27 ± 0.06 |
| Leaves | Aqueous | 431.11 ± 0.65 | 497.75 ± 0.14 | 251.73 ± 0.02 |
| | Methanol | 341.89 ± 0.94 | 378.85 ± 0.26 | 258.27 ± 0.08 |
| Roots | Aqueous | 586.22 ± 0.84 | 651.08 ± 0.35 | 225.00 ± 0.01 |
| | Methanol | 428.64 ± 0.49 | 262.80 ± 0.51 | 278.46 ± 0.02 |
| Seeds | Aqueous | 670.31 ± 0.24 | 725.45 ± 0.42 | 217.73 ± 0.02 |
| | Methanol | 282.05 ± 0.60 | 205.11 ± 0.89 | 296.46 ± 0.08 |
| Trolox[a] | | 124.84 ± 0.78 | 164.09 ± 0.21 | 349.18 ± 0.06 |

[a] Trolox was used as a positive control.

The results of this study showed that methanol crude extract of seeds displayed the highest antioxidant activities on DPPH, ABTS and FRAP assays. In the literature, acetone crude extract of *M. zapota* leaves showed more scavenger DPPH radical than ascorbic acid with $IC_{50}$ values of 7.6 and 11.4 μg/mL, respectively [18]. On the other hand, this study indicated that aqueous and methanol crude extracts of leaves showed lower scavenger DPPH radical than the previous research of Kaneria *et al* [18]. While, ethanol crude extract of *M. zapota* fruit pulps exhibited lower antioxidant activities on DPPH and ABTS radicals than ascorbic acid with $IC_{50}$ values of 37.63 ± 1.18 and 73.14 ± 2.84 μg/mL, respectively [106]. In this study, aqueous and methanol crude extracts of fruits showed less antioxidant activity on DPPH and ABTS radicals than that of reported by Pientaweeratch *et al* [106].

4.3.2 Antityrosinase activities

4.3.2.1 Monophenolase inhibitory activity

The results of monophenolase inhibitory activity of crude extracts of six different parts of *M. zapota* are shown in Table 8. The methanol crude extracts of barks, roots, leaves and aqueous crude extract of roots showed high monophenolase inhibitory activity with $IC_{50}$ values of $0.81 \pm 0.06$, $0.81 \pm 0.92$, $0.94 \pm 0.38$ and $0.97 \pm 0.88$ mg/mL, respectively. While, methanol crude extract of fruits showed weak monophenolase inhibitory activity with $IC_{50}$ value of $4.84 \pm 0.49$ mg/mL. However, kojic acid showed the strongest tyrosinase activity on monophenolase inhibitory activity with $IC_{50}$ value of $0.09 \pm 0.11$ mg/mL.

4.3.2.2 Diphenolase inhibitory activity

The results of diphenolase inhibitory activity of crude extracts of six different parts of *M. zapota* are shown in Table 8. Methanol crude extracts of roots, flowers, leaves, barks and fruits displayed stronger diphenolase inhibitory activity than other crude extracts with $IC_{50}$ values of $0.55 \pm 0.50$, $0.60 \pm 0.97$, $0.62 \pm 0.87$, $0.71 \pm 0.39$ and $0.73 \pm 0.51$ mg/mL, respectively. Whereas, aqueous crude extract of barks exhibited diphenolase inhibitory activity less than other crude extracts with $IC_{50}$ value of $4.30 \pm 0.66$ mg/mL. The $IC_{50}$ value of kojic acid was $0.10 \pm 0.03$ mg/mL.

**Table 8** Antityrosinase activities of crude extracts of different parts of *M. zapota*

| Plant part | Solvent extract | $IC_{50}$ (mg/mL) | |
| --- | --- | --- | --- |
| | | Monophenolase inhibitory activity | Diphenolase inhibitory activity |
| Barks | Aqueous | $1.71 \pm 0.82$ | $4.30 \pm 0.66$ |
| | Methanol | $0.81 \pm 0.06$ | $0.71 \pm 0.39$ |
| Flowers | Aqueous | $1.66 \pm 0.88$ | $2.04 \pm 0.16$ |
| | Methanol | $1.66 \pm 0.38$ | $0.60 \pm 0.97$ |
| Fruits | Aqueous | $2.60 \pm 0.55$ | $3.12 \pm 0.69$ |
| | Methanol | $4.84 \pm 0.49$ | $0.73 \pm 0.51$ |
| Leaves | Aqueous | $1.28 \pm 0.43$ | $3.04 \pm 0.50$ |
| | Methanol | $0.94 \pm 0.38$ | $0.62 \pm 0.87$ |
| Roots | Aqueous | $0.97 \pm 0.88$ | $1.01 \pm 0.39$ |
| | Methanol | $0.81 \pm 0.92$ | $0.55 \pm 0.50$ |
| Seeds | Aqueous | $2.06 \pm 0.69$ | $1.02 \pm 0.14$ |
| | Methanol | $1.66 \pm 0.66$ | $1.35 \pm 0.17$ |
| Kojic acid[a] | | $0.09 \pm 0.11$ | $0.10 \pm 0.03$ |

[a] Kojic acid was used as a positive control.

A previous report indicated that methanol crude extract of *M. zapota* leaves showed antityrosinase activity on monophenolase inhibitory activity with percentage

inhibition value of 39% at a concentration of 40 μg/mL [96]. This study indicated that methanol crude extracts of barks, leaves and roots showed potent antityrosinase activity on monophenolase and diphenolase inhibitory activities. However, methanol crude extracts of flowers and fruits exhibited higher diphenolase inhibitory activity than monophenolase inhibitory activity.

4.4 Correlations

Quantity of total phenolic and total flavonoid contents were evaluated for correlations with antioxidant activities by DPPH, ABTS radical scavenging and FRAP assays and with antityrosinase activities on monophenolase and diphenolase inhibitory activities of crude extracts of six different parts of *M. zapota*.

4.4.1 Correlations between total phenolic content and antioxidant activities

The correlations between total phenolic content in crude extracts of *M. zapota* with their DPPH and ABTS radical scavenging activities and reducing capacity with FRAP were investigated (Table 9). Total phenolic content and ABTS radical scavenging activity of six different parts of *M. zapota* showed the highest positive correlation (r = 0.90). In addition, relationship of total phenolic content with DPPH radical scavenging activity and with FRAP assay also showed positive correlation with r values of 0.79 and 0.69, respectively (Figures 34-36). It was similar to the previous report that indicated the high correlation between total phenolic content and ABTS radical scavenging activity of *M. zapota* fruits with r value of 0.99 [112]. These results suggested that crude extracts of *M. zapota* which contained high total phenolic content lead to express high antioxidant activity. The indicator of correlation between total phenolic content and antioxidant activity could be used an electron transfer in mechanism [101].

**Table 9** Correlation values of total phenolic content and antioxidant activities of crude extracts of different parts of *M. zapota*

| | Antioxidant activities | | |
| --- | --- | --- | --- |
| | DPPH radical scavenging activity | ABTS radical scavenging activity | FRAP activity |
| Total phenolic content | 0.79 | 0.90 | 0.69 |

4.4.2 Correlations between total phenolic content and antityrosinase activities

The correlations between total phenolic content and antityrosinase activities of crude extracts of *M. zapota* were investigated (Table 10). Correlation between total phenolic content and monophenolase inhibitory activity was negative related (r = -0.01). Moreover, there was very low correlation between total phenolic content and diphenolase inhibitory activity with r value of 0.10 (Figures 37-38). These results suggested that classes of phenolic compound of crude extracts of *M. zapota* could not inhibit tyrosinase activities.

**Table 10** Correlation values of total phenolic content and antityrosinase activities of crude extracts of different parts of *M. zapota*

|  | Antityrosinase activities | |
| --- | --- | --- |
|  | Monophenolase inhibitory activities | Diphenolase inhibitory activity |
| Total phenolic content | -0.01 | 0.10 |

4.4.3 Correlations between total phenolic content and total flavonoid content

The correlations between total phenolic and total flavonoid contents of crude extracts of *M. zapota* were investigated. Total phenolic content showed low positive correlation with total flavonoid contents (r = 0.28) (Figure 39). Phenolic compounds are a large group of chemical compounds that contain antioxidant, anti-inflammatory, anti-carcinogenic and other biological properties. Phenolic compounds are divided into serveral groups, one of whnich is represented by flavonoids. Flavonoids are further divided in serveral subclassvones.es such as anthocyanins, flavonols, flavanones, flavanols, flavones and isoflavonones. These results suggested that amount of flavonoids in each crude extract of *M. zapota* might not depend on total phenolic content of crude extract of *M. zapota*.

4.4.4 Correlations between total flavonoid content and antioxidant activities

The correlations between total flavonoid content and antioxidant activities on DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP activity of crude extracts of *M. zapota* were investigated (Table 11). Correlations between total flavonoid content and antioxidant activities with DPPH, ABTS and FRAP assays were evaluated as r values of 0.35, 0.29 and 0.39, respectively (Figures 40-42).

These results indicated that flavonoids in crude extracts of *M. zapota* might not be acted as reducing agents for antioxidant activities.

Table 11 Correlation values of total flavonoid content and antioxidant activities of crude extracts of different parts of *M. zapota*

| | Antioxidant activities | | |
|---|---|---|---|
| | DPPH radical scavenging activity | ABTS radical scavenging activity | FRAP activity |
| Total flavonoid content | 0.35 | 0.29 | 0.39 |

4.4.5 Correlations between total flavonoid content and antityrosinase activities

The correlations between total flavonoid content and antityrosinase activities of crude extracts of *M. zapota* were investigated (Table 12). No correlation between total flavonoid content and monophenolase inhibitory activity was observed ($r = -0.07$). Relationship of total flavonoid content and monophenolase inhibitory activity was not correlated ($r = 0.14$) (Figures 43-44). These results indicated that flavonoids in crude extracts of *M. zapota* should not be connected to inhibit tyrosinase activity.

**Table 12** Correlation values of total flavonoid content and antityrosinase activities of crude extracts of different parts of *M. zapota*

| | Antityrosinase activities | |
|---|---|---|
| | Monophenolase inhibitory activities | Diphenolase inhibitory activity |
| Total flavonoid content | -0.07 | 0.14 |

4.4.6 Correlations between antityrosinase and antioxidant activities

The correlations between antityrosinase and antioxidant activities of crude extracts of *M. zapota* were investigated (Table 13). It was no correlation between antityrosinase and antioxidant activities. (Figures 45-50). These results were similar to previous report on the correlation between antityrosinase and antioxidant activities of ethanol crude extract of *Dioscorea opposita* root tubers [113]. In contrast, diphenolase inhibitory activity and antioxidant activity of *Macaranga* sp. extract showed positive correlation with $R^2$ value of 0.787 [114]. This study confirmed that mechanism tyrosinase activity is independent from mechanism of antioxidant activity as previously described [115].

**Table 13** Correlation values of antityrosinase and antioxidant activities of crude of
extracts of different parts of *M. zapota*

| | Antioxidant activities | | |
| --- | --- | --- | --- |
| | DPPH radical scavenging activity | ABTS radical scavenging activity | FRAP activity |
| Monophenolase inhibitory activity | -0.10 | -0.10 | -0.09 |
| Diphenolase inhibitory activity | 0.02 | 0.06 | 0.02 |

Among crude extracts of six different parts of *M. zapota*, the highest total phenolic content was found in methanol crude extract of flowers and the maximum of total flavonoid content was found in methanol crude extract of roots. Moreover, methanol crude extract of seeds showed potent antioxidant activities with DPPH and ABTS radical scavenging activities and reducing capacity of FRAP activity. Methanol crude extract of barks and seeds displayed significant monophenolase inhibitory activity and methanol crude extract of roots exhibited the most potent diphenolase inhibitory activity. Total phenolic content of *M. zapota* showed a positive relationship with antioxidant activities but showed weak correlation with antityrosinase activities. Total flavonoid content of *M. zapota* exhibited low correlation with both antioxidant and antityrosinase activities. Furthermore, this study indicated that no correlation between antityrosinase and antioxidant activities. So phenolic compounds of *M. zapota* do not relate to antityrosinase activity. Methanol crude extracts of six different parts of *M. zapota* exhibited potent tyrosinase inhibitory activity. For next step, barks of *M. zapota* were extracted by organic solvents and isolated using chromatography techniques.

4.5 Extraction of *M. zapota* barks

The fresh barks of *M. zapota* (175 kg) were dried in a hot air oven at 60 °C. The dried barks (7 kg) were ground and macerated with various polarity organic solvents such as *n*-hexane, ethyl acetate, methanol and water, respectively. Each extract was filtrated and evaporated under *in vacuo* to afford four crude extracts. *n*-Hexane crude extract was obtained as yellow green gum (139.60 g, 1.99% w/dried weight of barks). Ethyl acetate crude extract was dark green gum (138.13 g, 1.97% w/dried weight of barks). Methanol crude extract was dark brown gum (819.96 g, 11.71% w/dried weight

of barks) and aqueous crude extract was brown gum (96.79 g, 1.38% w/dried weight of barks). All crude extracts were screened for antityrosinase activity by DOPAchrome method (Table 14). *n*-Hexane, ethyl acetate and methanol crude extracts showed high tyrosinase inhibitory effect with percentage inhibition of $85.85 \pm 0.03\%$, $82.80 \pm 0.50\%$ and $77.23 \pm 0.94\%$, respectively at concentration of 1.0 mg/mL. Aqueous crude extract showed the lowest antityrosinase activity with percentage inhibition of $24.86 \pm 0.54\%$ at concentration of 1.0 mg/mL. Kojic acid exhibited tyrosinase inhibitory activity with percentage inhibition of $96.19 \pm 0.13\%$ at concentration of 0.1 mg/mL. Theses results indicated that *n*-hexane, ethyl acetate and methanol crude extracts might contain tyrosinase inhibitor. So, they were conducted to isolate active compounds by bioassay-guided fractionation on tyrosinase inhibitory activity.

**Table 14** Extraction yield and tyrosinase inhibitory activity of crude extracts of *M. zapota* barks

| Crude extract | Appearance | Yield (g) (%, w/dried weight of barks) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| *n*-Hexane | Yellow green gum | 139.60 (1.99) | $85.85 \pm 0.03$* |
| Ethyl acetate | Dark green gum | 138.13 (1.97) | $82.80 \pm 0.50$* |
| Methanol | Dark brown gum | 819.96 (11.71) | $77.23 \pm 0.94$* |
| Aqueous | Brown gum | 96.79 (1.38) | $24.86 \pm 0.54$* |
| Kojic acid[a] | | | $96.19 \pm 0.13$** |

[a] Kojic acid was used as a positive control.
* Concentration of crude extract was 1.0 mg/mL.
** Concentration of kojic acid was 0.1 mg/mL.

4.6 Isolation of crude extracts of *M. zapota* barks

4.6.1 Isolation of *n*-hexane crude extract

*n*-Hexane crude extract was chromatographed on a silica gel quick column chromatography and eluted with *n*-hexane, a mixture of *n*-hexane:ethyl acetate, ethyl acetate and a mixture of ethyl acetate:methanol to yield four fractions [108]. They were investigated for tyrosinase inhibitory activity using *L*-DOPA as a substrate. The results are shown in Table 15. Fraction A exhibited the strongest antityrosinase activity with percentage inhibition value of $82.63 \pm 0.31\%$. While, fraction B ($42.95 \pm 0.10\%$) showed inhibitory tyrosinase activity less than fraction C ($62.71 \pm 0.43\%$). Thus, fraction A was further purified to obtain tyrosinase inhibitors.

**Table 15** Tyrosinase inhibitory activity of fractions A-D of n-hexane crude extract

| Fraction | Appearance | Yield (g) (%, w/weight of *n*-hexane crude extract) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| A | Yellow gum | 63.05 (46.70) | 82.63 ± 0.31 |
| B | Yellow gum | 20.97 (15.53) | 42.95 ± 0.10 |
| C | Brown gum | 1.40 (1.04) | 62.71 ± 0.43 |
| D | Brown gum | 4.91 (3.64) | NA |

NA = No activity.

The fraction A was subjected to silica gel column chromatography and eluted with a mixture gradient of *n*-hexane:dichloromethane to obtain four subfractions (A1-A4). All subfractions were investigated for tyrosinase inhibitory activity using *L*-DOPA as a substrate (Table 16). Subfractions A1 and A2 showed potent tyrosinase inhibition activity with percentage inhibition values of 70.18 ± 0.10% and 87.60 ± 0.14%, respectively. Subfraction A2 was recrystallized in a mixture of dichloromethane:methanol to afford compound **I**.

**Table 16** Tyrosinase inhibitory activity of subfractions A1-A4 of fraction A

| Subfraction | Appearance | Yield (g) (%, w/weight of fraction A) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| A1 | White powder | 0.94 (1.49) | 70.18 ± 0.10 |
| A2 | Colorless crystal | 0.03 (0.05) | 87.60 ± 0.14 |
| A3 | Colorless gum | 47.92 (76.18) | NA |
| A4 | White powder | 14.01 (22.27) | NA |

NA = No activity.

Fraction A1 was subjected to silica gel column chromatography using a gradient system of *n*-heptane and *n*-hexane to obtain four subfractions (A11-A14). All four subfractions were tested for tyrosinase inhibitory activity using *L*-DOPA as a substrate (Table 17). Subfraction A11 showed lower tyrosinase inhibitory activity than subfraction A14. Subfraction 14 was recrystallized to give compound **I**. The ¹H-NMR of subfraction A14 is similar to that of compound **I**.

**Table 17** Tyrosinase inhibitory activity of subfractions A11-A14 of subfraction A1

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction A1) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| A11 | White powder | 17.0 (1.81) | 46.11 ± 0.14 |
| A12 | Yellow gum | 716.0 (76.17) | NA |
| A13 | Yellow gum | 105.2 (11.19) | NA |
| A14 | White powder | 60.8 (6.47) | 71.58 ± 0.18 |

NA = No activity.

Fraction C showed moderate antityrosinase activity so it was then chromatographed by column chromatography on silica gel and eluted with a gradient system of *n*-heptane:*n*-hexane and *n*-hexane:dichloromethane to yield three subfractions (C1-C3). All three subfractions were investigated for tyrosinase inhibitory activity (Table 18). Both subfractions C1 and C2 exhibited low antityrosinase activity with percentage inhibition values of 15.91 ± 0.84% and 25.72 ± 0.52%, respectively. While subfraction C3 showed moderate tyrosinase inhibitory activity with percentage inhibition value of 52.27 ± 0.39%. The results indicated that subfraction C3 might consist of moderate tyrosinase inhibitors.

**Table 18** Tyrosinase inhibitory activity of subfractions C1-C3 of fraction C

| Subfraction | Appearance | Yield [115] (%, w/weight of fraction C) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| C1 | Yellow gum | 617.1 (44.08) | 15.91 ± 0.84 |
| C2 | Yellow solid | 473.8 (33.84) | 25.72 ± 0.52 |
| C3 | White powder | 147.3 (10.52) | 52.27 ± 0.39 |

Subfraction C3 was subjected to silica gel column chromatography to give four subfractions (C31-C34). Each of subfraction was tested for tyrosinase inhibitory activity using *L*-DOPA as a substrate (Table 19). Subfraction C33 showed the highest antityrosinase activity when compared with the other subfractions of subfraction C3 with percentage inhibition value of 48.70 ± 0.33%. Unfortunately, subfraction C3 was not a pure compound.

**Table 19** Tyrosinase inhibitory activity of subfractions C31-C34 of subfraction C3

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction C3) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| C31 | Yellow gum | 39.7 (26.95) | 17.53 ± 0.23 |
| C32 | White solid | 19.7 (13.37) | 16.56 ± 0.32 |
| C33 | White solid | 45.8 (31.09) | 48.70 ± 0.33 |
| C34 | White solid | 30.3 (20.57) | 32.31 ± 0.23 |

4.6.2 Isolation of ethyl acetate crude extract

Ethyl acetate crude extract was chromatographed by a silica gel quick column chromatography to yield four fractions (E-H). Fractions E-H were determined for tyrosinase inhibitory activity (Table 20). Fraction F showed the highest tyrosinase inhibitory activity with percentage inhibition value of 82.10 ± 0.04%. Moreover, fraction G showed higher antityrosinase activity than fraction E with percentage inhibition values of 75.52 ± 0.04% and 55.73 ± 0.10%, respectively. Fraction H showed no tyrosinase inhibitory activity. The results indicated that fractions E-G might contain tyrosinase inhibitors.

**Table 20** Tyrosinase inhibitory activity of fractions E-H of ethyl acetate crude extract

| Fraction | Appearance | Yield (g) (%, w/weight of ethyl acetate crude extract ) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| E | Brown gum | 37.27 (28.67) | 55.73 ± 0.10 |
| F | Brown gum | 20.08 (15.45) | 82.10 ± 0.04 |
| G | Brown gum | 4.75 (3.65) | 75.52 ± 0.04 |
| H | Brown gum | 4.91 (3.78) | NA |

NA = No activity.

Fraction E was chromatographed over silica gel column chromatography. Isolation of fraction E was eluted with mixtures gradient of *n*-hexane and dichloromethane to give nine subfractions (E1-E9). Subfractions E2, E5, E6 and E8 were determined for tyrosinase inhibitory activity (Table 21). Subfractions E2, E5 and E6 showed weak antityrosinase activity with percentage inhibition values of 24.93 ± 0.99%, 30.40 ± 0.40% and 14.05 ± 0.40%, respectively. Subfraction E5 was rechromatographed, although it showed weak antityrosinase activity. Preparative TLC

method was used for isolation of subfraction E5 and eluent was petroleum ether:dichloromethane. Three bands of separation were collected and determined by ^{1}H-NMR. Subfractions E51 (18.3 mg, 23.19% w/w), E52 (28.6 mg, 36.25% w/w) and E53 (5.0 mg, 6.34% w/w) were obtain as white powders. Unfortunately, subfraction E5 was not a pure compound.

**Table 21** Tyrosinase inhibitory activity of subfractions E1-E9 of fraction E

| Subfraction | Appearance | Yield [115] (%, w/weight of fraction E) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| E1 | Yellow oil | 8.9 (0.02) | ND |
| E2 | Yellow oil | 26.9 (0.07) | 24.93 ± 0.99 |
| E3 | White powder | 2.8 (0.01) | ND |
| E4 | White powder | 8.0 (0.02) | ND |
| E5 | White powder | 78.9 (0.21) | 30.40 ± 0.40 |
| E6 | White powder | 26.8 (0.07) | 14.05 ± 0.40 |
| E7 | White powder | 2.7 (0.01) | ND |
| E8 | Yellow oil | 84.8 (0.23) | NA |
| E9 | Yellow oil | 9.4 (0.03) | ND |

NA = No activity.
ND = No detection.

Fraction F was fractionated by column chromatography to yield six subfractions (F1-F6). Six subfractions were determined for tyrosinase inhibitory activity (Table 22). Subfractions F2, F3, F4 and F5 exhibited significant tyrosinase inhibitory activity with percentage inhibition values of 70.91 ± 0.30%, 71.34 ± 1.91%, 74.90 ± 0.07% and 80.15 ± 1.15%, respectively. While, subfractions F1 and F6 showed moderate tyrosinase inhibitory activity with percentage inhibition values of 64.11 ± 0.14% and 51.20 ± 1.45%, respectively.

**Table 22** Tyrosinase inhibitory activity of subfractions F1-F6 of fraction F

| Subfraction | Appearance | Yield (g) (%, w/weight of fraction F) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F1 | White powder | 1.26 (6.27) | 64.11 ± 0.14 |
| F2 | White powder | 0.03 (0.15) | 70.91 ± 0.30 |
| F3 | White powder | 10.63 (52.94) | 71.34 ± 1.91 |
| F4 | Green gum | 1.43 (7.12) | 74.90 ± 0.07 |
| F5 | Brown gum | 4.40 (21.91) | 80.15 ± 1.15 |
| F6 | Brown gum | 1.24 (6.18) | 51.20 ± 1.45 |

Subfraction F1 was fractionated by column chromatography. Fractionation was used gradient elution of *n*-hexane and dichloromethane to give six subfractions (F11-F16). Subfractions F11-F13 were determined for tyrosinase inhibitory activity (Table 23). Subfractions F14-F16 were not tested tyrosinase inhibitory activity because amount of subfraction F14-F16 was not enough to test tyrosinase inhibitory activity. Subfractions F11 and F13 showed strong tyrosinase inhibitory activity with percentage inhibition values of $81.62 \pm 0.91\%$ and $81.90 \pm 0.27\%$, respectively.

**Table 23** Tyrosinase inhibitory activity of subfractions F11-F16 of subfraction F1

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F1) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F11 | White powder | 126.3 (12.63) | $81.62 \pm 0.91$ |
| F12 | Yellow gum | 115.4 (11.54) | $29.21 \pm 0.89$ |
| F13 | White crystal | 5.4 (0.54) | $81.90 \pm 0.27$ |
| F14 | Yellow gum | 2.4 (0.24) | ND |
| F15 | Yellow gum | 4.3 (0.43) | ND |
| F16 | Yellow gum | 4.3 (0.43) | ND |

ND = No detection.

Subfraction F11 was rechromatographed and eluted with mixture gradient of *n*-hexane and dichloromethane to afford six subfractions (F111-F116). Both subfractions F112 and F113 were evaluated for antityrosinase activity (Table 24). Subfraction F113 showed higher antityrosinase activity than subfraction F112 with percentage inhibition values of $62.62 \pm 5.22\%$ and $12.25 \pm 0.48\%$, respectively.

**Table 24** Tyrosinase inhibitory activity of subfractions F111-F116 of subfraction F11

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F11) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F111 | Yellow oil | 2.5 (2.08) | ND |
| F112 | White powder | 30.6 (25.50) | $12.25 \pm 0.48$ |
| F113 | White powder | 71.8 (54.67) | $62.62 \pm 5.22$ |
| F114 | White powder | 2.5 (2.08) | ND |
| F115 | White powder | 9.7 (8.08) | ND |
| F116 | White powder | 9.1 (7.58) | ND |

ND = No detection.

Subfraction F113 was further purified by silica gel column chromatography and eluted with mixture of dichloromethane:ethyl acetate in increasing of polarity to yield five subfractions (F1131-F1135). Subfractions F1131 and F1132 showed strong

antityrosinase activity with percentage inhibition values of $71.79 \pm 0.22\%$ and $87.31 \pm 0.15\%$, respectively (Table 25). Subfractions F1133-F1135 did not evaluated for antityrosinase activity because of limitation of amount of them.

**Table 25** Tyrosinase inhibitory activity of subfractions F1131-F1135 of subfraction F113

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F113) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F1131 | White powder | 30.7 (51.17) | $71.79 \pm 0.22$ |
| F1132 | White powder | 5.8 (9.67) | $87.31 \pm 0.15$ |
| F1133 | Yellow oil | 6.9 (11.5) | ND |
| F1134 | Yellow oil | 7.2 (12.0) | ND |
| F1135 | Yellow oil | 3.2 (5.33) | ND |

ND = No detection.

Subfraction F1131 was rechromatographed by silica gel and eluted with mixture gradient of petroleum ether:*n*-hexane and *n*-hexane:dichloromethane to provide eight subfractions (F11311-F11318). Subfractions F11313 and F11316 showed potent antityrosinase activity with percentage inhibition values of $72.22 \pm 0.22\%$ and $70.62 \pm 0.36\%$, respectively. Antityrosinase activity of other subfractions was not tested (Table 26). As the attempted isolation of fraction F1, it was unsuccessful to obtain pure compounds.

**Table 26** Tyrosinase inhibitory activity of subfractions F11311-F11315 of subfraction F1131

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F1131) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F11311 | Yellow oil | 1.3 (42.35) | ND |
| F11312 | Clear oil | 4.2 (13.68) | ND |
| F11313 | White powder | 4.1 (13.36) | $72.22 \pm 0.22$ |
| F11314 | Yellow oil | 4.1 (13.36) | ND |
| F11315 | Yellow oil | 0.9 (2.93) | ND |
| F11316 | White powder | 5.0 (16.29) | $70.62 \pm 0.36$ |
| F11317 | White powder | 3.7 (12.05) | ND |
| F11318 | White powder | 5.1 (16.61) | ND |

ND = No detection.

Subfraction F2 was chromatographed by silica gel column chromatography with gradient of *n*-hexane and dichloromethane as eluent to obtain three subfractions

(F21-F23). Subfraction F21 showed strong antityrosinase activity with percentage inhibition value of $81.39 \pm 0.89\%$ (Table 27). Its R_f value exhibited subfraction F21 was compound **I**. Moreover, ^{1}H-NMR spectrum of subfraction F21 was compound **I**.

**Table 27** Tyrosinase inhibitory activity of subfractions F21-F23 of subfraction F2

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F2) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F21 | Clear crystal | 27.0 (23.40) | $81.39 \pm 0.89$ |
| F22 | Yellow gum | 3.7 (3.21) | ND |
| F23 | White powder | 1.4 (1.21) | ND |

ND = No detection.

Subfraction F3 was applied to silica gel column chromatography and eluted with gradient of *n*-hexane and ethyl acetate to obtain four subfractions (F31-F34). Subfraction F31 showed strong antityrosinase activity with percentage inhibition value of $82.10 \pm 0.32\%$ (Table 28). ^{1}H-NMR spectra of subfraction F31 and compound **I** were compared. The results indicated that subfraction F31 was compound **I**.

**Table 28** Tyrosinase inhibitory activity of subfractions F31-F34 of subfraction F3

| Subfraction | Appearance | Yield (g) (%, w/weight of subfraction F3) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F31 | White crystal | 0.01 (0.09) | $82.10 \pm 0.32$ |
| F32 | Yellow gum | 0.04 (0.38) | ND |
| F33 | Yellow gum | 0.73 (6.87) | ND |
| F34 | Brown gum | 9.84 (92.57) | ND |

ND = No detection.

Subfraction F4 was subjected to silica gel column chromatography with a step gradient elution with *n*-hexane and ethyl acetate to yield six subfractions (F41-F46). Subfraction F43 showed the strongest antityrosinase activity with percentage inhibition value of $73.69 \pm 0.11\%$. Subfractions F41, F44, F45 and F46 showed weak antityrosinase activity with percentage inhibition values of $20.60 \pm 0.72\%$, $12.74 \pm 0.97\%$, $24.31 \pm 0.25\%$ and $10.54 \pm 0.93\%$, respectively (Table 29). Thus, subfraction F43 was selected for further isolation of tyrosinase inhibitors.

**Table 29** Tyrosinase inhibitory activity of subfractions F41-F46 of subfraction F4

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F4) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F41 | Yellow powder | 90.2 (6.31) | $20.60 \pm 0.72$ |
| F42 | White powder | 2.0 (0.14) | ND |
| F43 | White powder | 133.7 (9.35) | $73.69 \pm 0.11$ |
| F44 | Yellow solid | 14.7 (1.03) | $12.74 \pm 0.97$ |
| F45 | Brown solid | 105.6 (7.38) | $24.31 \pm 0.25$ |
| F46 | Brown gum | 83.2 (5.82) | $10.54 \pm 0.93$ |

ND = No detection.

Subfraction F43 was further subjected to silica gel column chromatography to yield five subfractions (F431-F435). Subfraction F433 showed strong antityrosinase activity with percentage inhibition value of $91.59 \pm 0.87\%$ (Table 30). Therefore, subfraction F433 was collected for further isolation of active compounds.

**Table 30** Tyrosinase inhibitory activity of subfractions F431-F435 of subfraction F43

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F43) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F431 | White powder | 18.2 (18.20) | NA |
| F432 | Yellow gum | 5.8 (5.80) | ND |
| F433 | Yellow gum | 115.2 (70.20) | $91.59 \pm 0.87$ |
| F434 | Brown gum | 1.2 (1.20) | ND |
| F435 | Brown gum | 4.6 (4.60) | ND |

NA = No activity.
ND = No detection.

Subfraction F433 was separated by silica gel column chromatography and eluted with a stepwise mixture of *n*-hexane and ethyl acetate to obtain six subfractions (F4331-F4336). Subfractions F4332, F4334 and F4335 showed strong antityrosinase activity with percentage inhibition values of $87.22 \pm 0.41\%$, $90.21 \pm 0.95\%$ and $90.01 \pm 1.00\%$, respectively. Subfraction F4331 exhibited no activity against mushroom tyrosinase (Table 31). Subfractions F4332, F4334 and F4335 were collected for further tyrosinase-guided fractionation.

**Table 31** Tyrosinase inhibitory activity of subfractions F4331-F4336 of subfraction F433

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F433) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F4331 | Yellow oil | 4.4 (3.82) | NA |
| F4332 | White powder | 72.9 (63.28) | 87.22 ± 0.41 |
| F4333 | Clear gum | 2.0 (1.74) | ND |
| F4334 | White powder | 13.2 (11.46) | 90.21 ± 0.95 |
| F4335 | Yellow powder | 12.9 (11.20) | 90.01 ± 1.00 |
| F4336 | Brown gum | 9.7 (8.42) | ND |

NA = No activity.
ND = No detection.

Subfraction F4332 was separated by preparative TLC and eluted with a mixture of *n*-hexane and ethyl acetate to obtain two subfractions (F43321-F43322). Subfraction F43321 showed stronger antityrosinase activity than subfraction F43322 with percentage inhibition values of 93.00 ± 0.60% and 6.04 ± 0.42%, respectively (Table 32). Subfraction F43321 was obtained as white powder. Subfraction F43321 was identified as compound **I**.

**Table 32** Tyrosinase inhibitory activity of subfractions F43321-F43322 of subfraction F4332

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F4332) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F43321 | White powder | 8.9 (24.45) | 93.00 ± 0.60 |
| F43322 | White powder | 27.5 (75.55) | 6.04 ± 0.42 |

Subfraction F4334 was fractionated on an analytical $C_{18}$ column using an isocratic system of methanol and aqueous (3:2 v/v) in the flow rate of 1 mL/min to yield compound **II** (8 mg) at retention time 3.5 min. Compound **II** was obtain as yellow powder. Subfraction F4335 was submitted to Sephadex LH-20 column chromatography and used methanol as eluent to afford three subfractions (F43351-F43353). Subfraction F43353 showed high antityrosinase activity with percentage inhibition value of 95.94 ± 0.20% (Table 33). Subfraction F43353 (2.4 mg) was purified by HPLC using $C_{18}$ column and an isocratic system of methanol and water (7:3 v/v) in the flow rate of 0.5 mL/min to yield yellow powder (compound **III**, 2.4 mg) at retention time 5.9 min.

**Table 33** Tyrosinase inhibitory activity of subfractions F43351-F43353 of
subfraction F4335

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F4335) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F43351 | White powder | 9.5 (73.64) | NA |
| F43352 | Yellow powder | 1.0 (7.75) | ND |
| F43353 | Yellow powder | 2.4 (18.60) | 95.94 ± 0.20 |

NA = No activity.
ND = No detection.

Subfraction F5 was separated by silica gel column chromatography and eluted
with a mixture gradient of *n*-hexane:ethyl acetate to afford nine subfractions (F51-F59).
Subfraction F59 showed the highest tyrosinase inhibitory activity with percentage
inhibition value of 88.38 ± 0.76% when compared with other eluted subfractions from
subfraction F5. Subfraction F53 showed moderate tyrosinase inhibitory activity with
percentage inhibition value of 61.90 ± 0.85%. Subfractions F52, F54 and F55 showed
weak tyrosinase inhibitory activity with percentage inhibition values of 20.12 ± 0.97%,
40.98 ± 0.42% and 22.22 ± 0.51%, respectively (Table 34).

**Table 34** Tyrosinase inhibitory activity of all subfractions F51-F59 of subfraction
F5

| Subfraction | Appearance | Yield (g) (%, w/w) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F51 | Yellow gum | 0.05 (1.14) | NA |
| F52 | Yellow gum | 2.38 (54.09) | 20.12 ± 0.97 |
| F53 | Yellow gum | 0.08 (1.82) | 61.90 ± 0.85 |
| F54 | Brown gum | 0.11 (2.50) | 40.98 ± 0.42 |
| F55 | Brown gum | 0.22 (5.0) | 22.22 ± 0.51 |
| F56 | Brown gum | 0.13 (2.95) | NA |
| F57 | White powder | 0.04 (0.91) | NA |
| F58 | Brown gum | 0.45 (10.23) | NA |
| F59 | Brown gum | 0.12 (2.73) | 88.38 ± 0.76 |

NA = No activity.

Subfraction F53 was separated by preparative TLC and eluted with a mixture
gradient of *n*-hexane and ethyl acetate to afford two subfractions (F531-F532). The
results of subfractions F531-F532 are shown in Table 35. As the attempted isolation of
subfraction F53, it could not get pure compounds.

**Table 35** Isolation of subfractions F531-F532 of subfraction F53

| Fraction | Appearance | Yield [115] (%, w/weight of subfraction F53) |
|----------|------------|----------------------------------------------|
| F531 | White powder | 68.8 (86.00) |
| F532 | White powder | 1.2 (1.50) |

Subfraction F54 was separated by silica gel column chromatography to afford six subfractions (F541-F546). The results of subfractions F541-F546 are shown in Table 36. Unfortunately, isolation of subfraction F54 was not successful to get pure compounds.

Subfraction F59 was separated by silica gel column chromatography and eluted with *n*-hexane, a mixture gradient of *n*-hexane:acetone, acetone and acetone:methanol to afford ten subfractions (F591-F5910). Subfraction F596 showed high antityrosinase activity with percentage inhibition values of $84.21 \pm 0.44\%$. Subfraction F596 was recrystallized to obtain brown powder (compound **IV**). The results of subfractions F591-F5910 are shown in Table 36.

**Table 36** Tyrosinase inhibitory activity of subfractions F591-F5910 of subfraction F59

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F59) | Percentage of inhibitory activity (%) |
|-------------|------------|-----------------------------------------------|----------------------------------------|
| F591 | Yellow gum | 7.4 (3.43) | ND |
| F592 | Yellow gum | 6.4 (2.96) | ND |
| F593 | White powder | 5.1 (2.36) | ND |
| F594 | Yellow gum | 6.0 (2.78) | ND |
| F595 | Yellow gum | 9.1 (4.21) | ND |
| F596 | Brown powder | 60.0 (27.78) | $84.21 \pm 0.44$ |
| F597 | Yellow gum | 7.1 (3.29) | ND |
| F598 | Yellow gum | 8.0 (3.70) | ND |
| F599 | Yellow gum | 9.4 (4.35) | ND |
| F5910 | Yellow gum | 6.2 (2.87) | ND |

ND = No detection.

Subfraction F6 (42.1 mg) was further separated by silica gel column chromatography and eluted with hexane and a mixture gradient of *n*-hexane:ethyl acetate to give three subfractions (F61-F63). Subfraction F62 showed moderated antityrosinase activity with percentage inhibition value of $52.76 \pm 0.60\%$. While, subfraction F63 showed weak antityrosinase activity with percentage inhibition value of $15.89 \pm 0.81\%$. The results of subfractions F61-F63 are shown in Table 37.

**Table 37** Tyrosinase inhibitory activity of subfractions F61-F63 of subfraction F6

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction F6) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| F61 | Yellow gum | 3.7 (0.30) | ND |
| F62 | White powder | 24.9 (2.01) | $52.76 \pm 0.60$ |
| F63 | Brown gum | 12.5 (1.01) | $15.89 \pm 0.81$ |

ND = No detection.

Subfraction F62 was separated by silica gel column chromatography and eluted with *n*-hexane, a mixture gradient of *n*-hexane:dichloromethane and dichloromethane to give six subfractions (F621-F626). The results of subfractions F621-F626 are shown in Table 38. Unfoetunately, isolation of subfraction F62 did not succeed to obtain pure compounds.

**Table 38** Isolation of subfractions F621-F626 of subfraction F62

| Fraction | Appearance | Yield [115] (%, w/weight of subfraction F62) |
|---|---|---|
| F621 | Yellow gum | 1.6 (6.43) |
| F622 | White powder | 4.8 (19.28) |
| F623 | White powder | 2.8 (11.24) |
| F624 | White powder | 1.3 (5.22) |
| F625 | White powder | 8.2 (32.93) |
| F626 | Brown gum | 1.3 (5.22) |

Fraction G was separated by silica gel column chromatography to give five subfractions (G1-G5). Subfractions G3 and G5 exhibited strong tyrosinase inhibitory activity with percentage inhibition values of $80.10 \pm 0.90$ and $81.30 \pm 0.51\%$, respectively. Subfractions G1, G2 and G4 showed weak tyrosinase inhibitory activity with percentage inhibition values of $16.97 \pm 0.15\%$, $11.58 \pm 0.71\%$ and $31.09 \pm 0.54\%$, respectively. The results of subfractions G1-G5 are shown in Table 39.

**Table 39** Tyrosinase inhibitory activity of subfractions G1-G5 of fraction G

| Subfraction | Appearance | Yield (g) (%, w/weight of fraction G) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| G1 | Yellow gum | 0.77 (16.21) | $16.97 \pm 0.15$ |
| G2 | Yellow gum | 2.06 (43.37) | $11.58 \pm 0.71$ |
| G3 | White powder | 0.20 (4.21) | $80.10 \pm 0.90$ |
| G4 | Green gum | 0.14 (2.95) | $31.09 \pm 0.54$ |
| G5 | Green gum | 0.20 (4.21) | $81.30 \pm 0.51$ |

Subfraction G3 was further separated by silica gel column chromatography and eluted with *n*-hexane, a mixture gradient of *n*-hexane:ethyl acetate and ethyl acetate:methanol to give four subfractions (G31-G34). Subfraction G33 showed higher antityrosinase activity than subfraction G32 with percentage inhibition values of 70.60 ± 0.03% and 30.50 ± 0.03%, respectively. The results of subfractions G31-G34 are shown in Table 40.

**Table 40** Tyrosinase inhibitory activity of subfractions G31-G34 of subfraction G3

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction G3) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| G31 | White powder | 61.1 (30.55) | NA |
| G32 | Clear gum | 41.6 (20.80) | 30.50 ± 0.03 |
| G33 | White powder | 51.1 (25.55) | 70.60 ± 0.03 |
| G34 | White powder | 41.8 (20.90) | NA |

NA = No activity.

Subfraction G33 was separated by silica gel column chromatography and eluted with a mixture gradient of *n*-heptane:dichloromethane to give three subfractions (G331-G333). Subfractions G332 and G333 showed strong antityrosinase activity with percentage inhibition values of 84.90 ± 0.22% and 90.12 ± 0.42%. The results of subfractions G331-G333 are shown in Table 41.

**Table 41** Tyrosinase inhibitory activity of subfractions G331-G333 of subfraction G33

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction G33) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| G331 | White powder | 11.1 (21.72) | NA |
| G332 | White powder | 24.6 (4.81) | 84.90± 0.22 |
| G333 | Colorless crystal | 15.4 (30.14) | 90.12± 0.42 |

NA = No activity.

Subfraction G332 was purified by preparative TLC and eluted with a mixture of petroleum ether and dichloromethane to obtain two subfractions (G3321-G3322). Subfraction G3322 exhibited antityrosinase activity with percentage inhibition value of 84.27 ± 0.12%. Subfraction G3322 (compound **V**) was obtained as white powder (10.5 mg). The results of subfractions G3321-G3322 are shown in Table 42. Subfraction

G333 was recrystallized with methanol to give colorless crystal (15.4 mg, compound **VI**).

**Table 42** Tyrosinase inhibitory activity of subfractions G3321-G3322 of subfraction G332

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction G332) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| G3321 | White powder | 13.4 (55.83) | NA |
| G3322 | White powder | 10.5 (43.75) | 84.27 ± 0.12 |

NA = No activity.

Subfraction G5 was separated by silica gel column chromatography and eluted with a mixture of *n*-hexane:ethyl acetate to obtain two subfractions (G51-G52). The results of subfractions G51-G52 are shown in Table 43.

**Table 43** Tyrosinase inhibitory activity of subfractions G51-G52 of subfraction G5

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction G5) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| G51 | White powder | 100.0 (50.00) | 62.66 ± 0.33 |
| G52 | Green gum | 71.3 (35.60) | 38.68 ± 0.45 |

Subfraction G51 was separated by silica gel column chromatography and eluted with a mixture of dichloromethane and ethyl acetate to obtain two subfractions (G511-G512). Subfraction G511 was obtained as white powder (compound **VII**). The results of subfractions G511-G512 are shown in Table 44.

**Table 44** Tyrosinase inhibitory activity of subfractions G511-G512 of subfraction G51

| Subfraction | Appearance | Yield [115] (%, w/weight of subfraction G51 ) | Percentage of inhibitory activity (%) |
|---|---|---|---|
| G511 | White powder | 20.0 (20.00) | 75.31 ± 0.04 |
| G512 | White powder | 60.0 (60.00) | 10.71 ± 0.11 |

Among the isolation of crude extracts of *M. zapota* barks, compound **I** was isolated from *n*-hexane crude extract and compounds **II-VII** were isolated from ethyl acetate crude extract. Moreover, compounds **I-VII** were elucidated by spectroscopic data (NMR and HR-EI-MS), analyzed by physical properties (melting point and the optical rotation) and compared with previously reported. Compounds **I-VII** were evaluated for antityrosinase activities on monophenolase and diphenolase inhibitory

activities, antioxidant activities with DPPH, ABTS and FRAP assays and cytotoxic activity. Isolated compounds could reveal with these biological activities.

4.7 Elucidation of isolated compounds I-VII

4.7.1 Elucidation of compound **I**

Compound **I** was obtained as white powder (132.9 mg, 0.06% w/w of hexane crude extract and 0.03% of ethyl acetate crude extract) with melting point of 278-280 °C. The optical rotation was as $[\alpha]_D^{28}$ +8.3 (CHCl$_3$; c 0.63). The $^1$H-NMR spectrum (Figure 51) showed seven signals of methyl proton at δ 1.06 (3H, s, H-27), 0.94 (3H, s, H-29), 0.93 (3H, s, H-23), 0.90 (3H, s, H-24), 0.89 (6H, s, H-28 and H-30), 0.80 (3H, s, H-26) and 0.76 (3H, s, H-25) ppm. One signal of methoxy proton showed at δ 3.33 (s, H-1′) ppm. One signal of olefinic proton showed at δ 5.51 (1H, dd, $J$ = 8.1, 3.3 Hz, H-15) ppm. Four signals of methine proton showed at 2.61 (1H, dd, $J$ = 11.7, 4.2 Hz, H-3), 1.42 (1H, m, H-9), 1.39 (1H, m, H-18) and 0.85 (1H, d, $J$ = 3.6 Hz, H-5), ppm. Sixteen signals of methylene proton showed at δ 2.00 (1H, dt, $J$ = 12.0, 3.0 Hz, H-19a), 1.89 (1H, dd, $J$ = 15.0, 3.0 Hz, H-1a), 1.65 (1H, m, H-7a), 1.64 (1H, m, H-7b), 1.60 (1H, m, H-1b), 1.57 (2H, m, H-6a and H-21a), 1.51 (2H, m, H-11a and H-21b), 1.44 (1H, m, H-11b), 1.37 (1H, m, H-6b), 1.36 (1H, m, H-12a and H-22a), 1.34 (1H, m, H-19b), 1.31 (1H, m, H-16a), 1.25 (2H, m, H-16b and H-22b), 1.00 (1H, m, H-12b), 0.96 (1H, m, H-2a), 0.95 (1H, m, H-2b) ppm.

The $^{13}$C-NMR spectrum (Figure 52) showed thirty one carbon atoms. DEPT90 and DEEP135 experiments (Figures 53-54), two signals of olefinic carbon showed signal δ 158.3 and 116.8 ppm suggested that the chemical shift were C-14 and C-15. A signal carbon at 88.9 ppm indicated a C-3 substitued methoxy carbon (OCH$_3$, C-1′). Eight signals of methyl carbon displayed at δ 33.5 (C-29), 30.1 (C-28), 30.0 (C-26), 28.2 (C-23), 26.1 (C-27), 21.5 (C-30), 15.5 (C-24) and 16.3 (C-25) ppm. Nine signals of methylene carbon showed at δ 37.9 (C-7), 37.8 (C-1), 36.8 (C-16), 35.3 (C-12), 33.8 (C-21), 33.2 (C-22), 28.2 (C-2), 18.8 (C-6) and 17.7 (C-11) ppm. Five signals of methine carbon showed at δ 88.9 (C-3), 55.9 (C-5), 49.1 (C-18), 48.9 (C-9) and 41.5 (C-19) ppm. Six signals of quaternary carbon appeared at δ 39.2 (C-8 and C-10), 38.8 (C-4), 37.9 (C-13 and C-17) and 28.9 (C-20) ppm.

Experiments of HSQC, HMBC and COSY analysis (Figures 55-57) were confirmed the structure of compound **I.** HMBC spectrum (Figure 56) showed correlation peaks of H-3/C-1, 2 and 3, H-5/C-4, 6 and 10, H-6/C-4, 5 and 7, H-9/C-6, 8 and 11, H-11/C-9, 12 and 13, H-15/C-13, 14 and 16, H-19/C-18, 20 and 30, H-23/C-3, 4 and 5, H-24/C-4 and 5, H-25/C-1, 5 and 10, H-26/C-6, 7 and 8, H-27/C-12, 13 and 15, H-28/C-16, 17 and 18, H-29/C-18, 19 and 20 and H-30/C-17, 21 and 22 (Figure 17).

Comparison of NMR data of compound **I** and reported literature [116] are shown in Table 45.

**Table 45** Comparison of NMR data of compound **I** and taraxerol methyl ether

| Position | Chemical shift (ppm) | | | |
| --- | --- | --- | --- | --- |
| | Taraxerol methyl ether | | Compound **I** | |
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | 1.56 (m), 1.61 (m) | 33.9 | 1.89 (dd, 15.0, 3.0), 1.60 (m) | 37.8 |
| 2 | 1.40 (m), 1.76 (m) | 22.1 | 0.96 (m), 0.96 (m) | 28.2 |
| 3 | 2.63 (dd, 11.7, 4.3) | 88.9 | 2.61 (dd, 11.7, 4.2) | 88.9 |
| 4 | - | 39.2 | - | 38.8 |
| 5 | 0.75 (m) | 56.3 | 0.85 (d, 3.6) | 55.9 |
| 6 | 1.48 (m), 1.58 (m) | 18.8 | 1.57 (m), 1.37 (m) | 18.8 |
| 7 | 1.34 (m), 2.04 (m) | 41.5 | 1.65 (m), 1.64 (m) | 37.9 |
| 8 | - | 38.8 | - | 39.2 |
| 9 | 1.40 (m) | 49.4 | 1.42 (m) | 48.9 |
| 10 | - | 37.8 | - | 39.2 |
| 11 | 1.48 (m), 1.63 (m) | 17.7 | 1.51 (m), 1.44 (m) | 17.7 |
| 12 | 0.93 (m), 0.91 (m) | 29.0 | 1.36 (m), 1.00 (m) | 35.3 |
| 13 | - | 38.2 | - | 37.9 |
| 14 | - | 158.3 | - | 158.3 |
| 15 | 5.53 (dd, 8.3, 3.3) | 117.0 | 5.51 (dd, 8.1, 3.3) | 116.8 |
| 16 | 1.63 (m), 1.90 (m) | 37.9 | 1.31 (m), 1.25 (m) | 36.8 |
| 17 | - | 35.9 | - | 37.9 |
| 18 | 0.94 (m) | 48.9 | 1.39 (m) | 49.1 |
| 19 | 1.03 (m), 1.36 (m) | 35.3 | 2.00 (dt, 12.0, 3.0), 1.34 (m) | 41.5 |
| 20 | - | 37.7 | - | 28.9 |
| 21 | 0.99 (m), 1.31 (m) | 36.8 | 1.57 (m), 1.51 (m) | 33.8 |
| 22 | 1.26 (m), 1.34 (m) | 33.2 | 1.36 (m), 1.25 (m) | 33.2 |
| 23 | 0.96 (s) | 28.2 | 0.93 (s) | 28.2 |
| 24 | 0.78 (s) | 16.4 | 0.90 (s) | 15.5 |
| 25 | 0.93 (s) | 15.6 | 0.76 (s) | 16.3 |
| 26 | 1.08 (s) | 26.1 | 0.80 (s) | 30.0 |
| 27 | 0.91 (s) | 21.5 | 1.06 (s) | 26.1 |
| 28 | 0.91 (s) | 30.1 | 0.89 (s) | 30.1 |
| 29 | 0.95 (s) | 33.5 | 0.94 (s) | 33.5 |
| 30 | 0.82 (s) | 30.0 | 0.89 (s) | 21.5 |
| 1′ | 3.35 (s) | 57.7 | 3.33 (s) | 56.3 |

The HR-EI-MS spectrum (Figure 58) of compound **I** showed [M+Na]$^+$ at m/z 463.3249. A molecular formula was predicted as $C_{31}H_{52}ONa$ and it was calculated as 463.3916. Therefore, compound **I** was identified as taraxerol methyl ether. The

structure of compound **I** is shown in Figure 18.
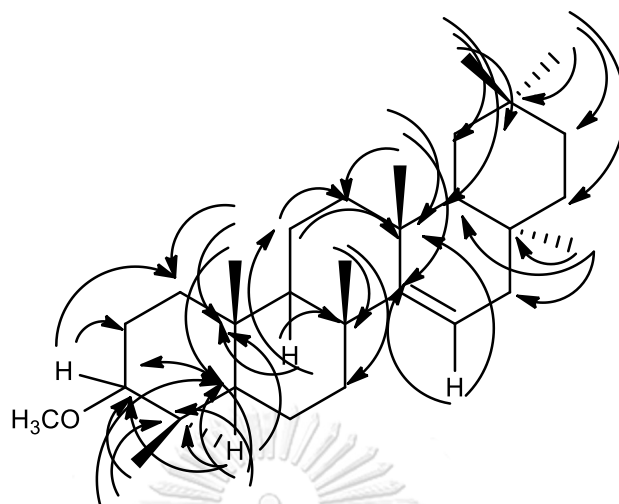


**Figure 17** HMBC correlations of compound **I**
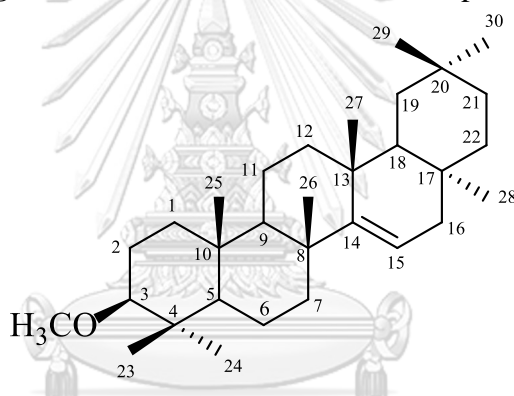


**Figure 18** The structure of compound **I**

Taraxerol methyl ether was isolated from *Echinochloa crusgallis* L. [116], leaves of *Bosistoa sapindiformis* [117] and stem barks of *Mimusops obtusifolia* [104]. It showed cytotoxic activity against HeLa, HL-60 and MCF-7 with $IC_{50}$ value > 50 μM [116] and displayed antiplasmodial activity with $IC_{50}$ value > 100 μg/mL [104].

4.7.2 Elucidation of compound **II**

Compound **II** was afforded as yellow powder (8.0 mg, 0.0006% w/w of ethyl acetate crude extract) with melting point of 180-182 °C. The optical rotation was determined as $[\alpha]_D^{28}$-1.2 (DMSO; c 1.05). The $^1$H-NMR spectrum (Figure 59) showed five aromatic protons at δ 7.53 (1H, dd, $J$ = 8.1, 1.5 Hz, H-2′ and H-6′), δ 7.39 (1H, m, H-3′, H-4′ and H-5′), 7.23 (1H, d, $J$ = 3.3 Hz, H-5), 7.06 (1H, dd, $J$ = 9.0, 3.3 Hz, H-7) and 6.96 (1H, dd, $J$ = 9.0, 3.3 Hz, H-8) ppm. Two signals of methylene proton displayed

at 3.08 (1H, $J$ = 16.8, 12.9 Hz, H-3) and 2.83 (1H, $J$ = 16.8, 3.0 Hz, H-3) ppm. One signal of methine proton showed at δ 5.48 (1H, dd, $J$ = 13.2, 3.0 Hz, H-2) ppm.

The $^{13}$C-NMR spectrum (Figure 60) showed one carbonyl carbon signal at δ 194.3 ppm (C-4) and one signal of methylene carbon showed at δ 45.6 (C-3) ppm. One signal of methine carbon showed at δ 81.0 (C-2) ppm. The remaining signals displayed aromatic carbons at δ 156.8 (C-9), 153.1 (C-6), 140.8 (C-1′), 129.7 (C-3′ and C-5′), 129.5 (C-4′), 127.3 (C-2′ and C-6′), 126.0 (C-7) and 122.4 (C-10) ppm.

Experiments of HSQC, HMBC and COSY (Figures 61-63) analysis were confirmed the structure of compound **II** and HMBC spectrum (Figure 62) showed correlation peaks of H-2/C-3 and 1′, H-3/C-2, 4 and 5, H-5/C-6 and 10, H-7/C-6 and 8, H-8/C-7 and 9, H-2′/C-1′ and 3′, H-3′/C-2′ and 4′, H-4′/C-3′ and 5′, H-5′/C-4′ and 6′ and H-6′/C-1′ and 5′ (Figure 19).

Compound **II** was identified by comparison of NMR data with reported in the literature [118] (Table 46).

**Table 46** Comparison of NMR data of compound II and 6-hydroxyflavanone

| Position | Chemical shift (ppm) | | | |
|---|---|---|---|---|
| | 6-hydroxyflavanone | | Compound **II** | |
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | - | - | - | - |
| 2 | 5.51 (dd, $J$ = 13.0, 2.8 Hz) | 79.2 | 5.48 (dd, $J$ = 13.2, 3.0 Hz) | 81.0 |
| 3 | 3.13 (dd, $J$ = 16.9, 13.0 Hz) | 44.2 | 3.08 ($J$ = 16.8, 12.9 Hz) | 45.6 |
| | 2.75 (dd, $J$ = 16.9, 2.9 Hz) | | 2.83 ($J$ = 16.8, 3.0 Hz) | |
| 4 | - | 192.2 | - | 194.3 |
| 5 | 7.09 (d, $J$ = 2.9 Hz) | 110.4 | 7.23 (d, $J$ = 3.3 Hz) | 111.5 |
| 6 | - | 152.1 | - | 153.1 |
| 7 | 7.00 (dd, $J$ = 8.8, 3.0 Hz) | 124.9 | 7.06 (dd, $J$ = 9.0, 3.3 Hz) | 125.9 |
| 8 | 6.92 (d, $J$ = 8.8 Hz) | 119.5 | 6.96 (dd, $J$ = 9.0, 3.3 Hz) | 120.2 |
| 9 | - | 154.9 | - | 156.8 |
| 10 | - | 121.4 | - | 122.4 |
| 1′ | - | 139.7 | - | 140.8 |
| 2′ | 7.35 (d, $J$ = 8.4 Hz) | 127.0 | 7.53 (dd, $J$ = 8.1, 1.5 Hz) | 127.3 |
| 3′ | 7.36 (m) | 129.0 | 7.39 (m) | 129.7 |
| 4′ | 7.36 (m) | 128.9 | 7.39 (m) | 129.5 |
| 5′ | 7.36 (m) | 129.0 | 7.39 (m) | 129.7 |
| 6′ | 7.35 (d, $J$ = 8.4 Hz) | 127.0 | 7.53 (dd, $J$ = 8.1, 1.5 Hz) | 127.3 |

The HR-EI-MS spectrum (Figure 64) of compound **II** displayed [M+H]$^+$ at m/z 263.0685. A molecular formula was assigned and calculated as $C_{15}H_{12}O_3Na$ (263.0684). Thus, compound **II** was elucidated as 6-hydroxyflavanone. The structure of compound **II** is shown in Figure 20.
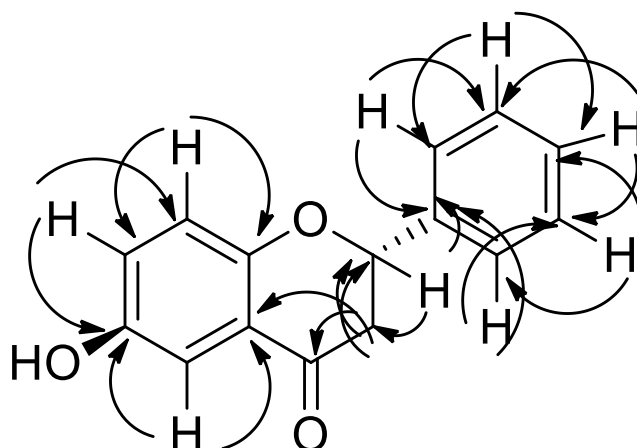
**Figure 19** HMBC correlations of compound **II**
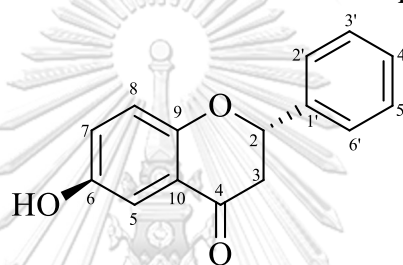


**Figure 20** The structure of compound **II**

This study, 6-hydroxyflavanone was isolated for the first time in *M. zapota* barks. Herath *et al* reported that 6-hydroxyflavone was fermented with *Beauveria bassiana* to afford 6-hydroxyflavanone [118]. Mikell *et al* studied that 6-hydroxyflavanone was fermented by *Cunninghamella blakesleeana* to yield 6-hydroxyflavanone derivatives such as flavanone 6-*O*-*β*-*D*-glucopyranoside, flavanone 6-sulfate and 6-hydroxyflavanone-7-sulfate [119]. 6-Hydroxyflavanone showed cytotoxic activity against human epithelial adenocarcinoma (HeLa) cell lines and human breast cancer cell lines (BT474) [120, 121].

### 4.7.3 Elucidation of compound **III**

Compound **III** was obtained as yellow powder (2.4 mg, 0.0017% w/w of ethyl acetate crude extract) with melting point of 228-230 °C. The optical rotation was recorded as $[\alpha]_D^{28}$ +26.4 (CH₃OH; c 0.12). The ¹H-NMR spectrum (Figure 65) showed four aromatic protons at δ 7.41 (2H, d, *J* = 8.4 Hz, H-2′ and 6′), 6.89 (2H, d, *J* = 8.7 Hz, H-3′ and 5′), 5.98 ppm (1H, d, *J* = 2.1 Hz, H-8) and 5.94 (1H, d, *J* = 2.1 Hz, H-6) ppm. Two signals methine proton showed at 5.08 (1H, d, *J* = 11.4 Hz, H-2) and 4.64 (1H, d,

$J = 11.7$ Hz, H-3) ppm. Hydroxy proton substitution signal displayed at δ 11.67 (4H, s, OH) ppm.

The $^{13}$C-NMR spectrum (Figure 66) showed one ketone carbon signal at δ 198.3 (C-4) ppm. Two signals of methine carbon showed at δ 84.3 (C-2) and 73.1 (C-3) ppm. Nine signals of aromatic carbon displayed at δ 168.7 (C-7), 165.0 (C-5), 164.2 (C-9), 158.8 (C-4′), 130.3 (C-2′ and C-6′), 129.1 (C-1′), 115.9 (C-3′ and C-5′), 101.5 (C-10), 97.0 (C-8) and 96.0 (C-6) ppm.

Experiments of HSQC, HMBC and COSY (Figures 67-69)analysis were confirmed the structure of compound **III** and HMBC spectrum (Figure 68) showed correlation peaks of H-3/C-3 and 4, H-6/C-5 and 7, H-8/C-7 and 9, H-2′/C-1′, 3′ and 6′, H-3′/C-2′, 4′ and 5′, H-5′/C-3′, 4′ and 6′ and H-6′/C-1′, 2′ and 5′ (Figure 21).

Compound **III** was identified by comparison of NMR data with reported in the literature [122] (Table 47).

**Table 47** Comparison of NMR data of compound **III** and dihydrokaempferol

| Position | Chemical shift (ppm) | | | |
|---|---|---|---|---|
| | dihydrokaempferol | | Compound **III** | |
| | δ$_H$ | δ$_C$ | δ$_H$ | δ$_C$ |
| 1 | - | - | - | - |
| 2 | 5.09 (1H, d, $J$ = 11.6 Hz) | 84.4 | 5.08 (1H, d, $J$ = 11.4 Hz) | 84.3 |
| 3 | 4.66 (1H, d, $J$ = 11.6 Hz) | 73.1 | 4.64 (1H, d, $J$ = 11.7 Hz) | 73.1 |
| 4 | - | 198.2 | - | 198.3 |
| 5 | - | 165.0 | - | 165.0 |
| 6 | 5.96 (1H, d, $J$ = 2.0 Hz) | 97.2 | 5.94 (1H, d, $J$ = 2.1 Hz) | 96.0 |
| 7 | - | 167.9 | - | 168.7 |
| 8 | 6.00 ppm (1H, d, $J$ = 2.0 Hz) | 96.1 | 5.98 ppm (1H, d, $J$ = 2.1 Hz) | 97.0 |
| 9 | - | 164.2 | - | 164.2 |
| 10 | - | 101.5 | - | 101.5 |
| 1′ | - | 129.1 | - | 129.1 |
| 2′ | 7.42 (2H, d, $J$ = 8.6 Hz) | 130.2 | 7.41 (2H, d, $J$ = 8.4 Hz) | 130.3 |
| 3′ | 6.90 (2H, d, $J$ = 8.6 Hz) | 115.9 | 6.89 (2H, d, $J$ = 8.7 Hz) | 115.9 |
| 4′ | - | 158.8 | - | 158.8 |
| 5′ | 6.90 (2H, d, $J$ = 8.6 Hz) | 115.9 | 6.89 (2H, d, $J$ = 8.7 Hz) | 115.9 |
| 6′ | 7.42 (2H, d, $J$ = 8.6 Hz) | 130.2 | 7.41 (2H, d, $J$ = 8.4 Hz) | 130.3 |

The HR-EI-MS spectrum (Figure 69) of compound **III** displayed [M+H]$^+$ at m/z 311.0387. A molecular formula was assigned and calculated as $C_{15}H_{13}O_6$ (311.0532). Thus, compound **III** was elucidated as (+)-dihydrokaempferol. The structure of compound **III** is shown in Figure 22.
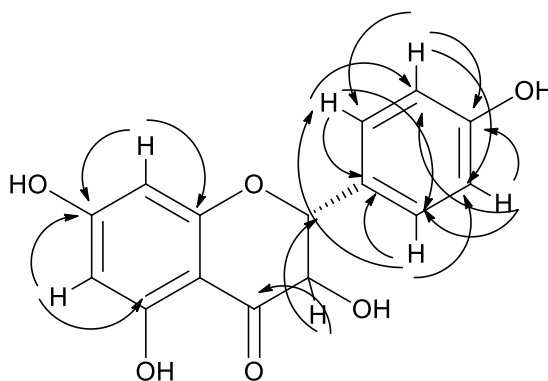
**Figure 21** HMBC correlations of compound **III**
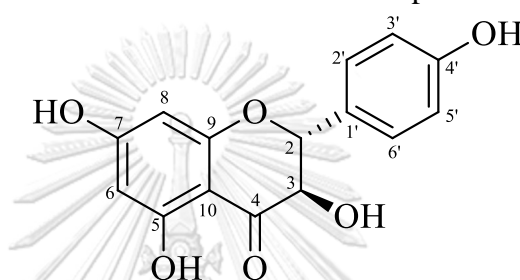


**Figure 22** The structure of compound III

This is the first report on isolation of (+)-dihydrokaempferol from *M. zapota* barks. However, flavonoid compounds were found in *M. zapota* such as (+)-catechin, dihydromiricetin and quercitrin. (+)-Dihydrokaempferol was isolated from roots of *Polygonum amplexicaule* [122], barks of *Juglans mandshurica* [36] and twigs of *Morus alba* [123]. (+)-Dihydrokaempferol displayed antityrosinase activity on monophenolase inhibitory activity with $IC_{50}$ value of > 200 μM [123]. In addition, it showed antityrosinase activity on diphenolase inhibitory activity with $IC_{50}$ value of 2.7 ± 0.7 mM [36].

### 4.7.4 Elucidation of compound **IV**

Compound **IV** was obtained as brown powder (60.0 mg, 0.0434% w/w of ethyl acetate crude extract) with melting point of 220-222 °C. The optical rotation was determined as $[\alpha]_D^{28}$ +13.3 (CH₃COCH₃; c 1.33). The ¹H-NMR spectrum (Figure 70) showed three aromatic proton signals at δ 7.52 (1H, d, *J* = 2.1 Hz, H-2), 7.47 (1H, d, *J* = 8.1, 1.8 Hz, H-6) and 6.89 (1H, d, *J* = 8.4 Hz, H-5) ppm.

The $^{13}C$-NMR spectrum (Figure 71) showed carboxylic carbon signal appeared at δ 167.5 (C-1′) ppm. Three signals of methine aromatic carbon showed at δ 123.6 (C-6), 117.5 (C-2) and 115.6 (C-5) ppm. Three signals of quaternary aromatic carbon appeared at δ 150.7 (C-4), 145.6 (C-3) and 123.1 (C-1) ppm.

Experiments of HSQC, HMBC and COSY (Figures 72-74) analysis were confirmed the structure of compound **IV** and HMBC spectrum (Figure 73) showed correlation peaks of H-2/C-1 and 6, H-3/C-2 and 4 and H-6/C-1 and 5 (Figure 23).

Compound **IV** was elucidated by comparison of NMR data with reported in the literature [124] (Table 48).

**Table 48** Comparison of NMR data of compound **IV** and 3,4-dihydroxybenzoic acid

| Position | 3,4-dihydroxybenzoic acid | | Compound **IV** | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | - | 122.2 | - | 123.1 |
| 2 | 7.54 (1H, d, $J$ = 2.4 Hz) | 116.8 | 7.52 (1H, d, $J$ = 2.1 Hz) | 117.5 |
| 3 | - | 150.3 | - | 145.6 |
| 4 | - | 144.9 | - | 150.7 |
| 5 | 6.88 (1H, d, $J$ = 8.0 Hz) | 123.1 | 6.89 (1H, d, $J$ = 8.4 Hz) | 115.6 |
| 6 | 7.45 (1H, d, $J$ = 10.27 Hz) | 115.1 | 7.47 (1H, d, $J$ = 8.1, 1.8 Hz) | 123.6 |
| 1′ | - | 207.0 | - | 167.5 |

The HR-EI-MS spectrum (Figure 75) of compound **IV** displayed [M+H]$^+$ at m/z 177.0125. A molecular formula was assigned and calculated as $C_7H_6O_4$ (177.0164). Thus, compound **IV** was elucidated as 3,4-dihydroxybenzoic acid. The structure of compound **IV** is shown in Figure 24.
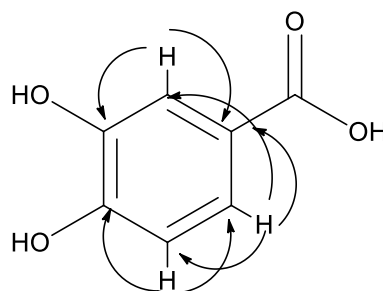


**Figure 23** HMBC correlations of compound IV
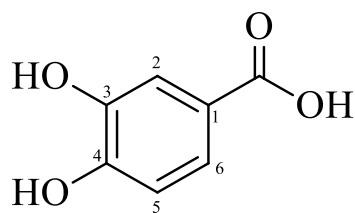
**Figure 24** The structure of compound **IV**

In this study, 3,4-dihydroxybenzoic acid was isolated for the first time from *M. zapota* barks. 3,4-Dihydroxybenzoic acid was found in leaves of *Ageratum conyzoides* [125] and stem barks of *Musanga cecropioides* [124]. Benzoic acid derivatives showed biological activities such as antimicrobial and antioxidant activities [126].

4.7.5 Elucidation of compound **V**

Compound **V** was obtained as white crystal (10.5 mg, 0.00076% w/w of ethyl acetate crude extract) with melting point of 284-286 °C. The optical rotation was determined as $[\alpha]_D^{28}$ +0.7 (CH$_3$Cl$_3$; c 3.24). The $^1$H-NMR spectrum (Figure 76) showed seven signals of methyl proton at δ 1.09 (3H, s, H-27), 0.98 (1H, s, H-23), 0.95 (1H, s, H-29), 0.93 (1H, s, H-24), 0.91 (2H, s, H-26 and H-30), 0.82 (1H, s, H-28) and 0.80 (1H, s, H-25) ppm. One signal of olefinic proton showed at δ 5.53 (1H, dd, *J* = 8.1, 3.3 Hz, H-15) ppm. Four signals of methine proton showed at δ 0.76 (1H, d, *J* = 2.7 Hz, H-5), 3.19 (1H, dd, *J* = 8.1, 3.3 Hz, H-3), 1.44 (m, H-18) and 0.76 (1H, d, *J* = 2.7 Hz, H-5) ppm. Thirteen signals of methylene proton displayed at δ 1.92 ppm (1H, dd, *J* = 14.7, 3.0 Hz, H-16a), 2.03 (1H, dt, *J* = 11.7, 3.0 Hz, H-19a), 1.65 (2H, m, H-2a and H-21a), 1.64 (2H, m, H-6a and H-11a), 1.62 (2H, m, H-1a and H-22a), 1.58 (3H, m, H-1b, H-6b and H-21b), 1.39 (1H, m, H-11b), 1.38 (1H, m, H-22b), 1.33 (1H, m, H-19b), 1.31 (2H, m, H-7a and H-12a), 1.30 (1H, m, H-16b), 1.25 (1H, m, H-2b) and 1.02 (1H, m, H-7b, H-9 and H-12b) ppm.

The $^{13}$C-NMR spectrum (Figure 77) showed thirty carbon atoms. Two signals of olefinic carbons showed at δ 158.2 and 117.0 ppm and suggested that these chemical shifts were C-14 and C-15. A signal carbon at 79.2 ppm indicated a C-3 substitued hydroxyl group (OH). Seven signals of methyl carbon displayed at δ 33.5 (C-29), 30.1 (C-28), 30.0 (C-26), 28.1 (C-23), 26.1 (C-27), 21.5 (C-30) and 15.6 (C-24 and C-25) ppm. Eight signals of methylene carbon showed at δ 37.9 (C-1), 36.8 (C-16), 35.3 (C-

7 and C-12), 33.8 (C-21), 33.2 (C-22), 27.3 (C-2), 18.9 (C-6) and 17.7 (C-11) ppm. Five signals of methine carbon showed at δ 79.2 (C-3), 55.7 (C-5), 49.4 (C-18), 48.9 (C-9) and 41.5 (C-19) ppm. Six signals of quaternary carbon appeared at δ 39.1 (C-8), 38.9 (C-4), 38.2 (C-17), 37.7 (C-13), 35.9 (C-10) and 29.0 (C-20) ppm.

Experiments of HSQC, HMBC and COSY analysis (Figures 80-82) were confirmed the structure of compound **V** and HMBC spectrum (Figure 81) showed correlation peaks of H-3/C-1, 2 and 4, H-5/C-6 and 10, H-7/C-6 and 8, H-9/C-10 and 11, H-15/C-14 and 16, H-18/C-13 and 19, H-23/C-3, 4 and 5, H-24/C-3, 4 and 5, H-25/C-1, 5 and 10, H-26/C-7, 8 and 9, H-27/C-11, 12 and 13, H-28/C-16, 17 and 22, H-29/C-18, 19 and 20 and H-30/C-20, 21 and 22 (Figure 25).

Comparison of NMR data of compound **V** and reported literature [116] are shown in Table 49.

**Table 49** Comparison of NMR data of compound **V** and taraxerol

| Position | Chemical shift (ppm) | | | |
|---|---|---|---|---|
| | Taraxerol | | Compound **V** | |
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | | 38.0 | 1.62 (m), 1.58 (m) | 37.9 |
| 2 | | 27.2 | 1.65 (m), 0.96 (m) | 27.3 |
| 3 | | 79.1 | 3.19 (dd, 8.1, 3.3) | 79.2 |
| 4 | | 39.0 | - | 38.9 |
| 5 | | 55.6 | 0.76 (d, 2.7) | 55.7 |
| 6 | | 18.8 | 1.57 (m), 1.58 (m) | 18.9 |
| 7 | 2.0 (dt, 12.6, 3.1) | 35.1 | 1.31 (m), 1.64 (m) | 35.3 |
| 8 | | 38.8 | - | 39.1 |
| 9 | | 48.8 | 1.42 (m) | 48.9 |
| 10 | | 37.6 | - | 35.9 |
| 11 | | 17.5 | 1.64 (m), 1.39 (m) | 17.7 |
| 12 | | 35.8 | 1.31 (m), 1.00 (m) | 35.3 |
| 13 | | 37.6 | - | 37.7 |
| 14 | | 158.1 | - | 158.3 |
| 15 | 5.53 (dd, 8.2, 3.2) | 116.9 | 5.53 (dd, 8.1, 3.3) | 116.8 |
| 16 | 1.9 (dd, 14.6, 3.0) | 36.7 | 1.64 (m), 1.30 (m) | 36.8 |
| 17 | | 37.7 | - | 38.2 |
| 18 | | 49.3 | 1.44 (m) | 49.4 |
| 19 | | 41.3 | 2.03 (dt, 11.7, 3.0), 1.33 (m) | 41.5 |
| 20 | | 28.8 | - | 29.0 |
| 21 | | 33.7 | 1.65 (m), 1.58 (m) | 33.8 |
| 22 | | 33.1 | 1.62 (m), 1.38 (m) | 33.2 |
| 23 | 0.98 (s) | 28.0 | 0.98 (s) | 28.1 |
| 24 | 0.80 (s) | 15.4 | 0.93 (s) | 15.6 |
| 25 | 0.93 (s) | 15.5 | 0.80 (s) | 15.6 |
| 26 | 1.09 (s) | 29.8 | 0.91 (s) | 30.0 |
| 27 | 0.91 (s) | 25.9 | 1.09 (s) | 26.1 |
| 28 | 0.82 (s) | 39.9 | 0.82 (s) | 30.1 |

**Table 49** Comparison of NMR data of compound **V** and taraxerol (continue)

| Position | Taraxerol | | Compound **V** | |
|---|---|---|---|---|
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 29 | 0.95 (s) | 33.4 | 0.95 (s) | 33.5 |
| 30 | 0.90 (s) | 21.3 | 0.91 (s) | 21.5 |

The HR-EI-MS spectrum (Figure 83) of compound **V** displayed [M+H]$^+$ at m/z 449.3748. A molecular formula was assigned and calculated as $C_{30}H_{50}ONa$ (449.3759). Thus, compound **V** was elucidated as taraxerol. The structure of compound **V** is shown in Figure 26.
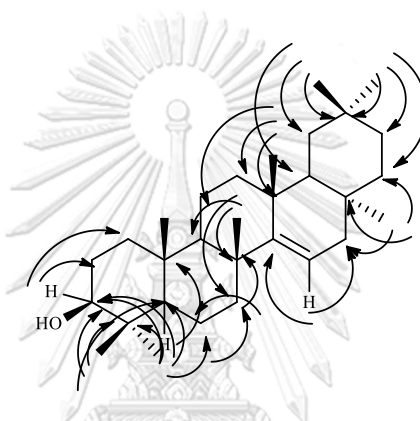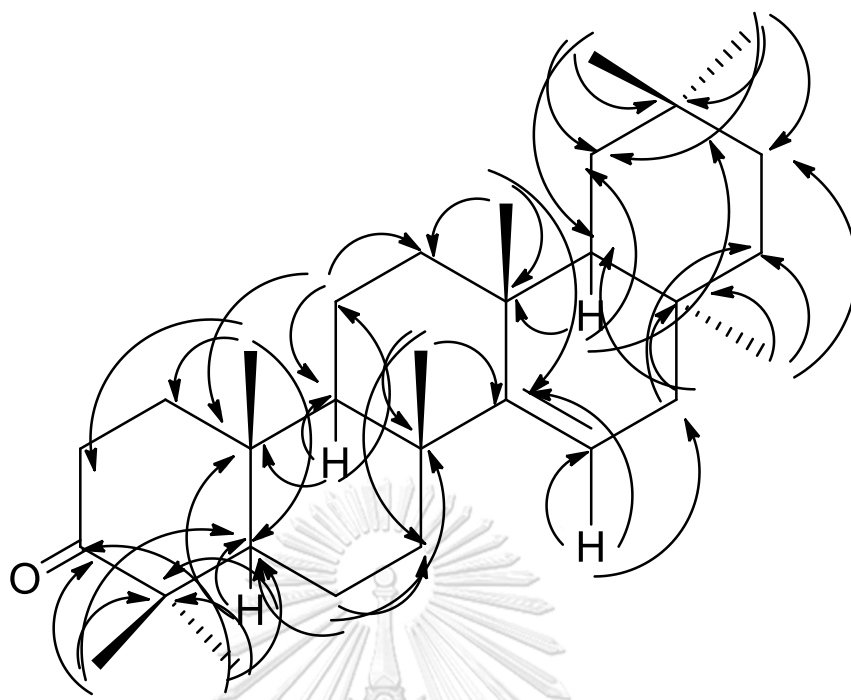


**Figure 25** HMBC correlations of compound V
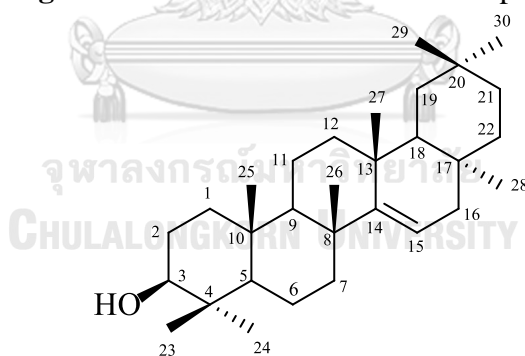


**Figure 26** The structure of compound V

Taraxerol was prevoiusly isolated from *M. obtusfolia* which showed antiplasmodial activity with IC$_{50}$ value > 100 μg/mL [104]. Moreover, taraxerol was produced from Agrobacterium rhizogenes transformed root cultured of *Clitoria ternatea* and showed anticancer activity [127]. Viswanadh *et al* reported that taraxerol was isolated from *Homonoia riparia* barks [128].

4.7.6 Elucidation of compounds **VI**

Compound **VI** was obtained as white crystal (15.4 mg, 0.0111% w/w of ethyl acetate crude extract) with melting point of 248-250 °C. The optical rotation was recorded as $[\alpha]_D^{28}$ +8.1 (CHCl$_3$; c 1.60). The $^1$H-NMR spectrum (Figure 84) showed six signals of methyl group at δ 1.14 (3H, s, H-27), 1.08 (6H, s, H-23 and H-25), 1.06 (3H, s, H-24), 0.95 (3H, s, H-29), 0.91 (6H, s, H-28 and H-30) and 0.83 (3H, s, H-26) ppm. One signal of olefinic proton showed at δ 5.56 (1H, dd, $J$ = 8.1, 2.1 Hz, H-15) ppm. Three signals of methine proton showed at δ 2.58 (1H, m, H-3), 1.50 (2H, m, H-9 and H-18) and 1.33 (1H, m, H-5) ppm. Eleven signals of methylene proton displayed at δ 2.58 (1H, m, H-2a), 2.33 (1H, ddd, $J$ = 15.9, 6.3, 3.3 Hz, H-2b), 2.31 (1H, m, H-21), 2.08 (1H, dt, $J$ = 12.9, 3.3 Hz, H-19), 1.87 (1H, m, H-1a), 1.65 (2H, m, H-7 and H-12), 1.59 (1H, m, H-6), 1.58 (1H, m, H-11), 1.37 (1H, m, H-16), 1.32 (1H, m, H-22) and 0.99 (1H, m, H-1b) ppm.

The $^{13}$C-NMR spectrum (Figure 85) showed thirty carbon atoms. Two signals of olefinic carbon showed at δ 157.6 (C-14) and 117.3 (C-15) ppm. One signal of carbonyl carbon showed at δ 217.6 (C-3) ppm. Eight signals of methyl carbon appeared at δ 33.5 (C-29), 30.0 (C-26), 30.1 (C-28), 26.2 (C-23), 25.7 (C-27), 21.6 (C-24), 21.5 (C-30) and 14.9 (C-25) ppm. Eight signals of methylene carbon displayed at δ 38.5 (C-1), 36.8 (C-16), 35.2 (C-7 and C-12), 33.7 (C-21), 33.2 (C-22), 28.2 (C-2), 20.1 (C-6) and 17.6 (C-11) ppm. Three signals of methine carbon showed at δ 55.9 (C-5), 48.9 (C-9 and C-18) and 40.8 (C-19) ppm. Five signals of quaternary carbon appeared at 47.7 (C-4), 39.0 (C-8), 37.7 (C-10), 37.9 (C-13 and C-17) and 28.9 (C-20) ppm.

Experiments of HSQC, HMBC and COSY (Figures 88-90) analysis were confirmed the structure of compound **VI** and HMBC spectrum (Figure 89) showed correlation peaks of H-5/C-4 and 10, H-6/C-5, 7 and 8, H-9/C-10 and 11, H-11/C-10 and 12, H-15/C-14 and 16, H-16/C-17, 18 and 22, H-18/C-13, 19 and 20, H-23/C-3, 4 and 5, H-24/C-3, 4 and 5, H-25/C-1, 2 and 5, H-26/C-7, 8 and 14, H-27/C-12, 13 and 14, H-28/C-17, 21 and 22, H-29/C-18, 19 and 20 and H-30/C-19, 20 and 21 (Figure 27).

Comparison of NMR data of compound **VI** and reported literature [128] are shown in Table 50.

**Table 50** Comparison of NMR data of compound **VI** and taraxerone

| Position | Chemical shift (ppm) | | | |
|---|---|---|---|---|
| | Taraxerone | | Compound **VI** | |
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | | 38.3 | 1.87 (1H, m) 0.99 (1H, m) | 38.5 |
| 2 | 2.52 (2H, m) | 34.1 | 2.33 (1H, ddd, $J$ = 15.9, 6.3, 3.3 Hz) 2.58 (1H, m) | 28.2 |
| 3 | | 217.5 | - | 217.6 |
| 4 | | 47.6 | - | 47.7 |
| 5 | | 55.6 | 1.33 (1H, m) | 55.9 |
| 6 | | 20.0 | 1.59 (1H, m) | 20.1 |
| 7 | | 35.8 | 1.65 (2H, m) | 35.2 |
| 8 | | 38.9 | - | 39.0 |
| 9 | | 48.7 | 1.50 (2H, m) | 48.9 |
| 10 | | 37.5 | - | 37.7 |
| 11 | | 17.4 | 1.58 (1H, m) | 17.6 |
| 12 | | 29.9 | 1.65 (2H, m) | 35.2 |
| 13 | | 37.7 | - | 37.9 |
| 14 | | 157.6 | - | 157.6 |
| 15 | 5.56 (1H, dd, $J$ = 8.4, 4.0 Hz) | 117.9 | 5.56 (1H, dd, $J$ = 8.1, 2.1 Hz) | 117.3 |
| 16 | | 33.1 | 1.37 (1H, m) | 36.8 |
| 17 | | 40.6 | - | 37.9 |
| 18 | | 47.8 | 1.50 (2H, m) | 48.9 |
| 19 | | 40.6 | 2.08 (1H, dt, $J$ = 12.9, 3.3 Hz) | 40.8 |
| 20 | | 28.8 | - | 28.9 |
| 21 | | 33.6 | 2.31 (1H, m) | 33.7 |
| 22 | | 28.8 | 1.32 (1H, m) | 33.2 |
| 23 | 0.98 (3H, s) | 26.1 | 1.08 (3H, s) | 26.2 |
| 24 | 0.88 (3H, s) | 21.5 | 1.06 (3H, s) | 21.6 |
| 25 | 1.08 (3H, s) | 14.6 | 1.08 (3H, s) | 14.9 |
| 26 | 1.05 (3H, s) | 29.9 | 0.83 (3H, s) | 30.0 |
| 27 | 1.05 (3H, s) | 25.6 | 1.14 (3H, s) | 25.7 |
| 28 | 0.81 (3H, s) | 29.9 | 0.91 (3H, s) | 30.1 |
| 29 | 0.88 (3H, s) | 33.6 | 0.95 (3H, s) | 33.5 |
| 30 | 0.94 (3H, s) | 21.4 | 0.91 (3H, s) | 21.5 |

The HR-EI-MS spectrum (Figure 91) of compound **VI** displayed [M+H]$^+$ at m/z 447.3590. A molecular formula was assigned and calculated as $C_{30}H_{48}ONa$ (447.3602). Thus, compound **VI** was elucidated as taraxerone. The structure of compound **VI** is shown in Figure 28.
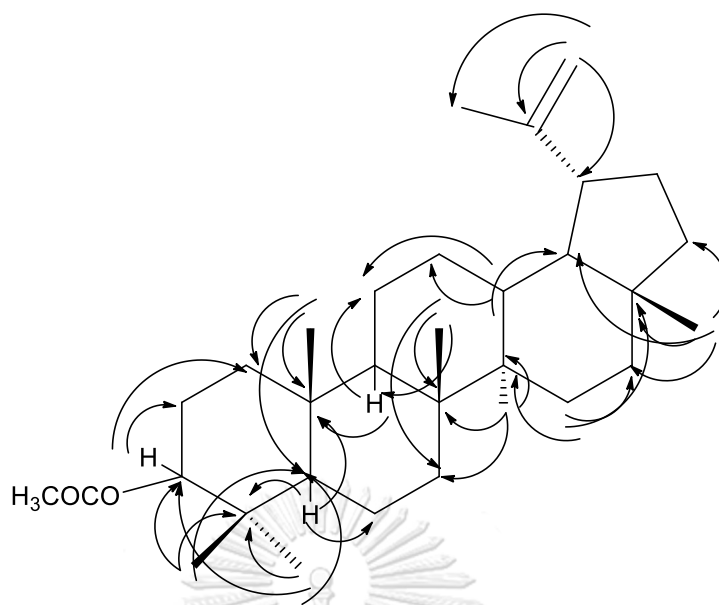
**Figure 27** HMBC correlations of compound **VI**
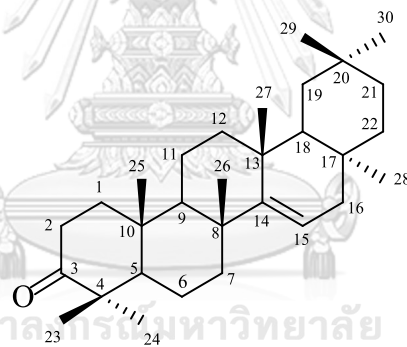


**Figure 28** The structure of compound **VI**

Taraxerone was previously reported that it was isolated from *M. zapota* barks and showed cytotoxic activity against the human Caucasian prostate adenocarcinoma with $IC_{50}$ value of $24.8 \pm 0.8$ μg/ml [94]. Furthermore, taraxerone was isolated from barks of *Homonoia riparia* [128] and leaves of *Sedum sarmentosum* [129].

4.6.7 Elucidation of compounds **VII**

Compound **VII** was obtained as white powder (20.0 mg, 0.0145% w/w of ethyl acetate crude extract) with melting point of 210-212 °C. The optical rotation was recorded as $[\alpha]_D^{28}$ +45.1 (CHCl₃; c 1.76). The ¹H-NMR spectrum (Figure 92) showed seven signals of methyl proton appeared at δ 2.04 (3H, s, H-2′), 1.03 (3H, s, H-23), 0.94

(3H, s, H-27), 0.85 (3H, s, H-26), 0.84 (3H, s, H-25), 0.83 (3H, s, H-24), 0.78 (3H, s, H-28). Two signals of olefinic proton appeared at δ 4.68 (1H, $J$ = 1.5 Hz, H-29a) and 4.58 (1H, dd, $J$ = 1.2, 0.6 Hz, H-29b) ppm. Six signals of methine proton showed at δ 4.47 (1H, dd, $J$ = 6.3, 3.6 Hz, H-3), 2.37 (1H, dt, $J$ = 9.6, 3.6 Hz, H-19), 1.34 (m, H-18), 1.30 (m, H-9), 0.98 (m, H-13) and 0.81 (1H, dd, $J$ = 4.5, 2.7 Hz, H-5) ppm. Seventeen signals of methylene proton appeared at δ 2.04 (s, H-2′), 1.91 (m, H-21a and H-30a), 1.67 (m, H-15a), 1.62 (m, H-2a and H-12a), 1.59 (m, H-1a), 1.51 (m, H-6a), 1.49 (m, H-16a), 1.41 (m, H-2b and H-11a), 1.40 (m, H-16b and H-22a), 1.39 (m, H-6b), 1.38 (m, H-7a), 1.35 (m, H-7b), 1.25 (m, H-21b and H-30b), 1.21 (m, H-11b and H-22b), 1.06 (m, H-12b), 0.96 (m, H-1b) and 0.86 (m, H-15b) ppm.

The $^{13}$C-NMR spectrum (Figure 93) showed thirty two carbon atoms. Two signals of olefinic carbon showed at δ 151.1 (C-20) and 109.5 (C-19) ppm. One signal of carbonyl carbon showed at δ 171.4 (C-1′) ppm. Eight signals of methyl carbon displayed at δ 28.1 (C-23), 21.5 (C-2′), 19.4 (C-30), 18.1 (C-28), 16.6 (C-26), 16.3 (C-24), 16.1 (C-25) and 14.6 (C-27) ppm. Ten signals of methylene carbon appeared at δ 40.1 (C-22), 38.5 (C-1), 35.7 (C-16), 34.4 (C-7), 30.0 (C-21), 27.6 (C-15), 25.2 (C-12), 23.9 (C-2), 21.1 (C-11) and 18.3 (C-6) ppm. Six signals of methine carbon showed at δ 81.2 (C-3), 55.5 (C-5), 50.5 (C-9), 48.2 (C-18), 48.4 (C-19) and 38.2 (C-13) ppm. Four signals of quaternary carbon appeared at δ 43.1 (C-14 and C-17), 41.0 (C-8), 37.9 (C-4) and 37.2 (C-10) ppm.

Experiments of HSQC, HMBC and COSY analysis (Figures 96-98) were confirmed the structure of compound **VII** and HMBC spectrum (Figure 99) showed correlation peaks of H-3/C-1 and 2, H-5/C-4, 6 and 10, H-9/C-10 and 11, H-13/C-11, 12 and 18, H-15/C-14, 16 and 17, H-23/C-3, 4 and 5, H-24/C-3, 4 and 5, H-25/C-1, 5 and 10, H-26/C-7, 8 and 9, H-27/C-7, 8 and 14, H-28/C-17, 18 and 22 and H-29/C-19, 20 and 30 (Figure 29).

Comparison of NMR data of compound **VII** and reported literature [130] are shown in Table 51.

**Table 51** Comparison of NMR data of compound **VII** and lupeol acetate

| Position | Chemical shift (ppm) | | | |
|---|---|---|---|---|
| | lupeol acetate | | Compound **VII** | |
| | $\delta_H$ | $\delta_C$ | $\delta_H$ | $\delta_C$ |
| 1 | | 38.3 | 1.59 (m), 0.96 (m) | 38.5 |
| 2 | | 23.7 | 1.62 (m), 1.41 (m) | 23.9 |
| 3 | 4.44 (1H, dd, $J$ = 10.8, 5.8 Hz) | 80.9 | 4.47 (1H, dd, $J$ = 6.3, 3.6 Hz) | 81.2 |
| 4 | | 37.7 | - | 37.9 |
| 5 | 0.76 (1H, dd, $J$ = 10.8, 5.8 Hz) | 55.3 | 0.81 (1H, dd, $J$ = 4.5, 2.7 Hz) | 55.5 |
| 6 | | 18.1 | 1.51 (m) 1.39 (m) | 18.3 |
| 7 | | 34.2 | 1.38 (m), 1.35 (m) | 34.4 |
| 8 | | 40.8 | - | 41.0 |
| 9 | | 50.3 | 1.30 (m) | 50.5 |
| 10 | | 37.0 | - | 37.2 |
| 11 | | 20.9 | 1.41 (m) 1.21 (m) | 21.1 |
| 12 | | 25.0 | 1.62 (m), 1.06 (m) | 25.2 |
| 13 | | 38.0 | 0.98 (m) | 38.2 |
| 14 | | 42.8 | - | 43.1 |
| 15 | | 27.4 | 1.67 (m), 0.86 (m) | 27.6 |
| 16 | | 35.5 | 1.49 (m) 1.40 (m) | 35.7 |
| 17 | | 42.9 | - | 43.1 |
| 18 | | 48.0 | 1.34 (m) | 48.2 |
| 19 | 2.33 (1H, dt, $J$ = 11.1, 5.6 Hz) | 48.2 | 2.37 (1H, dt, $J$ = 9.6, 3.6 Hz) | 48.4 |
| 20 | | 150.9 | - | 151.1 |
| 21 | 1.82-1.93 (2H, m) | 29.8 | 1.91 (m) 1.25 (m) | 30.0 |
| 22 | | 39.9 | 1.40 (m), 1.21 (m) | 40.1 |
| 23 | 0.82 (3H, s) | 28.2 | 1.03 (3H, s) | 28.1 |
| 24 | 0.82 (3H, s) | 15.9 | 0.83 (3H, s) | 16.3 |
| 25 | 0.82 (3H, s) | 16.1 | 0.84 (3H, s) | 16.1 |
| 26 | 1.00 (3H, s) | 16.4 | 0.85 (3H, s) | 16.6 |
| 27 | 0.91 (3H, s) | 14.5 | 0.94 (3H, s) | 14.6 |
| 28 | 0.81 (3H, s) | 17.9 | 0.78 (3H, s) | 18.1 |
| 29 | 4.66 (1H, s) 4.54 (1H, s) | 109.3 | 4.68 (1H, d, $J$ = 1.5 Hz) 4.58 (1H, dd, $J$ = 1.2, 0.6 Hz) | 109.5 |
| 30 | 1.66 (3H, s) | 19.0 | 1.91 (m), 1.25 (m) | 19.4 |
| 1′ | | 171.0 | - | 171.4 |
| 2′ | 2.01 (3H, s) | 21.3 | 2.04 (3H, s) | 21.5 |

The HR-EI-MS spectrum (Figure 100) of compound **VII** displayed [M+H]$^+$ at m/z 464.3328. A molecular formula was assigned and calculated as $C_{32}H_{53}ONa$ (464.3994). Thus, compound **VII** was elucidated as lupeol acetate. The structure of compound **VII** is shown in Figure 30.

**Figure 29** HMBC correlations of compound **VII**
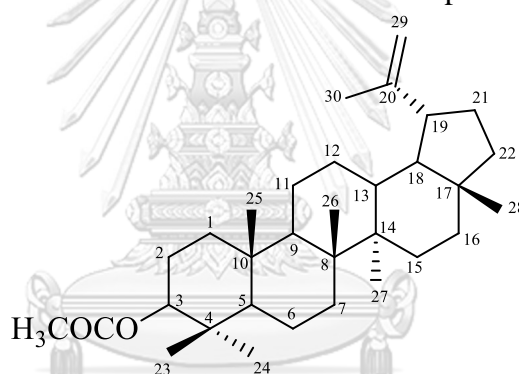


**Figure 30** The structure of compound **VII**

Lupeol acetate was previously isolated from *M. zapota* barks and showed cytotoxic activity against the human Caucasian prostate adenocarcinoma with $IC_{50}$ value of $61.2 \pm 0.7$ μg/mL [94]. Lupeol acetate was isolated from *Diospuros rubra* stem and showed antimicrobial activity against *Corynebacterium diphtheriae* (NCTC 10356) with minimum inhibitory concentrations (MIC) value of 64 μg/mL [130].

The investigation of the chemical constituents of *M. zapota* using tyrosinase activity guided fractionation was performed. It was found that three kinds of isolated compounds **I-VII** were distinguished as triterpenoid, flavonoid and phenolic compounds. Triterpenoid consisted of taraxerol methyl ether (**I**), taraxerol (**V**) and

taraxerone (**VI**). Flavonoid had two compounds as 6-hydroxyflavanone (**II**) and (+)-dihydrokaempferol (**III**). 3,4-dihydroxy benzoic acid (**IV**) was a phenolic compound.

4.8 Antityrosinase activities of isolated compounds I-VII

Tyrosinase inhibitory activity is the assay for skin whitening agent. The tyrosinase inhibitory activity of compounds **I-VII** was evaluated. Kojic acid and arbutin were used as positive controls. (+)-Dihydrokaempferol (**III**) showed the strongest tyrosinase inhibitory activity on monophenolase inhibitory activity with $IC_{50}$ value of $32.17 \pm 0.32$ μM and compared with the positive standard, kojic acid ($IC_{50}$ $40.21 \pm 0.63$ μM) and arbutin ($IC_{50}$ $116.30 \pm 0.45$ μM). 6-Hydroxyflavanone (**II**) and kojic acid exhibited similar inhibition of monophenolase activity with $IC_{50}$ values of $41.76 \pm 0.20$ and $40.21 \pm 0.63$ μM, respectively. While, 6-hydroxyflavanone (**II**) exhibited higher antityrosinase activity than arbutin ($IC_{50}$ $116.30 \pm 0.45$ μM). 3,4-Dihydroxybenzoic acid (**IV**), taraxerone (**VI**) and lupeol acetate (**VII**) displayed moderate monophenolase inhibitory activity with $IC_{50}$ values of $55.21 \pm 0.70$, $70.63 \pm 0.36$ and $71.17 \pm 0.33$ μM, respectively, when compared with kojic acid ($IC_{50}$ $40.21 \pm 0.63$ μM). Taraxerol methyl ether (**I**) and taraxerol (**V**) showed weak monophenolase inhibitory activity with $IC_{50}$ values of *106.53 ± 0.34 and* $103.37 \pm 0.22$ μM, respectively.

For diphenolase inhibitory activity, (+)-dihydrokaempferol (**III**) and kojic acid displayed similar antityrosinase activity with $IC_{50}$ values of $31.60 \pm 0.73$ and $30.07 \pm 0.32$ μM, respectively, while (+)-dihydrokaempferol (**III**) showed stronger against tyrosinase activity than the other compounds and arbutin ($IC_{50}$ $370.65 \pm 0.90$ μM). *6-*Hydroxyflavanone (**II**), 3,4-dihydroxybenzoic acid (**IV**), taraxerone (**VI**) and lupeol aceate (**VII**) showed moderate antityrosinase activity with $IC_{50}$ values of $63.10 \pm 0.73$, $43.91 \pm 0.21$, $90.60 \pm 0.26$ and $87.50 \pm 0.63$ μM, respectively. Taraxerol methyl ether (**I**) and taraxerol (**V**) exhibited weak diphenolase inhibitory activity with $IC_{50}$ values of *283.33 ± 0.59 and* $272.10 \pm 0.16$ μM, respectively. The $IC_{50}$ values of compounds **I-VII** for antityrosinase activity are shown in Table 4.43.

These results showed that (+)-dihydrokaempferol (**III**) showed more antityrosinase activity on diphenolase inhibitory activity with $IC_{50}$ value of $31.60 \pm 0.73$ μM than previous study by Hou *et al* [36]. Hou *et al* reported that flavanone, dihydrokaempferol showed diphenolase inhibitory activity with $IC_{50}$ value of *2.7 ± 0.7*

mM [36]. Flavanone, artocarpanone showed diphenolase inhibitory activity with $IC_{50}$ value of 147.55 µM [25]. The structure of artocarpanone had methoxy group that substituted at 7 position and three hydroxy groups at 5, 2′ and 4′ positions. It is suggested that methoxy group and the number of hydroxy group affected tyrosinase inhibitory activity.

**Table 52** The $IC_{50}$ values of compounds **I-VII** for antityrosinase activities

| Compound | $IC_{50}$ (µM) | |
|---|---|---|
| | Monophenolase inhibitory activity | Diphenolase inhibitory activity |
| Taraxerol methyl ether (**I**) | $106.53 \pm 0.34$ | $283.33 \pm 0.59$ |
| 6-Hydroxyflavanone (**II**) | $41.76 \pm 0.20$ | $63.10 \pm 0.73$ |
| (+)-Dihydrokaempferol (**III**) | $32.17 \pm 0.32$ | $31.60 \pm 0.73$ |
| 3,4-Dihydroxybenzoic acid (**IV**) | $55.21 \pm 0.70$ | $43.91 \pm 0.21$ |
| Taraxerol (**V**) | $103.37 \pm 0.22$ | $272.10 \pm 0.16$ |
| Taraxerone (**VI**) | $70.63 \pm 0.36$ | $90.60 \pm 0.26$ |
| Lupeol acetate (**VII**) | $71.17 \pm 0.33$ | $87.50 \pm 0.63$ |
| Kojic acid[a] | $40.21 \pm 0.63$ | $30.07 \pm 0.32$ |
| Arbutin[a] | $116.30 \pm 0.45$ | $370.65 \pm 0.90$ |

[a] Kojic acid and arbutin were used as positive controls.

4.9 Determination of antioxidant activities of isolated compounds I-VII

Antioxidant activities of isolated compounds **I-VII** were determined on DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP activity (Table 54).

4.9.1 DPPH radical scavenging activity

(+)-Dihydrokaempferol (**III**) showed the strongest scavenging DPPH capacity with $IC_{50}$ value of $2.21 \pm 0.77$ µM, followed by 6-hydroxyflavanone (**II**) and 3,4-dihydroxybenzoic acid (**IV**) with $IC_{50}$ values of $3.21 \pm 0.70$ and $4.71 \pm 0.10$ µM, respectively. While, taraxerol methyl ether (**I**), taraxerol (**V**), taraxerone (**VI**) and lupeol acetate (**VII**) displayed weak DPPH radical scavenging with $IC_{50}$ values of $77.31 \pm 0.60$, $16.28 \pm 0.33$, $10.20 \pm 0.40$ and $87.10 \pm 0.31$ µM, respectively. Trolox showed a significant free radical scavenging DPPH capacity with $IC_{50}$ value of $1.92 \pm 0.22$ µM.

4.9.2 ABTS radical scavenging activity

(+)-Dihydrokaempferol (**III**) displayed the highest free radical scavenging ABTS activity with $IC_{50}$ value of $214.83 \pm 0.51$ µM. 6-Hydroxyflavanone (**II**) exhibited stronger ABTS radical scavenging activity than 3,4-dihydroxybenzoic acid (**IV**) with $IC_{50}$ values of $225.53 \pm 0.95$ and $290.14 \pm 0.95$ µM, respectively. Whereas, taraxerol methyl ether (**I**), taraxerol (**V**), taraxerone (**VI**) and lupeol acetate (**VII**) showed low ABTS radical scavenging activity with $IC_{50}$ values of $520.22 \pm 0.30$, $630.84 \pm 0.54$, $334.83 \pm 0.99$ and $669.62 \pm 0.42$ µM, respectively. Trolox exhibited strong ABTS radical scavenging activity with $IC_{50}$ value of $188.39 \pm 0.43$ µM.

4.9.3 FRAP activity

(+)-Dihydrokaempferol (**III**) showed the highest reducing capacity with FRAP value of $6.23 \pm 0.10$ µM, followed by 6-hydroxyflavanone (**II**) and 3,4-dihydroxybenzoic acid (**IV**) with FRAP values of $4.12 \pm 0.12$ and $3.00 \pm 0.40$ µM, respectively. However, taraxerol methyl ether (**I**), taraxerol (**V**), taraxerone (**VI**) and lupeol acetate (**VII**) exhibited weak reducing capacity with FRAP values of $1.31 \pm 0.16$, $1.46 \pm 0.11$, $1.12 \pm 0.13$, and $1.28 \pm 0.30$ µM, respectively. Trolox exhibited the greatest reducing activity with FRAP value of $6.10 \pm 0.28$ µM.

**Table 53** Antioxidant activities of isolated compounds **I**-**VII**

| Compound | IC$_{50}$ (µM) | | FRAP (µM) |
| --- | --- | --- | --- |
| | DPPH | ABTS | |
| Taraxerol methyl ether (**I**) | $77.31 \pm 0.60$ | $520.22 \pm 0.30$ | $1.31 \pm 0.16$ |
| 6-Hydroxyflavanone (**II**) | $3.21 \pm 0.70$ | $225.53 \pm 0.95$ | $4.12 \pm 0.12$ |
| (+)-Dihydrokaempferol (**III**) | $2.21 \pm 0.77$ | $214.83 \pm 0.51$ | $6.23 \pm 0.10$ |
| 3,4-Dihydroxybenzoic acid (**IV**) | $4.71 \pm 0.10$ | $290.14 \pm 0.95$ | $3.00 \pm 0.40$ |
| Taraxerol (**V**) | $16.28 \pm 0.33$ | $630.84 \pm 0.54$ | $1.46 \pm 0.11$ |
| Taraxerone (**VI**) | $10.20 \pm 0.40$ | $334.83 \pm 0.99$ | $1.12 \pm 0.13$ |
| Lupeol acetate (**VII**) | $87.10 \pm 0.31$ | $669.62 \pm 0.42$ | $1.28 \pm 0.30$ |
| Trolox[a] | $1.92 \pm 0.22$ | $188.39 \pm 0.43$ | $6.10 \pm 0.28$ |

[a] Trolox was used as positive control.

From this study, 6-hydroxyflavanone (**II**), (+)-dihydrokaempferol (**III**) and 3,4-dihydroxybenzoic acid (**IV**) showed potent antioxidant activity. 6-Hydroxyflavanone (**II**) showed antioxidant activity on lipid peroxidation with $IC_{50}$ value of 33 µM [131]. (+)-Dihydrokaempferol (**III**) showed antioxidant activity with DPPH and ABTS

radical scavenging activities with $IC_{50}$ values of $24.6 \pm 0.41$ and $25.4 \pm 0.68$ μg/mL, respectively [132]. 3,4-dihydroxybenzoic acid (**IV**) showed antioxidant activity with DPPH radical scavenging activities with $EC_{50}$ value of 0.093 μM [126]. These results supported that phenolic compounds had property as electron donating agents or hydrogen donating agents. Ability to donate electrons of phenolic compounds depends on hydroxyl substitution.

4.10 Determination of cytotoxicity of isolated compounds I-VII

Many phytochemicals have been developed for anticancer drugs. However, mortality rate of cancer patients was increased. Compounds **I**-**VII** were evaluated for cytotoxicity against five human cancer cell lines including breast (BT474), lung (ChaGo-K-1), liver (HepG$_2$), stomach (KATO-III), colon (SW620) carcinoma cell lines using MTT assay. In addition, normal human diploid lung fibroblast (Wi-38) was used as the normal cell line. Doxorubicin was used as a positive control. The $IC_{50}$ values for inhibition of cytotoxicity are shown in Table 55.

Taraxerol methyl ether (**I**) and taraxerone (**VI**) displayed no cytotoxicity on tested carcinoma cell lines. 6-Hydroxyflavanone (**II**) exhibited cytotoxic activity against BT474, ChaGo-K-1, HepG$_2$, KATO-III and SW620 cell lines with $IC_{50}$ values of $86.16 \pm 0.45$, $57.73 \pm 1.08$, $65.76 \pm 2.37$, $88.78 \pm 3.70$ and $82.79 \pm 1.33$ μM, respectively. (+)-Dihydrokaempferol (**III**) showed cytotoxic activity against BT474, ChaGo-K-1, HepG$_2$, KATO-III and SW620 cell lines with $IC_{50}$ values of $11.66 \pm 0.42$, $12.32 \pm 0.73$, $13.67 \pm 0.38$, $39.79 \pm 0.38$ and $41.11 \pm 1.08$ μM, respectively. 3,4-Dihydroxybenzoic acid (**IV**) exhibited inhibitory effect on ChaGo-K-1 and BT474 cell lines with $IC_{50}$ values of $79.22 \pm 4.02$ and $85.21 \pm 3.96$ μM, respectively and displayed no cytotoxic activity against HepG$_2$, KATO-III and SW620 cell lines. *Taraxerol (V)* showed anticancer activity against BT474, ChaGo-K-1, HepG$_2$ and KATO-III cell lines with $IC_{50}$ values of $19.24 \pm 0.40$, $26.75 \pm 0.97$, $20.41 \pm 1.43$ and $26.49 \pm 0.57$ μM, respectively. In contrast, Toze *et al* reported that taraxerone (**VI**) showed cytotoxicity against the human Caucasian prostate adenocarcinoma cell line [93]. Lupeol acetate (**VII**) showed cytotoxic activity against BT474 cell line with $IC_{50}$ value of $60.20 \pm 0.90$ μM and showed weak anticancer activity against KATO-III, SW620 and ChaGo-K-1 cell lines. Taraxerol methyl ether (**I**) showed cytotoxic activity against HeLa, HL-60

and MCF-7 with IC$_{50}$ value > 50 μM [116]. 6-Hydroxyflavanone (**II**) was reported to show cytotoxic activity against HeLa and BT474 cell lines [120, 121]. This literature data supported that 6-hydroxyflavanone (**II**) showed anticancer activity against BT474 with IC$_{50}$ value of 184.95 ± 1.61 μM.

**Table 54** Cytotoxic activities of compounds **I**-**VII**

| Isolated compound | IC$_{50}$ of cell lines (μM) | | | | | |
|---|---|---|---|---|---|---|
| | BT474 | ChaGo-K-1 | HepG$_2$ | KATO-III | SW620 | Wi-38 |
| Taraxerol methyl ether (**I**) | 184.95 ± 1.61 | > 227.07 | > 227.07 | > 227.07 | > 227.07 | > 227.07 |
| 6-Hydroxyflavanone (**II**) | 86.16 ± 0.45 | 57.73 ± 1.08 | 65.76 ± 2.37 | 88.78 ± 3.70 | 82.79 ± 1.33 | > 416.22 |
| (+)-Dihydrokaempferol (**III**) | 11.66 ± 0.42 | 12.32 ± 0.73 | 13.67 ± 0.38 | 39.79 ± 0.38 | 41.11 ± 1.08 | > 346.92 |
| 3,4-Dihydroxybenzoic acid (**IV**) | 85.21 ± 3.96 | 79.22 ± 4.02 | 364.72 ± 2.27 | 507.53 ± 4.61 | 591.36 ± 0.71 | > 648.85 |
| Taraxerol (**V**) | 19.24 ± 0.40 | 26.75 ± 0.97 | 20.41 ± 1.43 | 26.49 ± 0.57 | > 234.34 | > 234.34 |
| Taraxerone (**VI**) | > 235.45 | > 235.45 | > 235.45 | > 235.45 | > 235.45 | > 235.45 |
| Lupeol acetate (**VII**) | 60.20 ± 0.90 | 199.87 ± 0.30 | >213.33 | 136.68 ± 0.66 | 182.67 ± 1.51 | > 213.33 |
| Doxorubicin[a] | 1.21 ± 0.20 | 1.58 ± 0.40 | 2.70 ± 0.83 | 1.78 ± 0.20 | 1.82 ± 0.39 | > 183.99 |

[a] Doxorubicin was used as a positive control.
BT474 = breast carcinoma cell line, ChaGo-K-1 = lung bronchus carcinoma cell line, HepG$_2$ = liver carcinoma cell line, KATO-III = gastric carcinoma cell line, SW620 = colon carcinoma cell line and Wi-38 = human diploid lung fibroblast.

# CHAPTER V
# CONCLUSION


The six different parts of *M. zapota* were extract with methanol and water for evaluation on correlation of total phenolic and total flavonoid contents with antioxidant and antityrosinase activities. Six different parts consisted of barks, flowers, fruits, leaves, roots and seeds. Methanol crude extract of flowers showed the highest amount of total phenolic content, followed by methanol crude extract of barks and methanol crude extract of seeds. Methanol crude extract of seeds showed the highest total flavonoid content, followed by methanol crude extract of roots and aqueous crude extract of seeds. Moreover, methanol crude extract of seeds showed potent antioxidant activities with DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP activity. The methanol crude extracts of barks, roots, leaves and aqueous crude extract of roots showed high monophenolase inhibitory activity. Methanol crude extracts of roots, flowers, leaves, barks and fruits displayed stronger diphenolase inhibitory activity than other crude extracts. Correlations between total phenolic content and antioxidant activities of crude extract of six different parts of *M. zapota* were high positive. Total phenolic content of crude extracts of different parts of *M. zapota* and ABTS radical scavenging activity showed high correlation (r = 0.90), followed by DPPH radical scavenging activity (r = 0.79) and FRAP assay (r = 0.69). These results indicated high total phenolic content of *M. zapota* revealed high radical scavenging activity and reducing capacity. While, no correlation between total phenolic content with antityrosinase activities and with total flavonoid content of crude extracts of different parts of *M. zapota*. A low correlations were found between total total flavonoid content of crude extracts of different parts of *M. zapota* and antioxidant activities with DPPH radical scavenging activity (r = 0.35), ABTS radical scavenging activity (r = 0.29) and FRAP assay (r = 0.39). Likewise, correlation between total flavonoid content with antityrosinase activities and correlation between antityrosinase and antioxidant activities were not correlated in crude extract of different parts of *M. zapota*.

In this study, methanol crude extract of roots, leaves and barks showed potent antityrosinase activity. Thus, tyrosinase inhibitors from *M. zapota* barks were isolated by activity-guided fractionation. *M. zapota* barks were extracted with *n*-hexane, ethyl acetate, methanol and water, respectively. *n*-Hexane, ethyl acetate and methanol crude extracts showed high tyrosinase inhibitory effect with percentage inhibition. They were conducted to isolate active compounds by bioassay-guided fractionation on tyrosinase inhibitory activity. Taraxerol methyl ether (**I**) was isolated from *n*-hexane crude extract of *M. zapota* barks. 6-Hydroxyflavanone (**II**) exhibited moderate antityrosinase activities on monophenolase and diphenolase inhibitory activities. (+)-dihydrokaempferol (**III**) showed potent against tyrosinase activities on both monophenolase and diphenolase inhibitory activities with $IC_{50}$ value of $32.17 \pm 0.32$ and $31.60 \pm 0.73$ μM, respectively. 3,4-dihydroxybenzoic acid (**IV**) displayed moderate antityrosinase activities on both monophenolase and diphenolase inhibitory activities. Taraxerol (**V**), taraxerone (**VI**) and lupeol acetate (**VII**) showed weak antityrosinase activities on monophenolase and diphenolase inhibitory activities. For antioxidant activities, taraxerol methyl ether (**I**), taraxerol (**V**), taraxerone (**VI**) and lupeol acetate (**VII**) exhibited weak antioxidant activities with DPPH radical scavenging activity, ABTS radical scavenging activity and FRAP capacity. 6-Hydroxyflavanone (**II**) and 3,4-dihydroxybenzoic acid (**IV**) showed moderate antioxidant activities on DPPH, ABTS and FRAP capacity assays. (+)-Dihydrokaempferol (**III**) displayed strong antioxidant activities with DPPH, ABTS radical scavenging activities with $IC_{50}$ values of $2.21 \pm 0.70$ and $225.53 \pm 0.95$ μM, respectively and showed high reducing capacity with FRAP value of $6.23 \pm 0.10$ μM. Comparison of isolated compounds **I-VII** for cytotoxic activity, taraxerol methyl ether (**I**) and taraxerone (**IV**) showed no cytotoxic activity against carcinoma cell lines. 6-Hydroxyflavanone (**II**), (+)-dihydrokaempferol (**III**) and 3,4-dihydroxybenzoic acid (**IV**) showed cytotoxic activity against BT474, ChaGo-K-1, HepG₂, KATO-III and SW620. Lupeol acetate (**VII**) displayed cytotoxic activity against gainst BT474 and ChaGo-K-1. From the results, (+)-dihydrokaempferol (**III**) could be attributed for active constituent in cosmetic ingredient.

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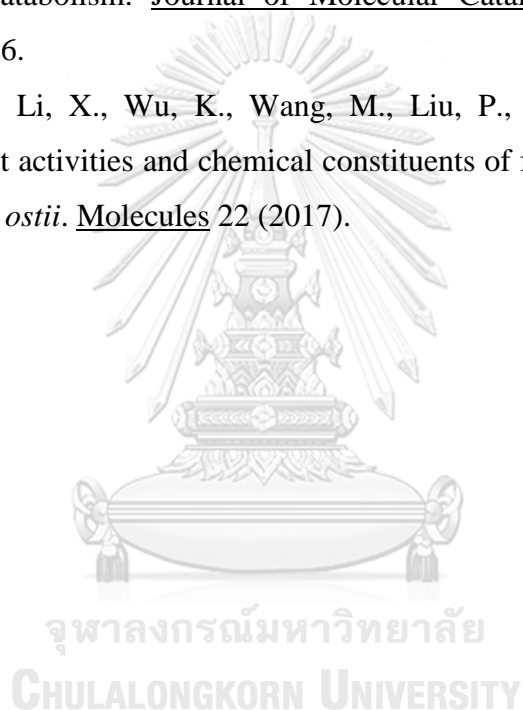
APPENDIX
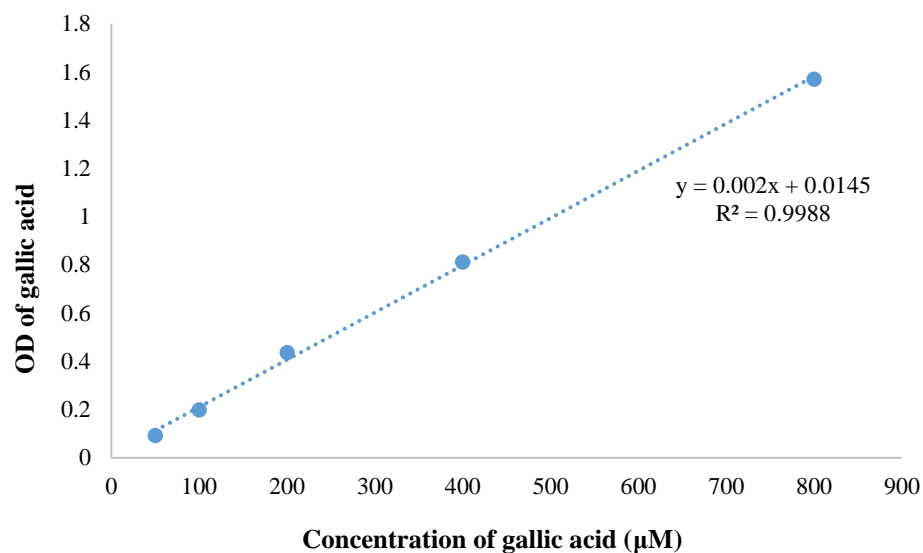
**Figure 31** Calibration curve of gallic acid for total phenolic content of crude extract of different parts of *M. zapota*
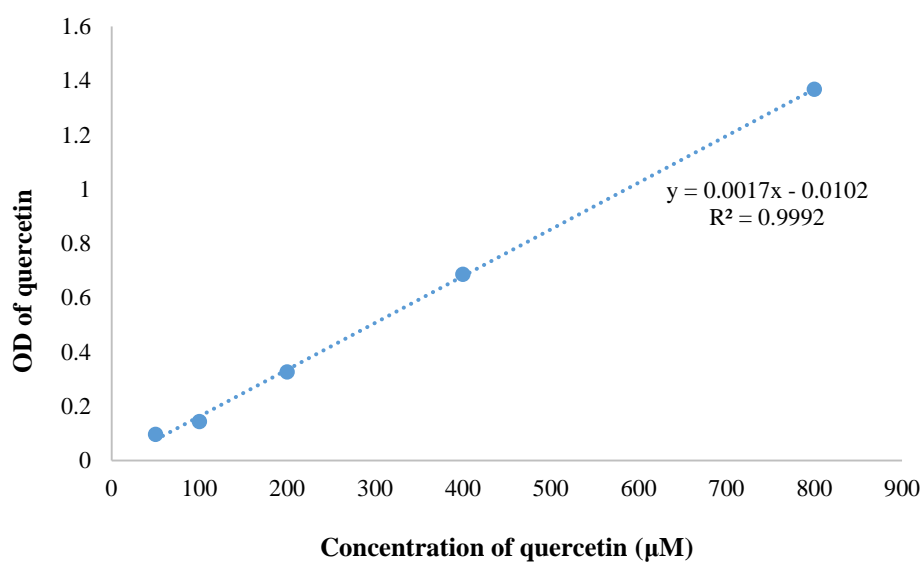


**Figure 32** Calibration curve of quercetin for total flavonoid content of crude extract of different parts of *M. zapota*
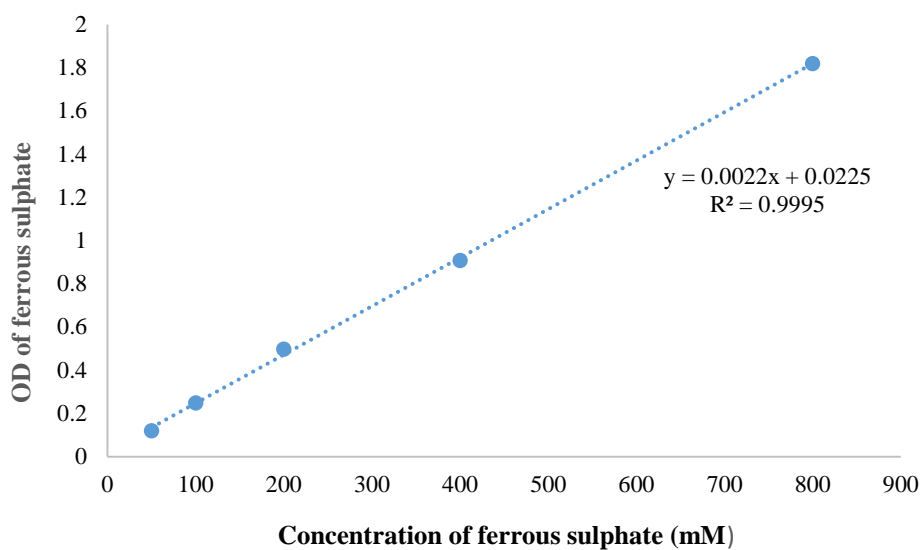
**Figure 33** Calibration curve of ferrous sulphate of crude extract of different parts of *M. zapota*
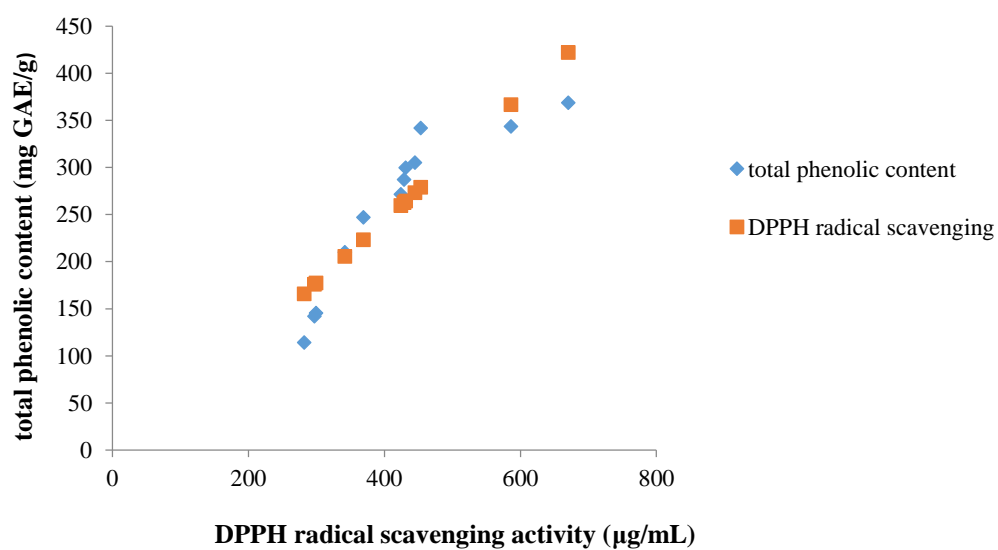


**Figure 34** Correlation between total phenolic content and DPPH radical scavenging activity of crude extract of different parts of *M. zapota*
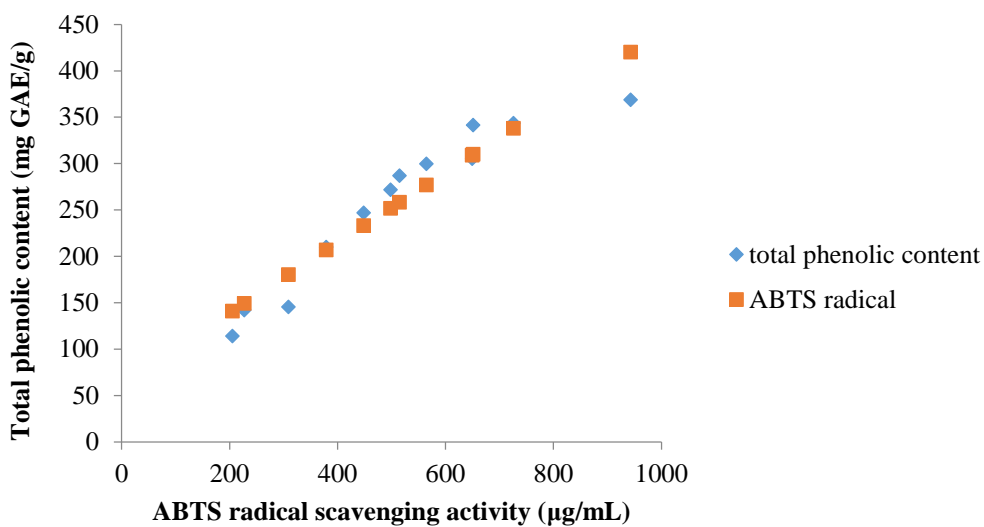
**Figure 35** Correlation between total phenolic content and ABTS radical scavenging activity of crude extract of different parts of *M. zapota*
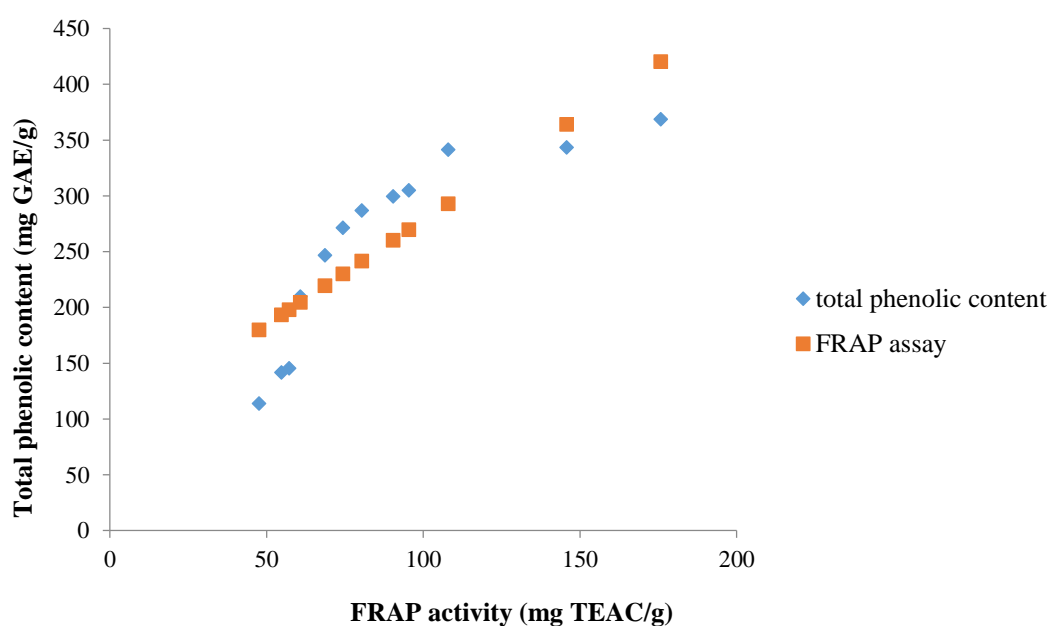


**Figure 36** Correlation between total phenolic content and FRAP activity of crude extract of different parts of *M. zapota*
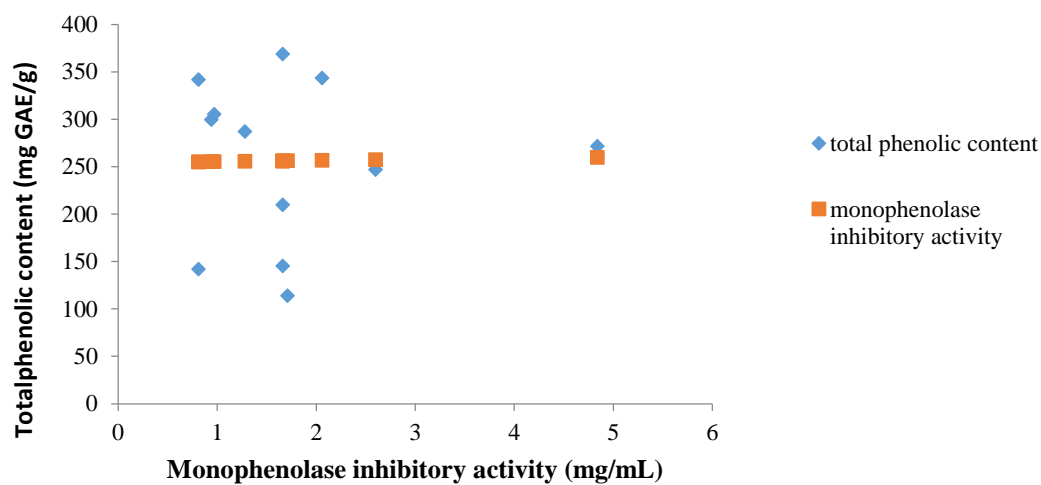
**Figure 37** Correlation between total phenolic content and monophenolase inhibitory activity of crude extract of different parts *of* M. zapota
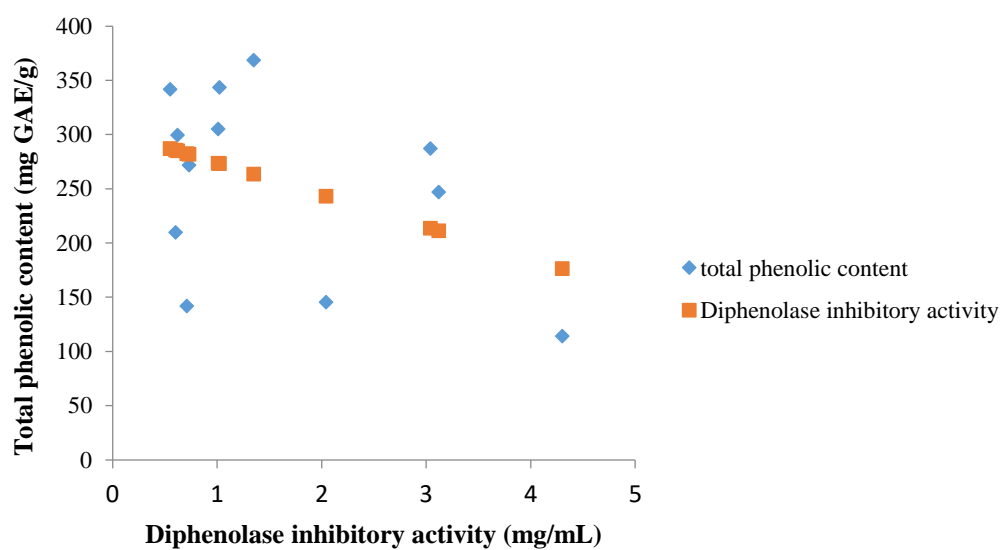


**Figure 38** Correlation between total phenolic content and diphenolase inhibitory activity of crude extract of different parts of *M. zapota*
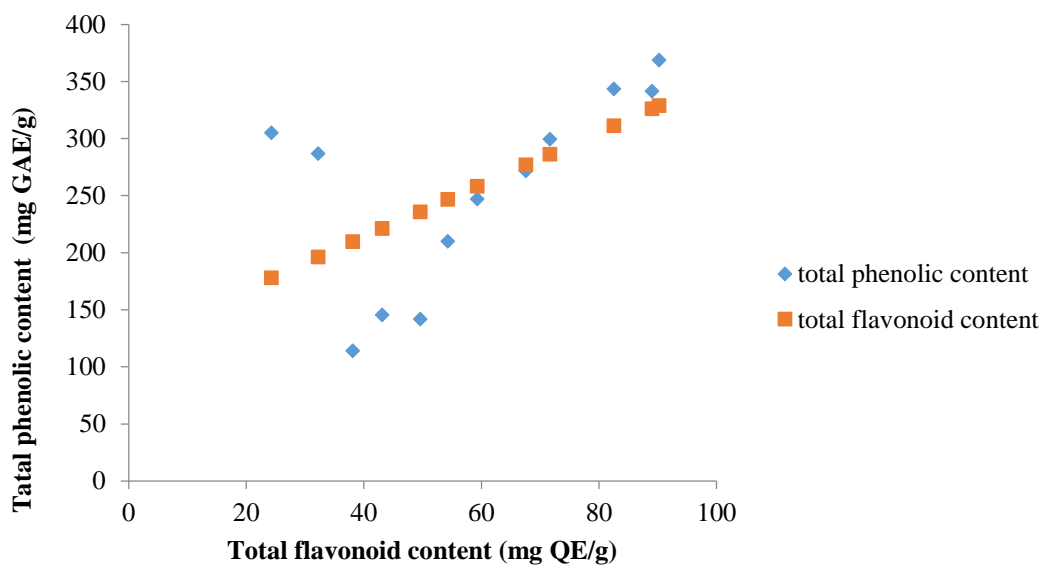
**Figure 39** Correlation between total phenolic content and total flavonoid content of crude extract of different parts of *M. zapota*
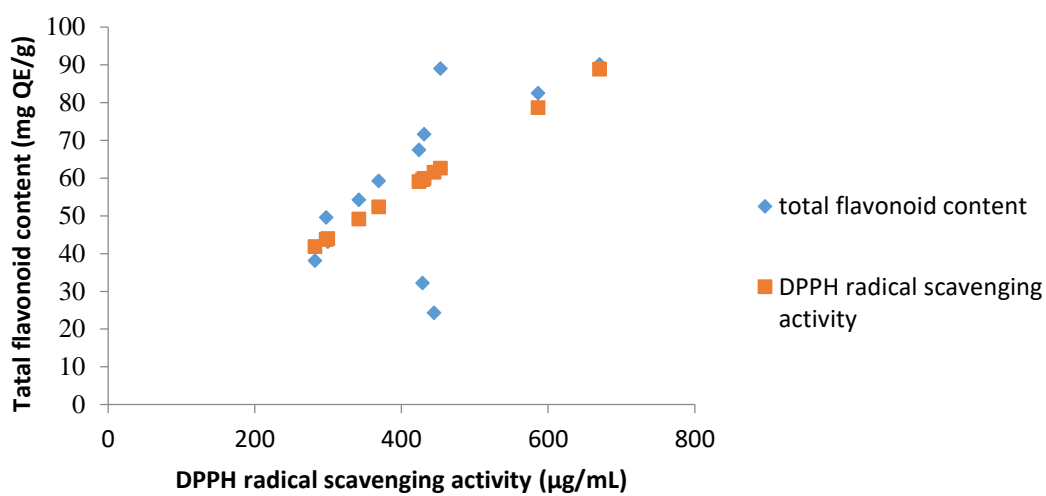


**Figure 40** Correlation between total flavonoid content and DPPH radical scavenging activity of crude extract of different parts of *M. zapota*
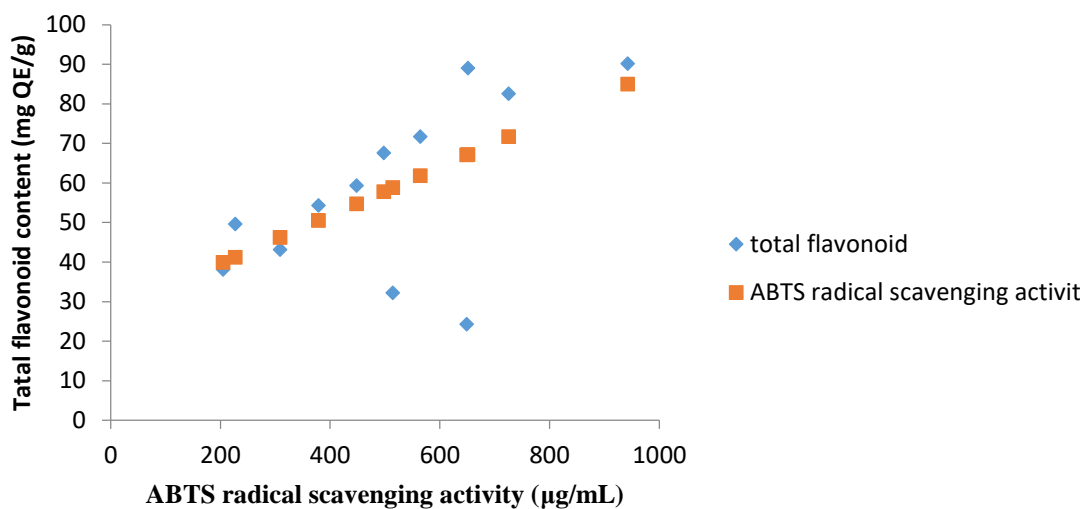
**Figure 41** Correlation between total flavonoid content and ABTS radical scavenging activity of crude extract of different parts of *M. zapota*
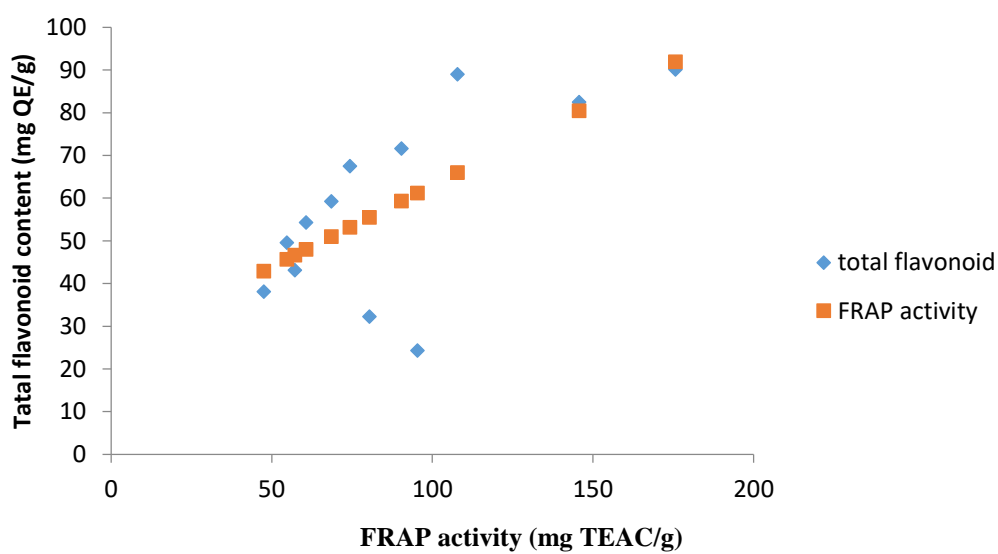


**Figure 42** Correlation between total flavonoid content and FRAP activity of crude extract of different parts of *M. zapota*
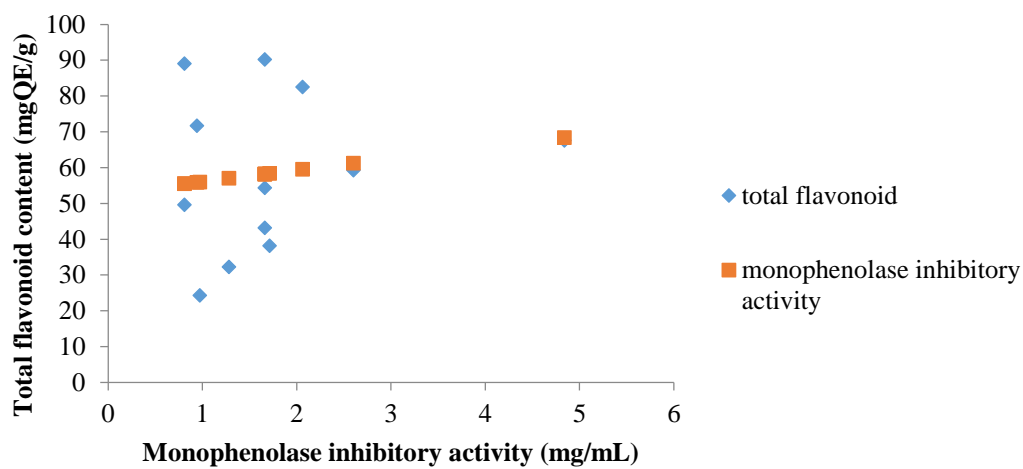
**Figure 43** *Correlation between total flavonoid content and monophenolase inhibitory activity of different parts of M. zapota*
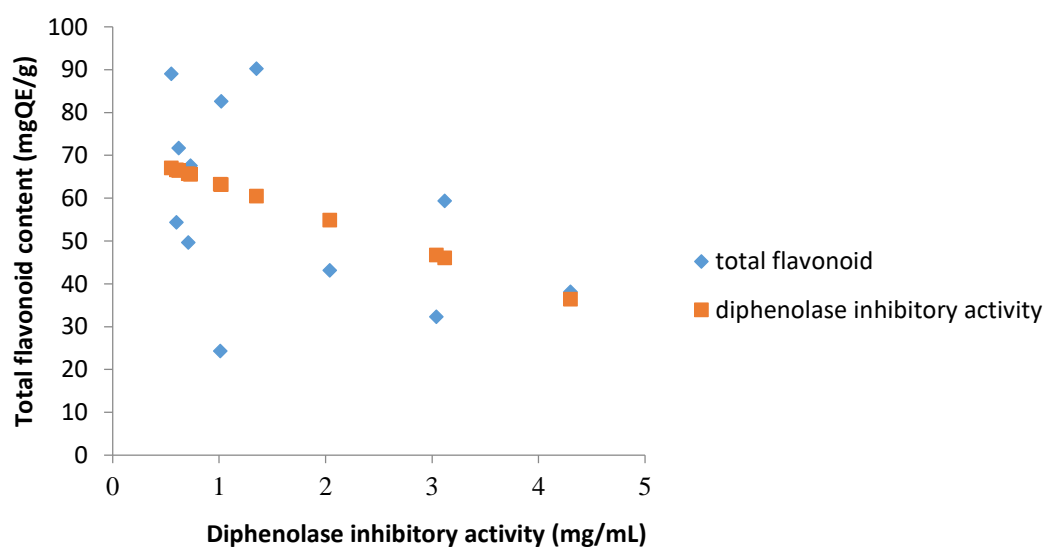


**Figure 44** Correlation between total flavonoid content and diphenolase inhibitory activity of different parts of *M. zapota*
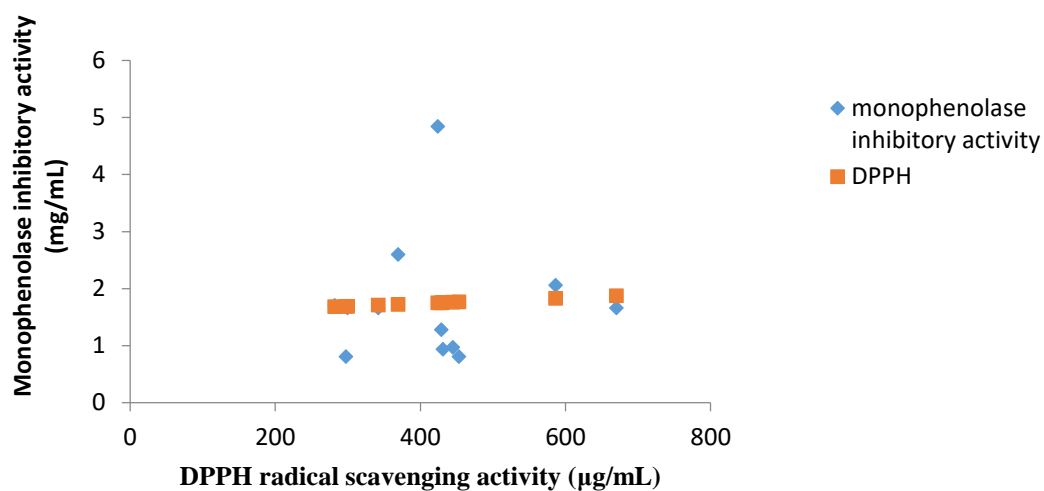
**Figure 45** Correlation between monophenolase inhibitory activity and DPPH radical
scavenging activity of different parts of *M. zapota*
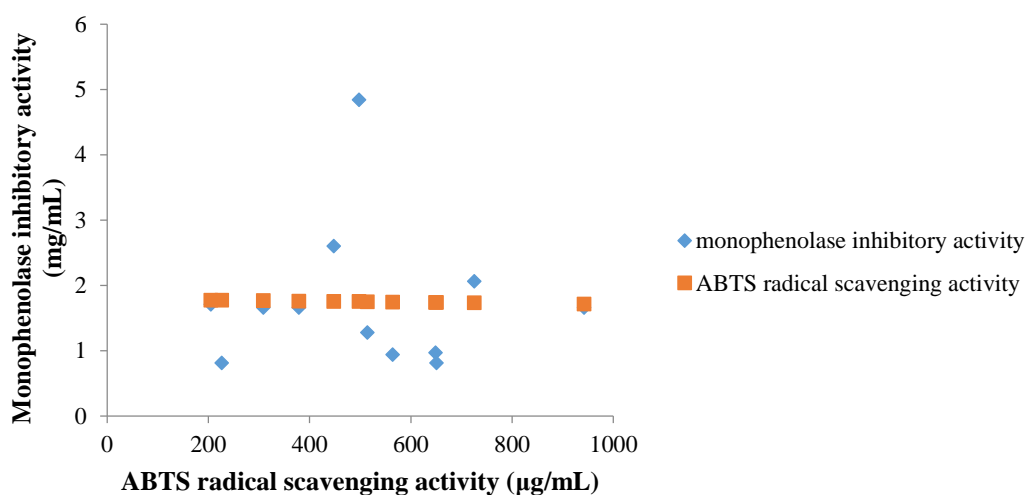


**Figure 46** Correlation between monophenolase inhibitory activity and ABTS radica*l*
scavenging activity of different parts of *M. zapota*
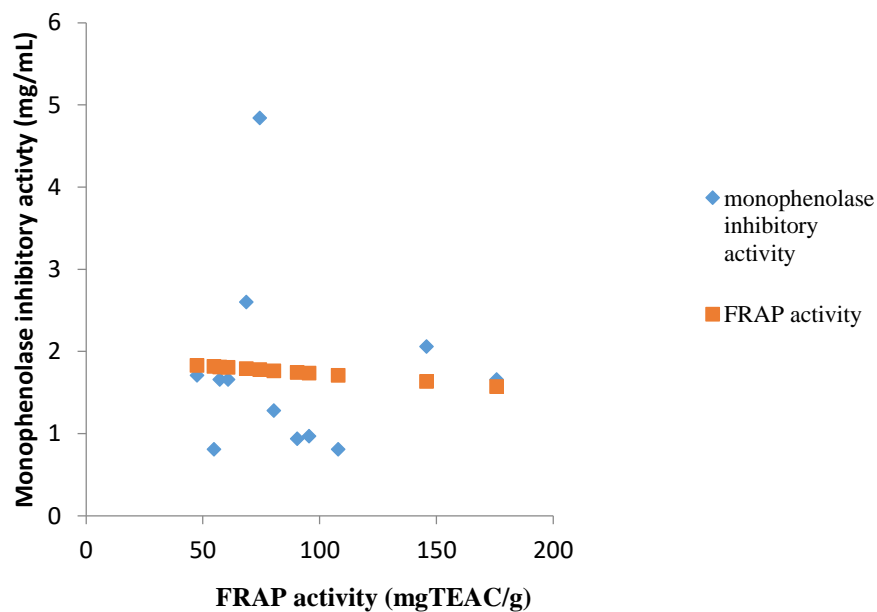
**Figure 47** Correlation between monophenolase inhibitory activity and FRAP activity
of different parts of *M. zapota*
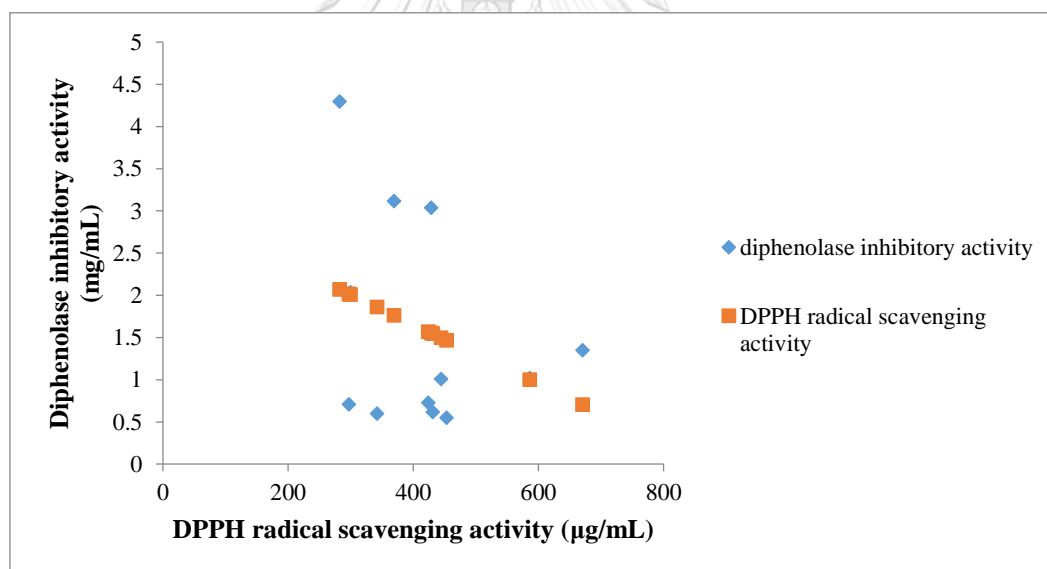


**Figure 48** Correlation between diphenolase inhibitory activity and DPPH radical
scavenging activity of different parts of *M. zapota*
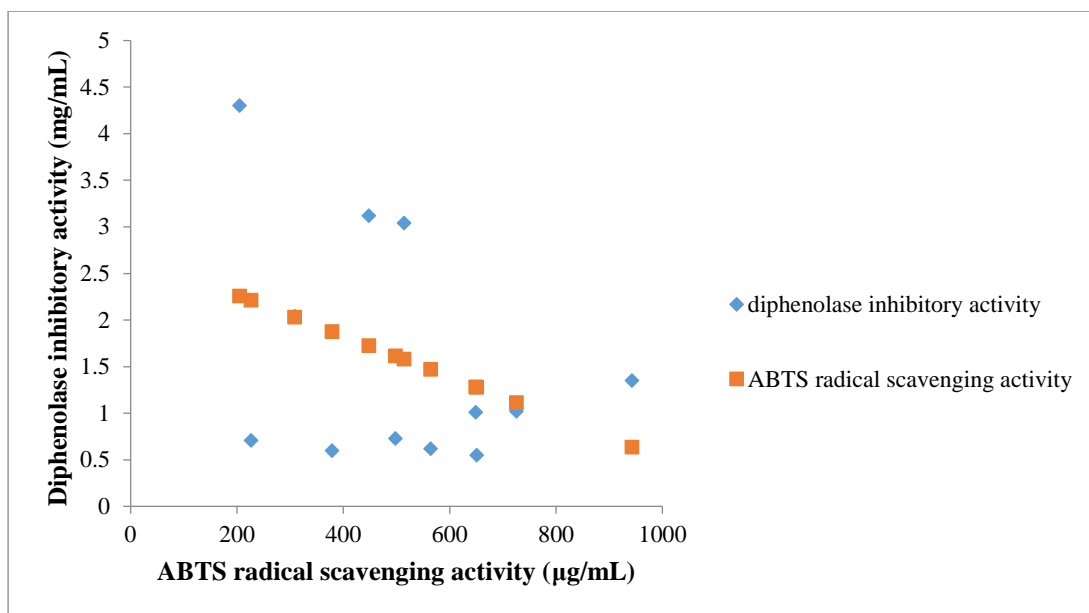
**Figure 49** Correlation between diphenolase inhibitory activity and ABTS radical scavenging activity of different parts of *M. zapota*
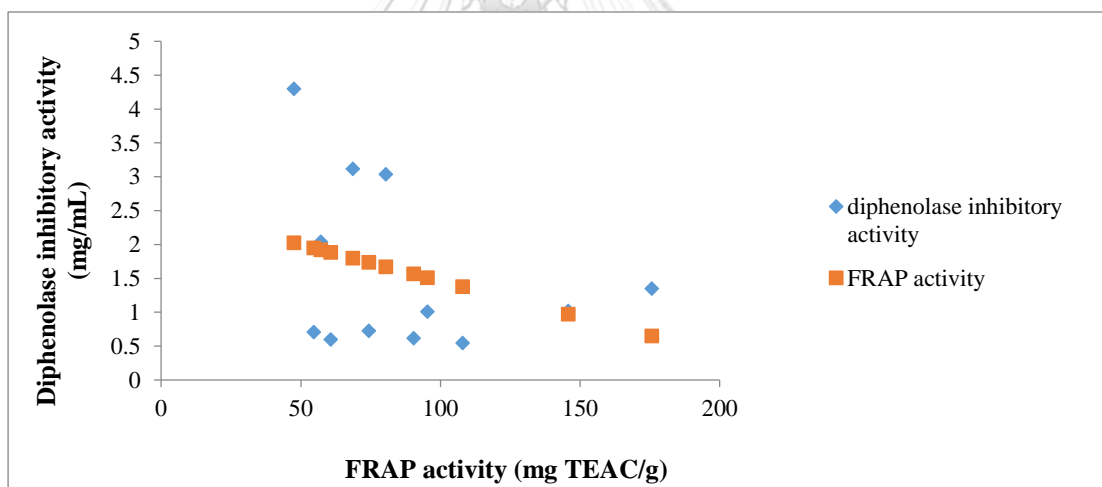


**Figure 50** Correlation between diphenolase inhibitory activity and FRAP activity of different parts of *M. zapota*
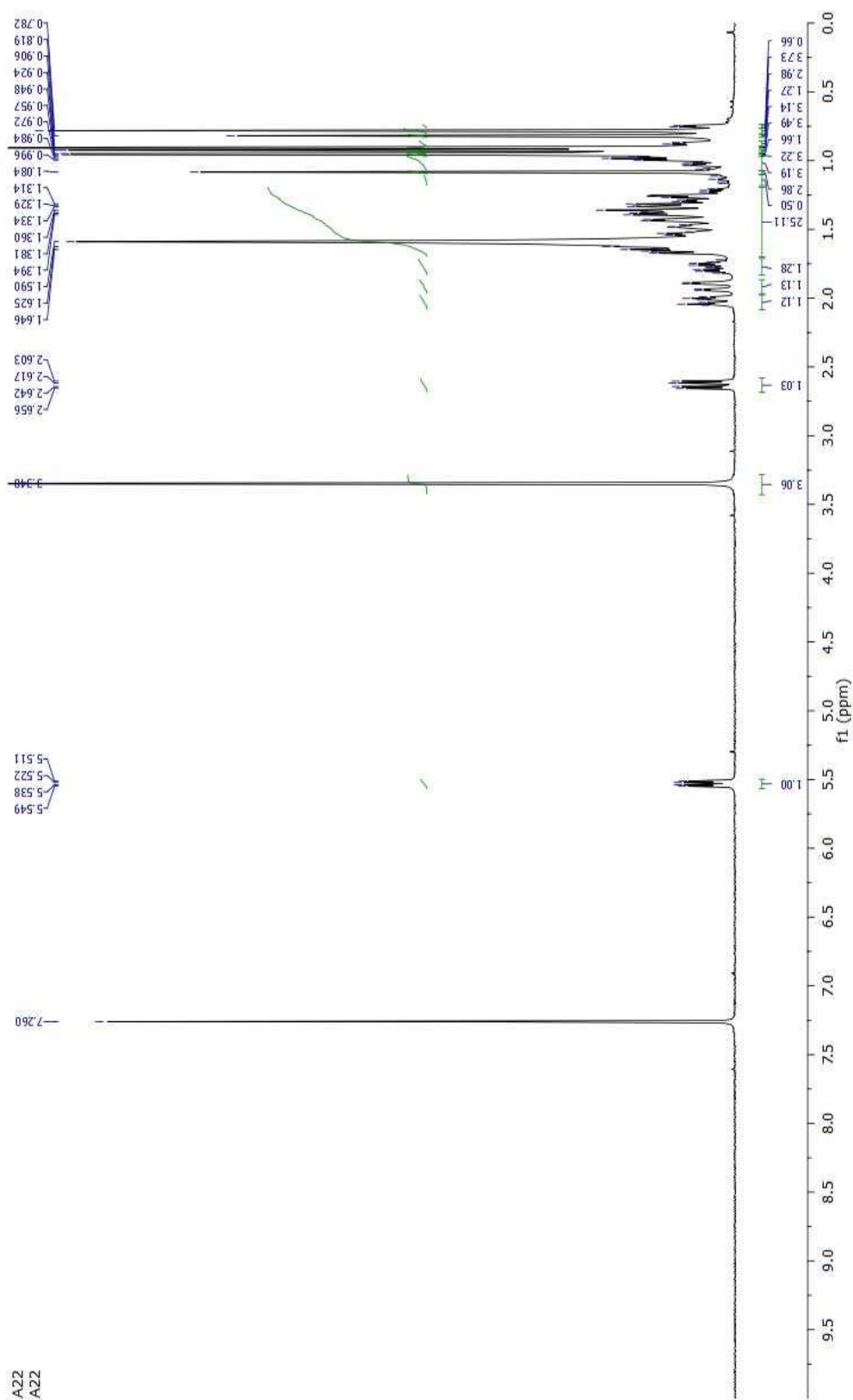
**Figure 51** The $^1$H-NMR spectrum of taraxerol methyl ether (**I**)
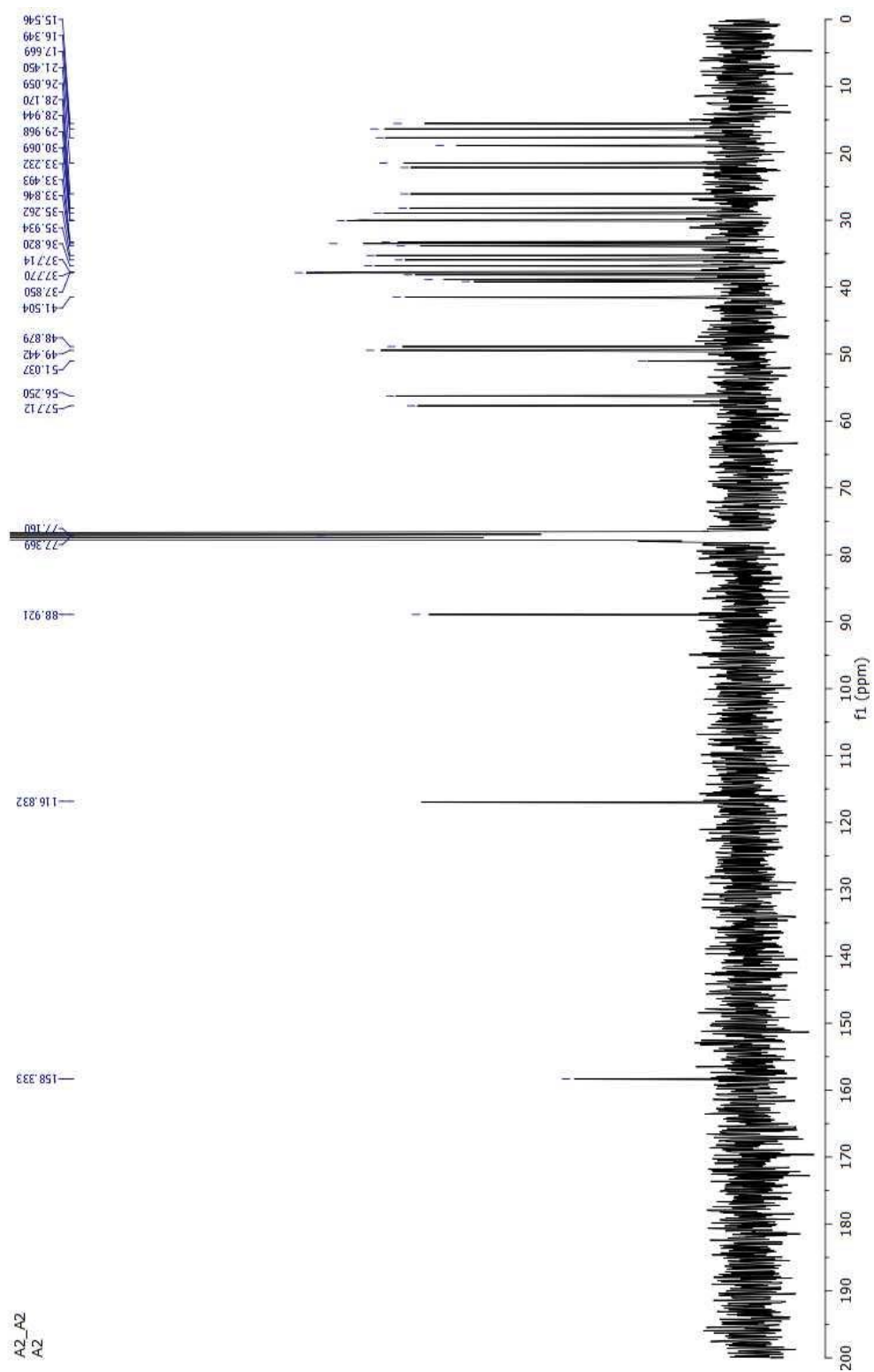
**Figure 52** The $^{13}$C-NMR spectrum of taraxerol methyl ether (**I**)
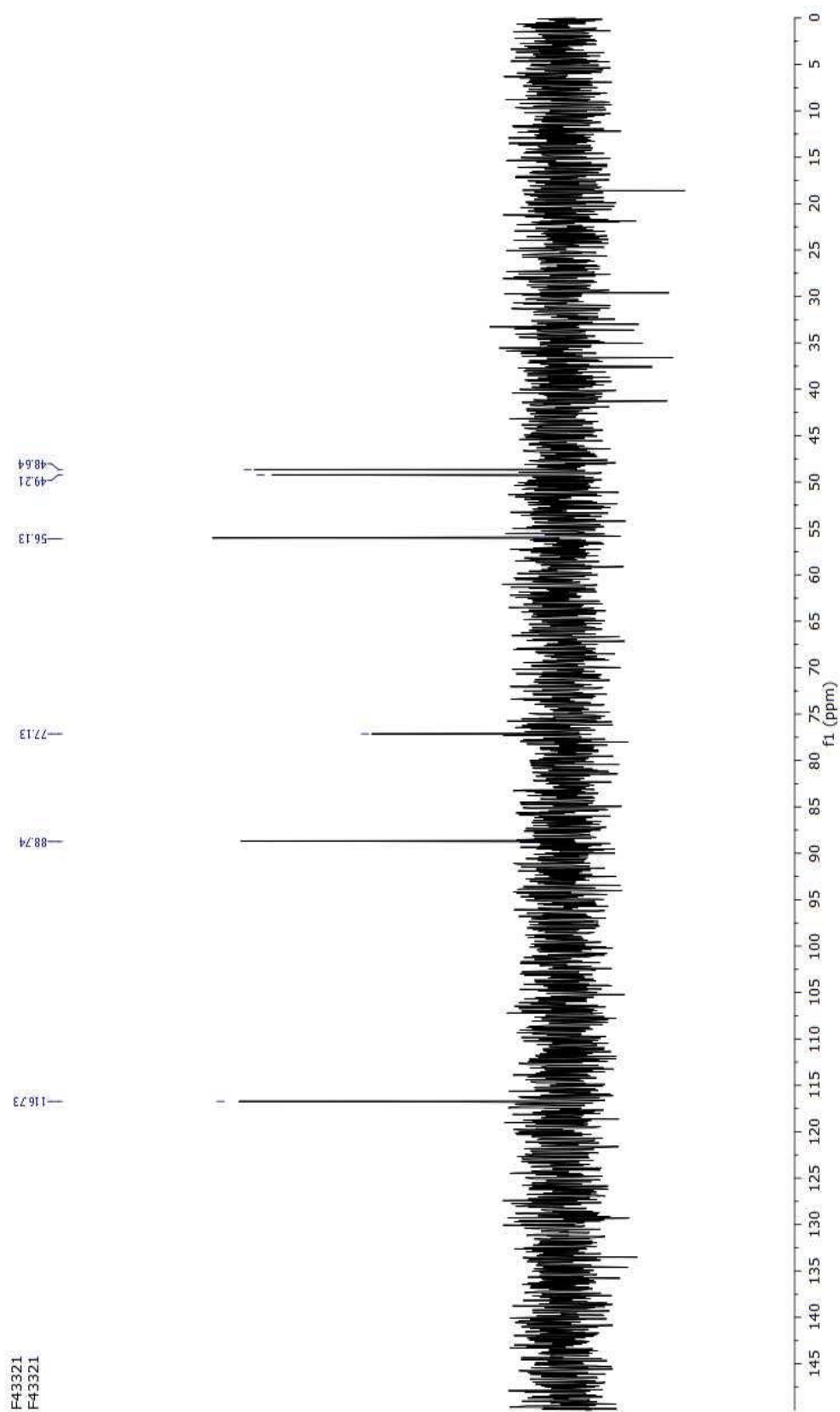
**Figure 53** The DEPT90 spectrum of taraxerol methyl ether (**I**)

15.32
16.13
17.44
18.60
21.23
21.88
25.83
27.95
29.63
29.74
29.84
33.01
33.27
33.62
35.03
36.59
37.55
41.27

48.65
49.21

56.08
57.48

77.13

88.67

116.73

F43321
F43321
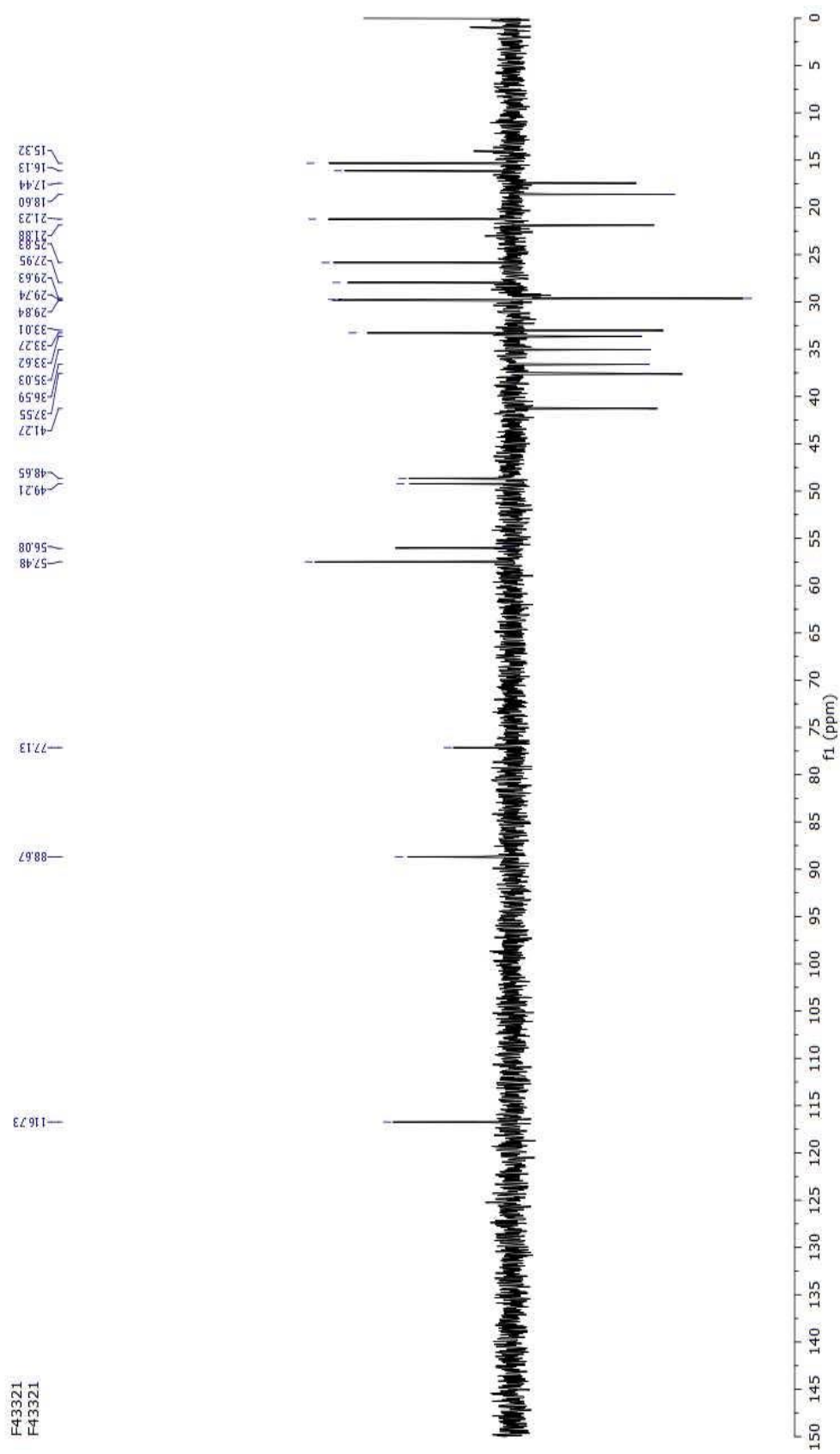
**Figure 54** The DEPT135 spectrum of taraxerol methyl ether (**I**)

f1 (ppm)
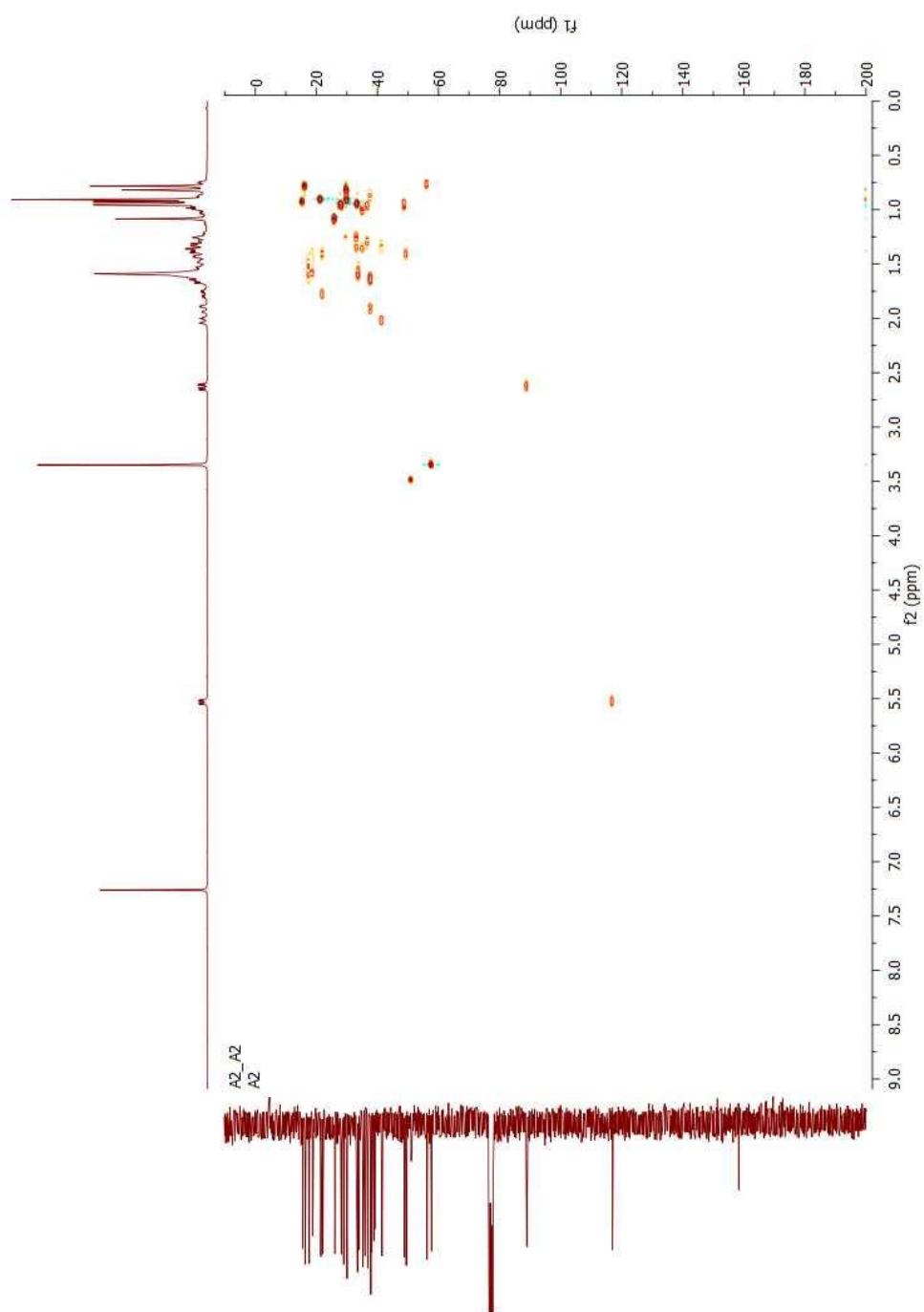
**Figure 55** The HSQC spectrum of taraxerol methyl ether (**I**)
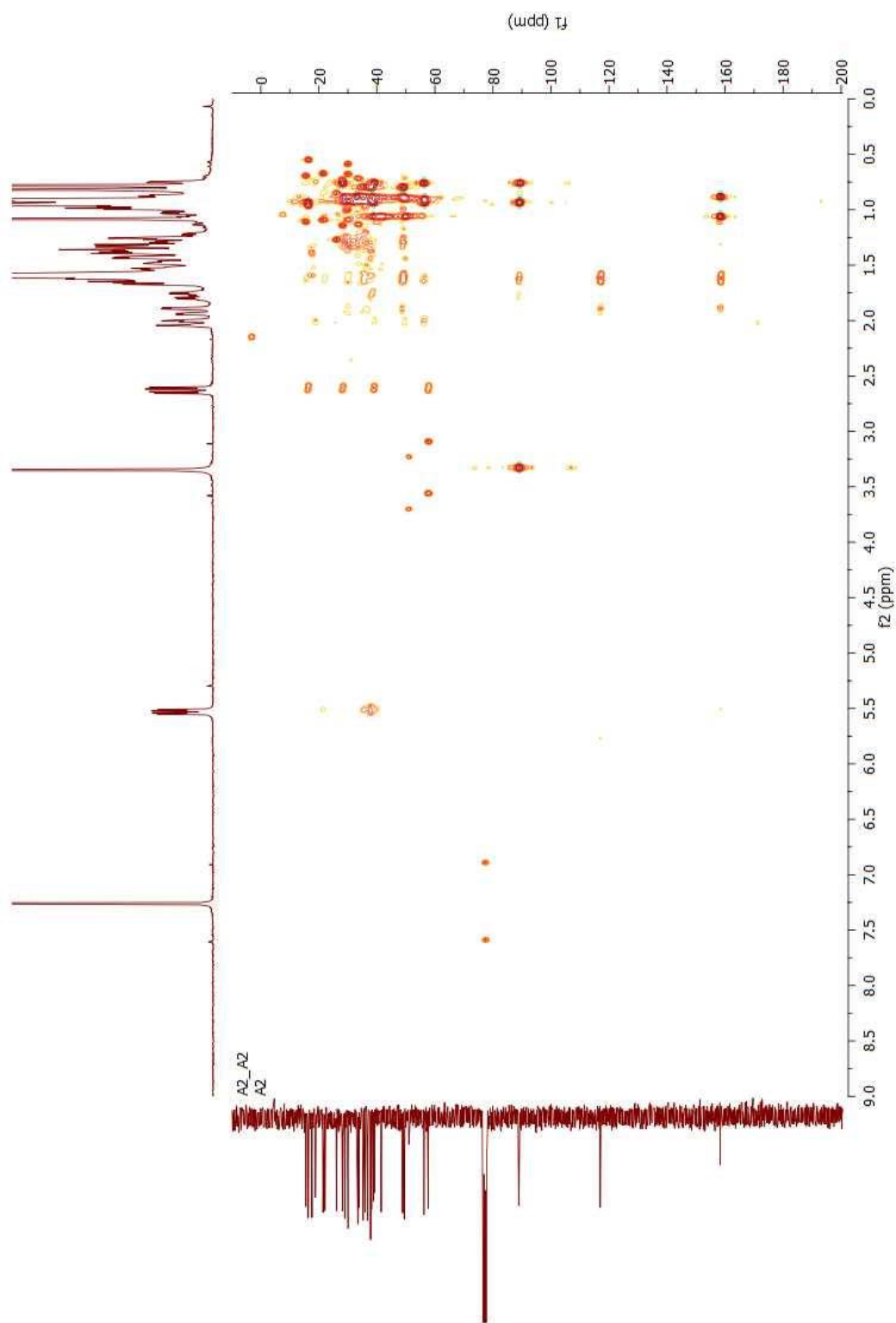
**Figure 56** The HMBC spectrum of taraxerol methyl ether (**I**)

**Figure 57** The COSY spectrum of taraxerol methyl ether (**I**)
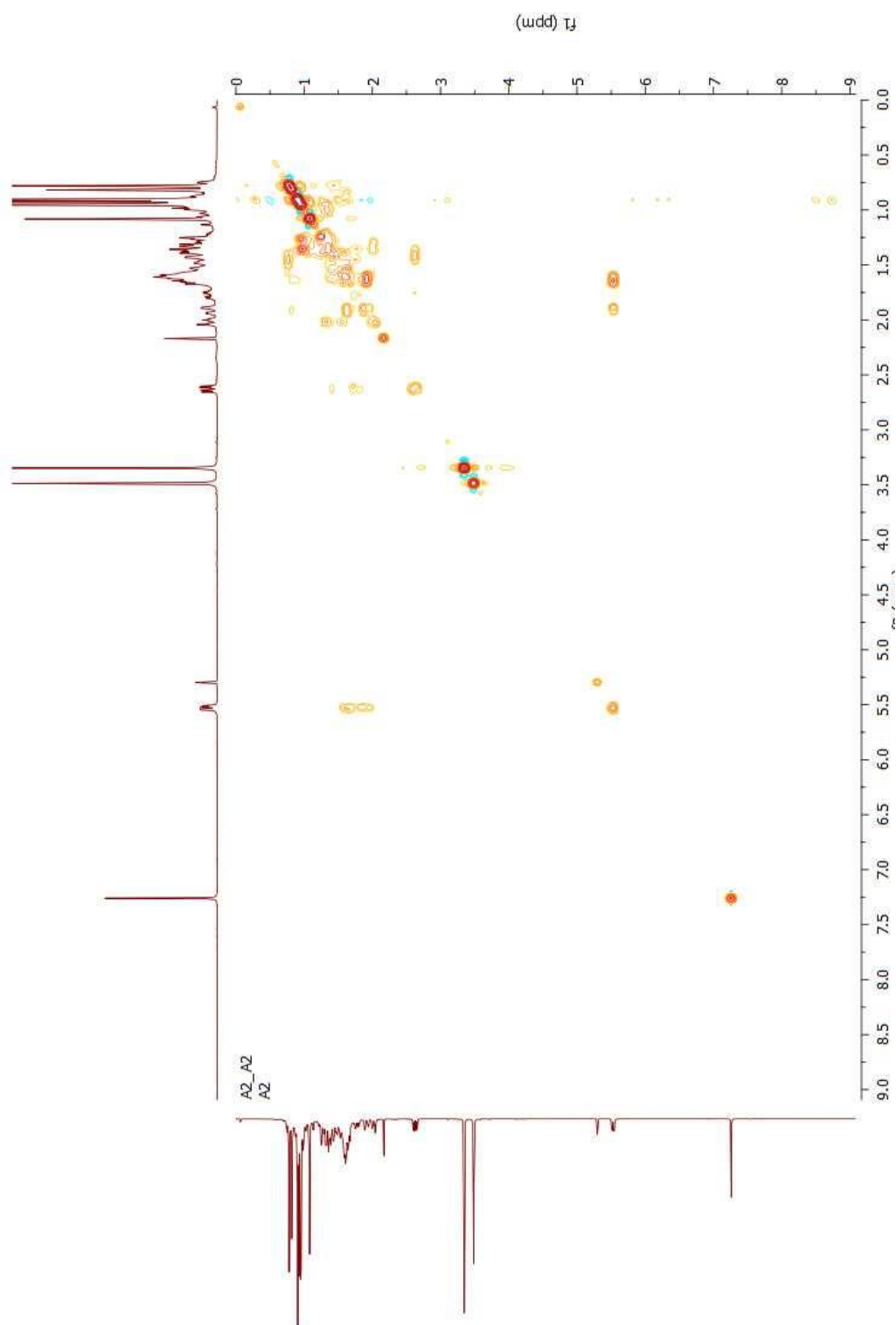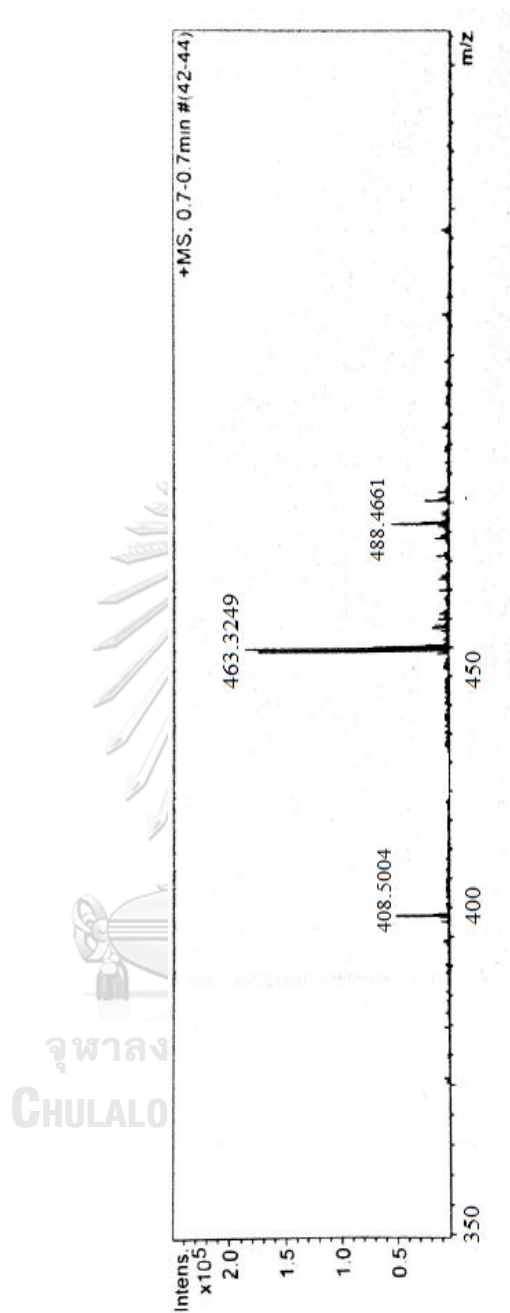
**Figure 58** The HR-EI-MS spectrum of taraxerol methyl ether (**I**)

**Figure 59** The $^1$H-NMR spectrum of 6-hydroxyflavanone (**II**)
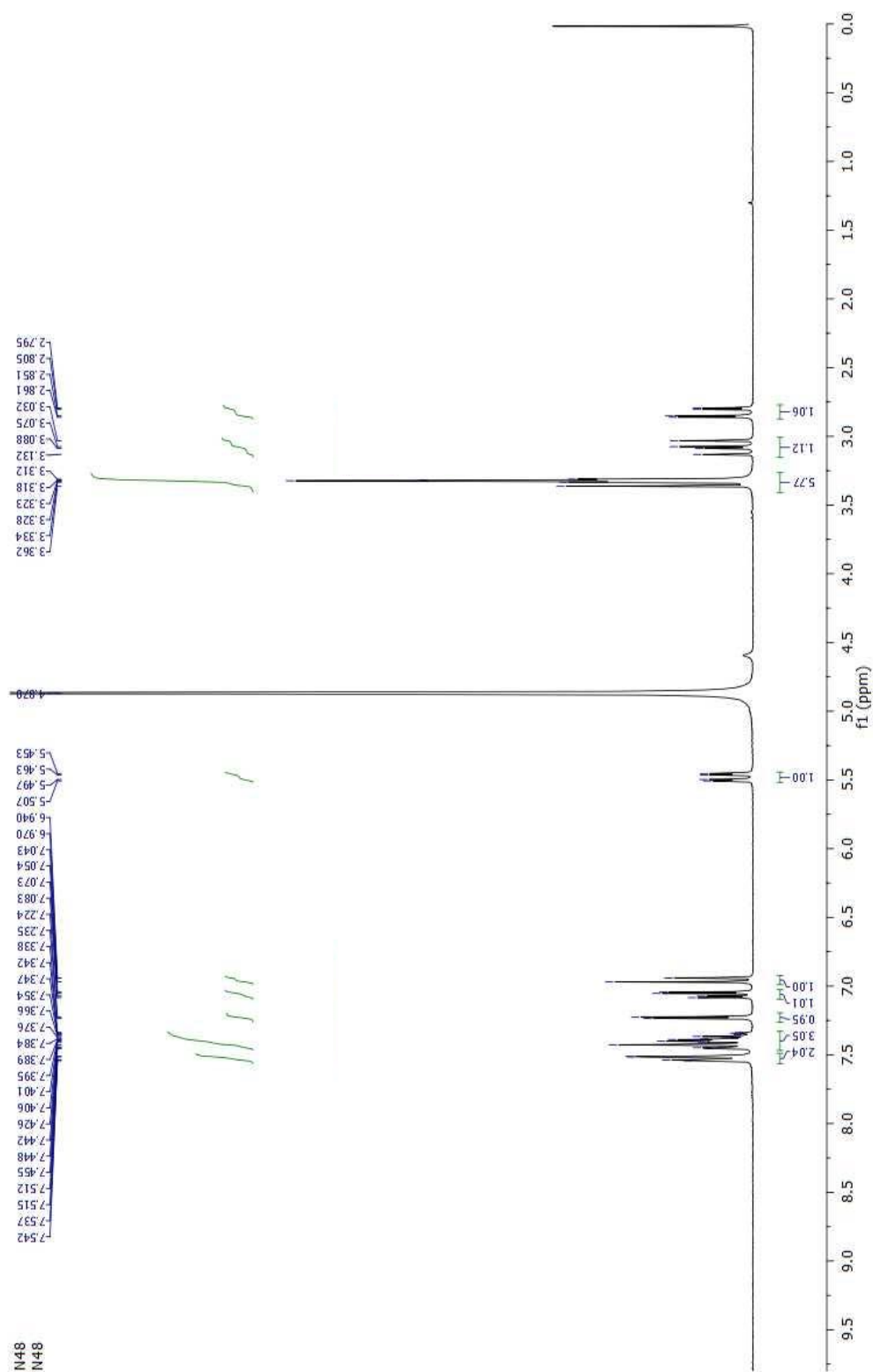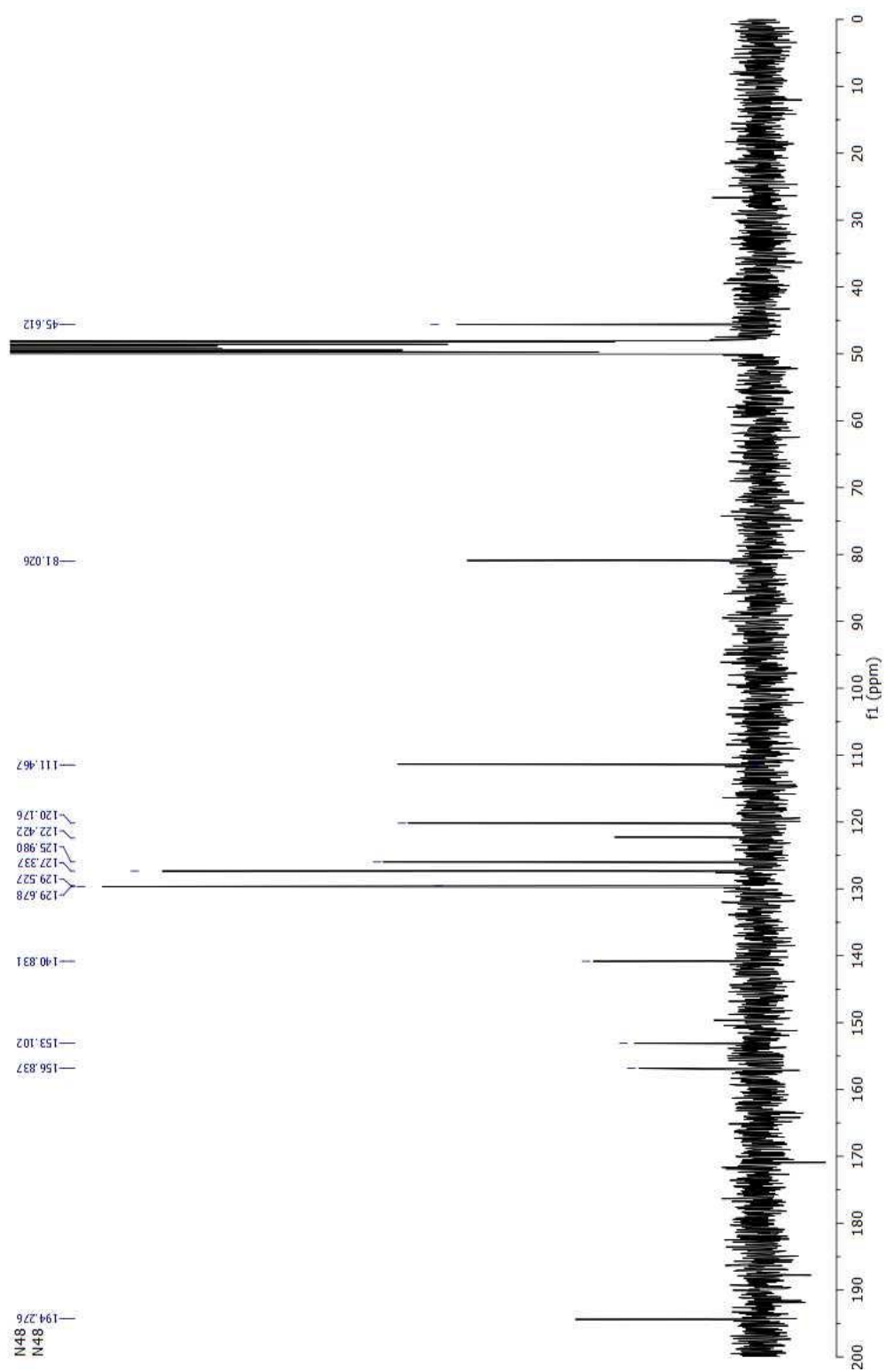
**Figure 60** The $^{13}$C-NMR spectrum of 6-hydroxyflavanone (**II**)
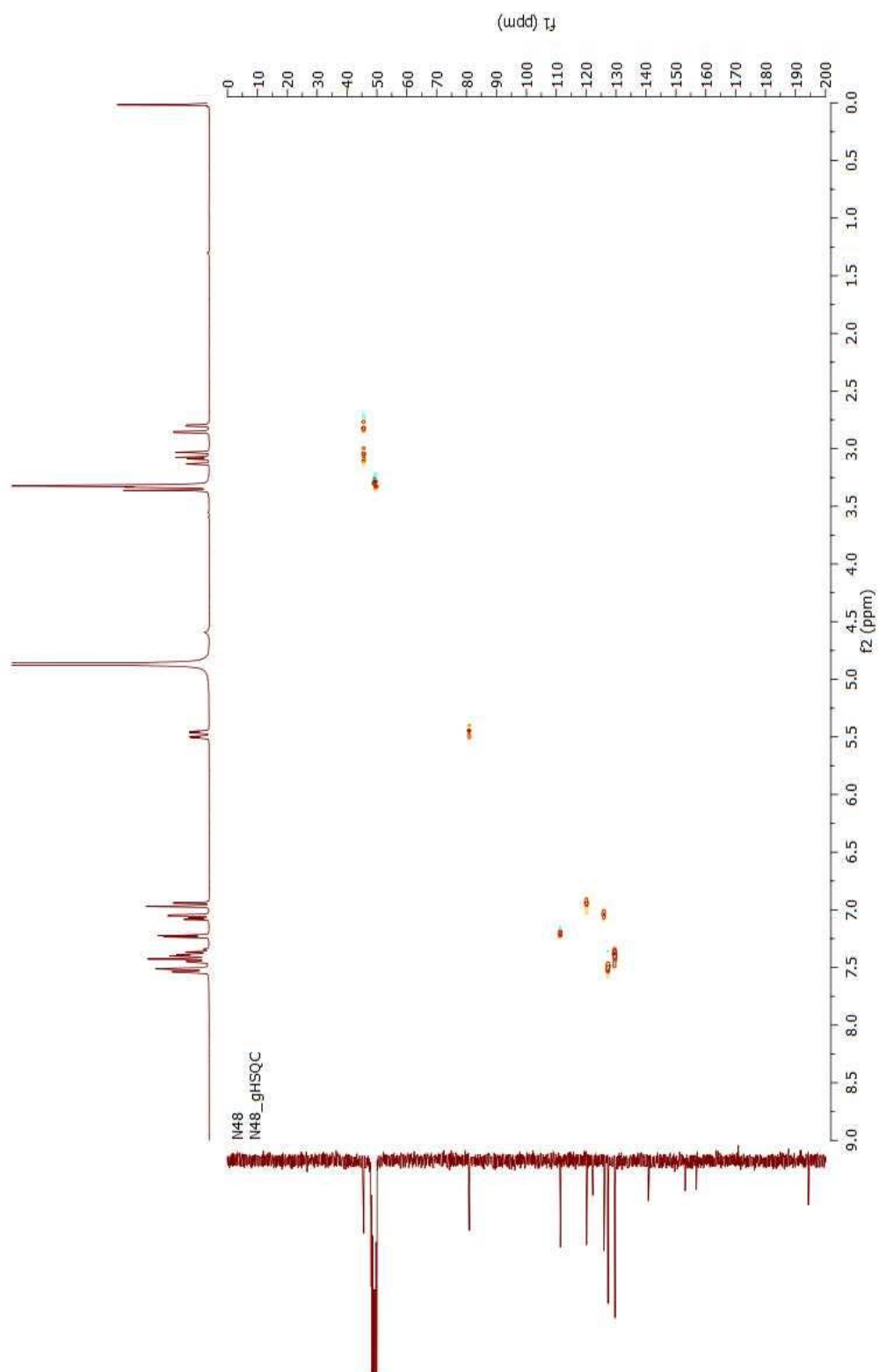
**Figure 61** The HSQC spectrum of 6-hydroxyflavanone (**II**)
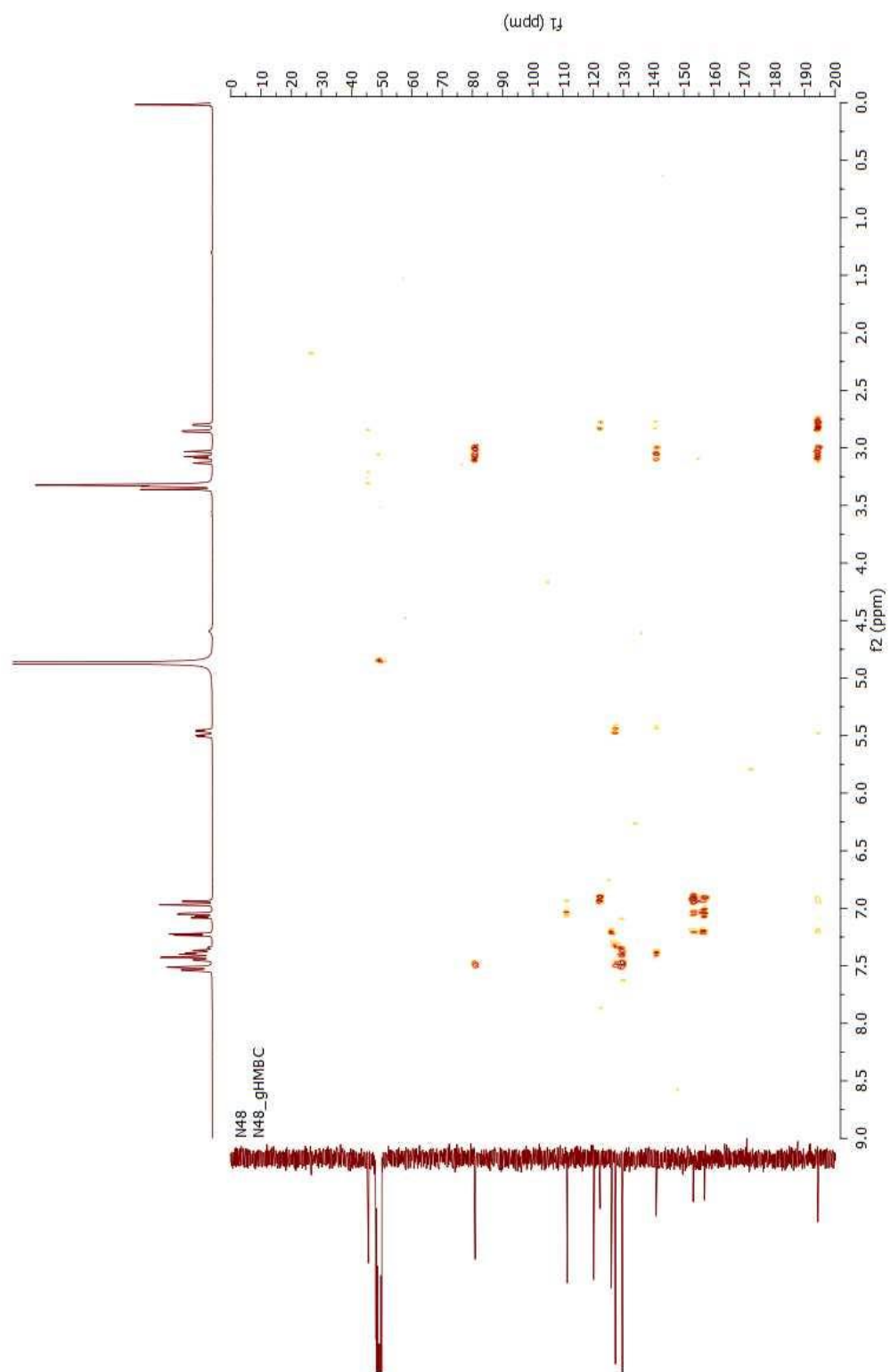
**Figure 62** The HMBC spectrum of 6-hydroxyflavanone (**II**)
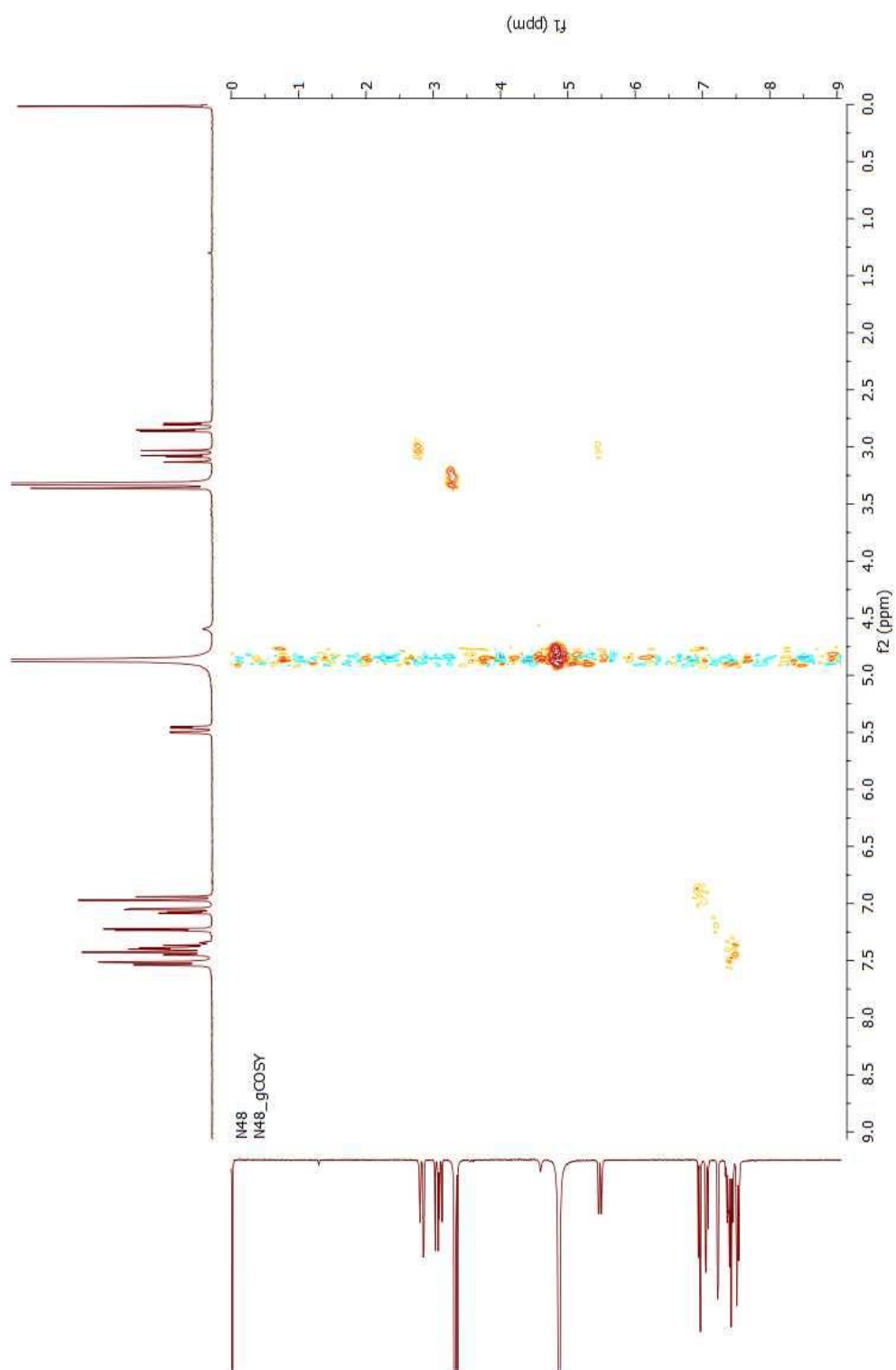
**Figure 63** The COSY spectrum of 6-hydroxyflavanone (**II**)
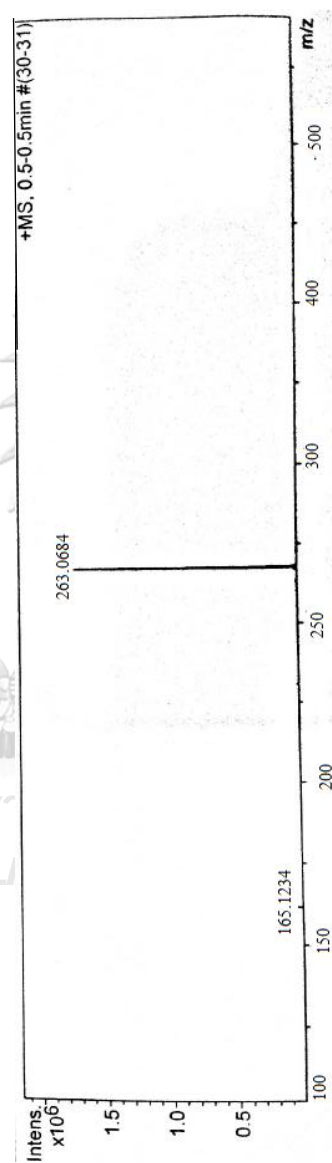
**Figure 64** The HR-EI-MS spectrum of 6-hydroxyflavanone (**II**)
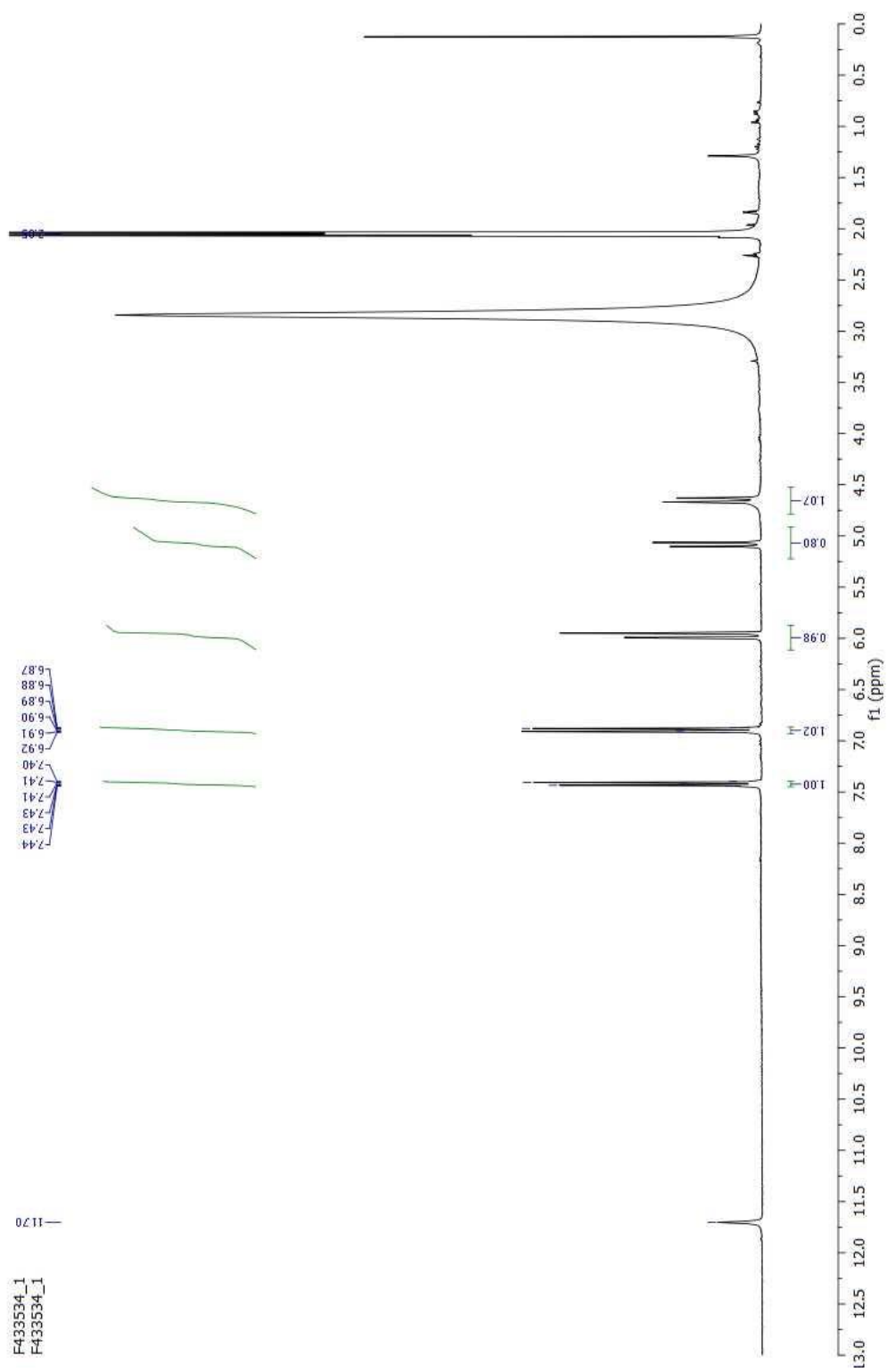
**Figure 65** The ${}^1$H-NMR spectrum of (+)-dihydrokeampferol (**III**)

206.26
197.99

167.61
164.71
163.90
158.58

130.03
128.82

115.66

101.37
96.84
95.78

84.07

72.84

29.85

N87_1
N87
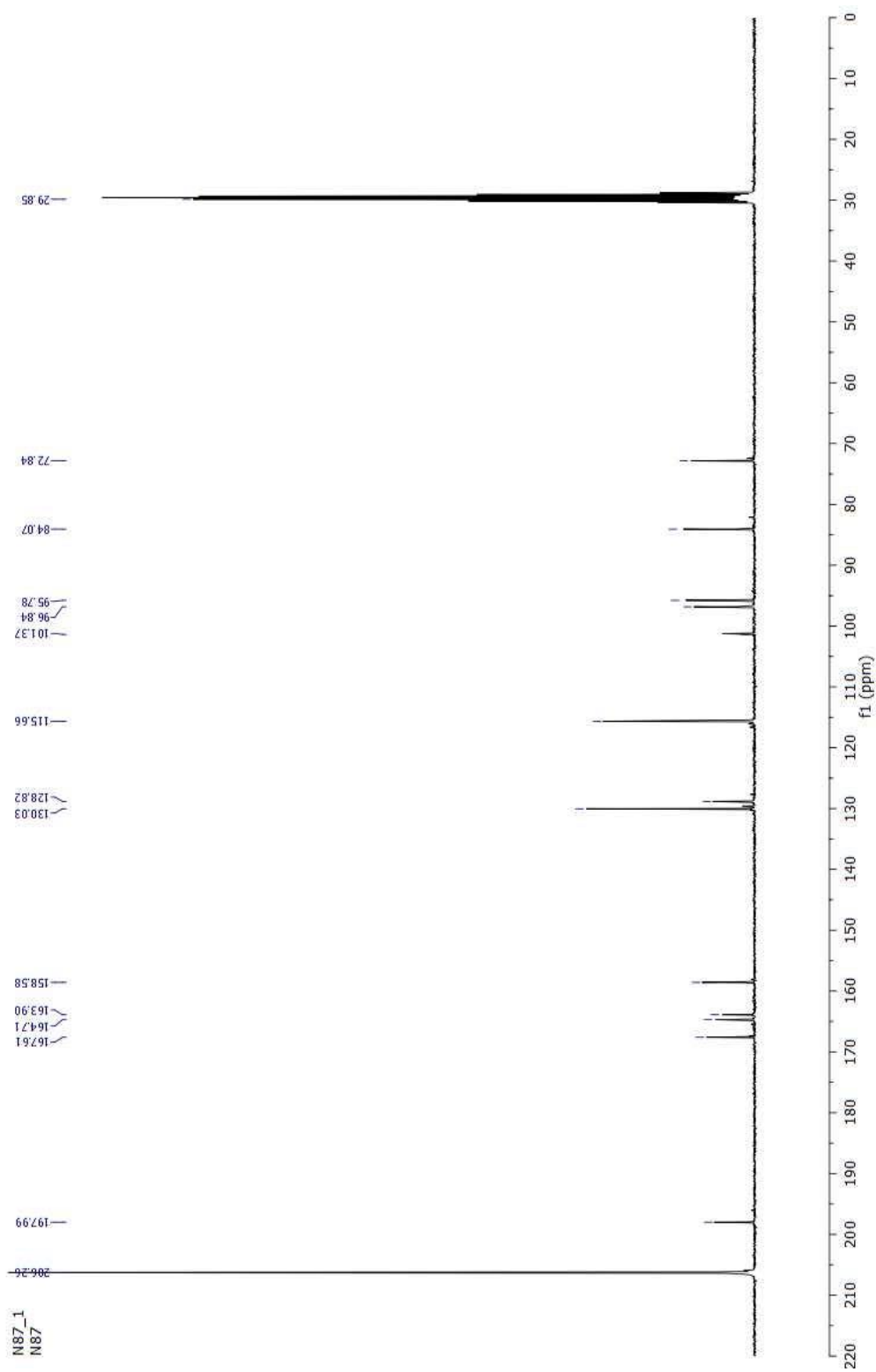
**Figure 66** The $^{13}$C-NMR spectrum of (+)-dihydrokeampferol (**III**)
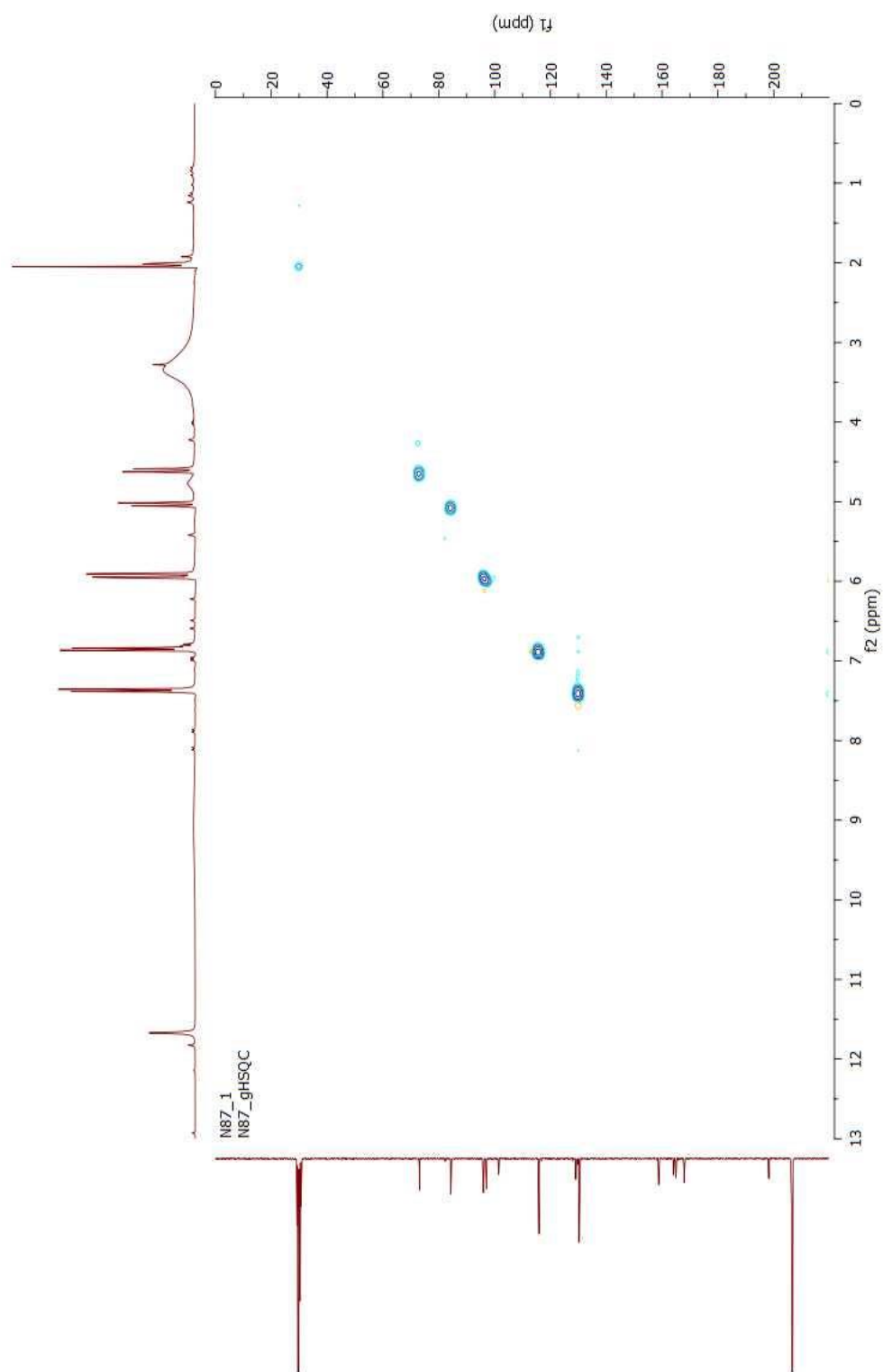
**Figure 67** The HSQC spectrum of (+)-dihydrokeampferol (**III**)
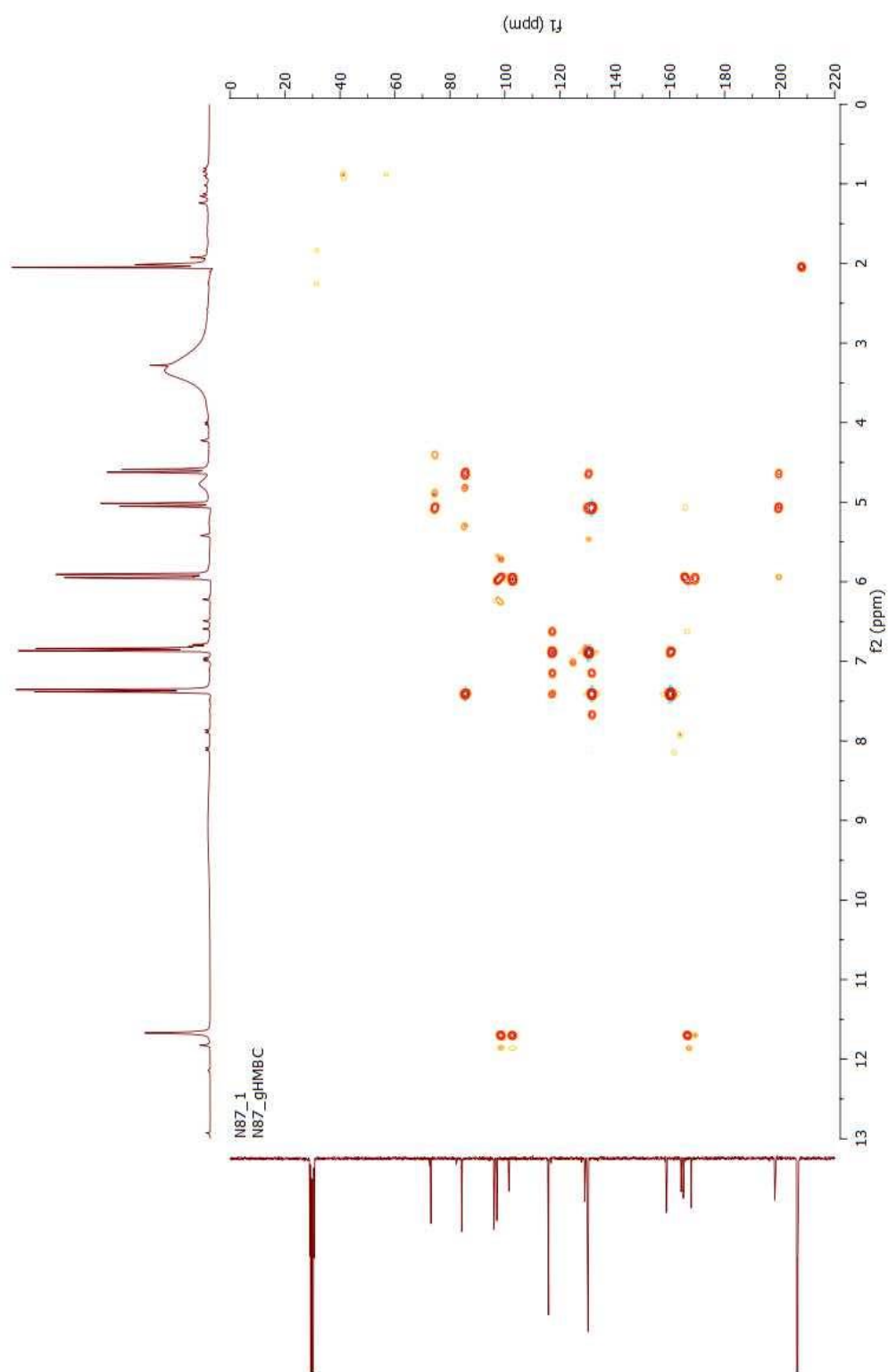
**Figure 68** The HMBC spectrum of (+)-dihydrokeampferol (**III**)
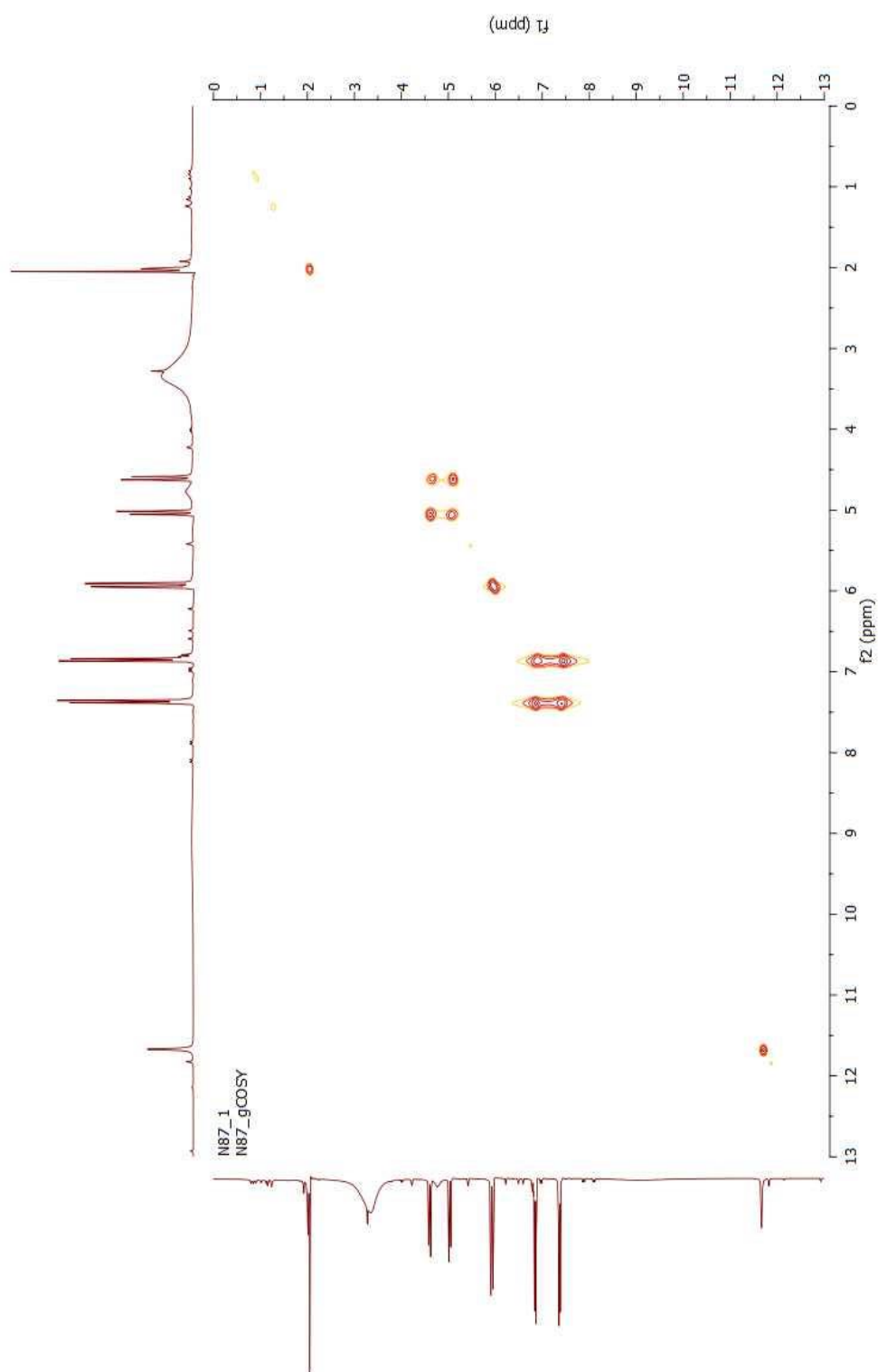
**Figure 69** The COSY spectrum of (+)-dihydrokeampferol (**III**)
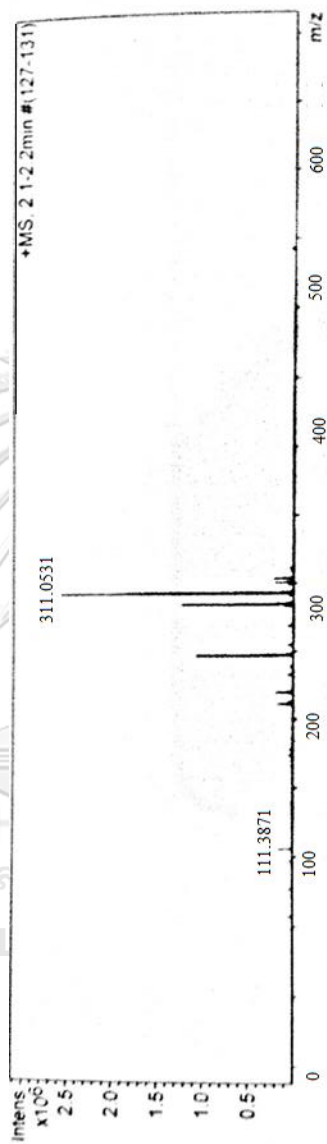
**Figure 70** The HR-EI-MS spectrum of (+)-dihydrokeampferol (**III**)

1.286

2.050

2.977

6.879
6.907

7.452
7.459
7.480
7.486
7.520
7.527

F59F1
F59F1

4.82

5.78

8.49

1.10

1.07
1.00
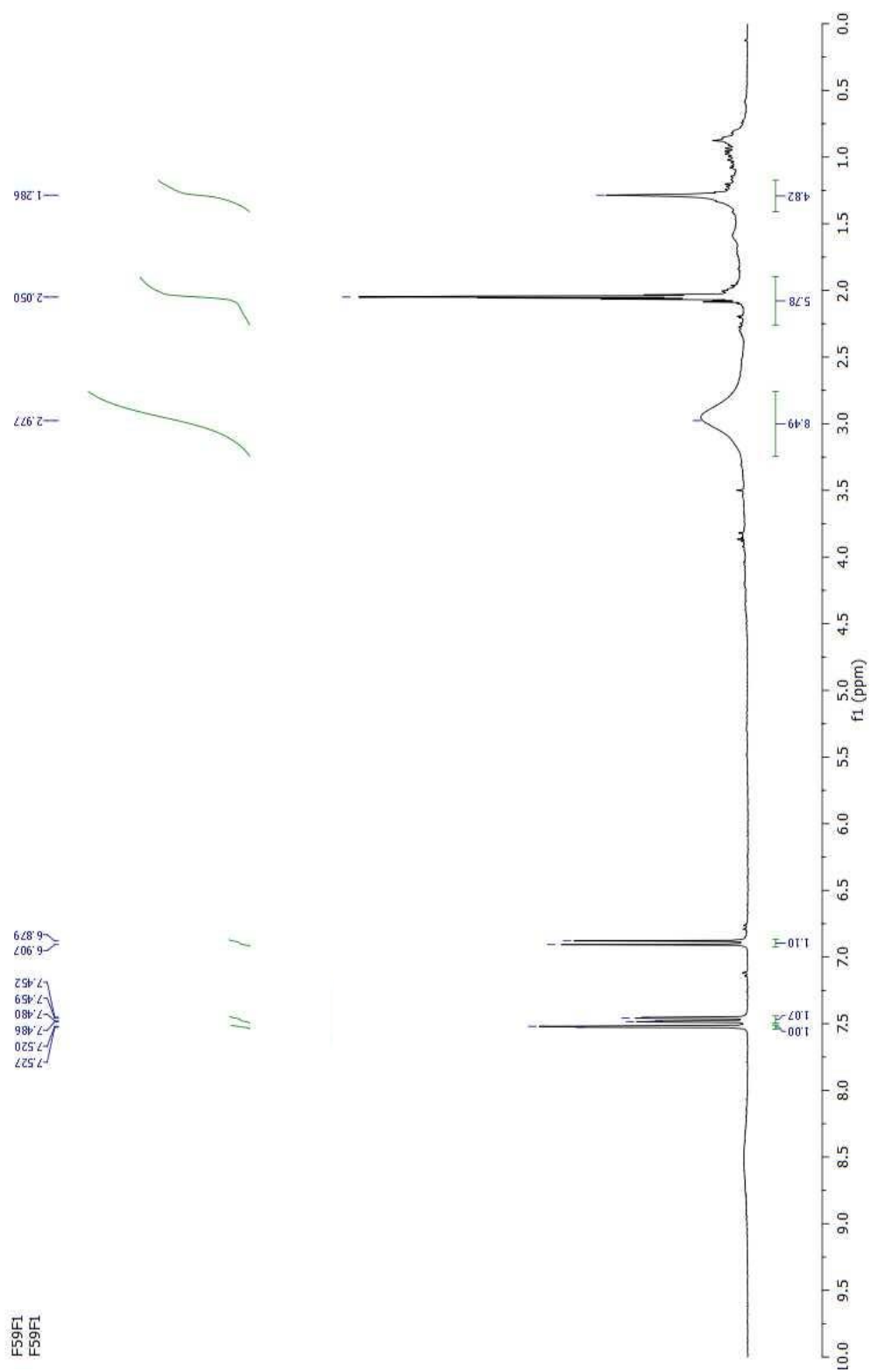
**Figure 71** The $^1$H-NMR spectrum of 3,4-dihydroxyflavanone (**IV**)
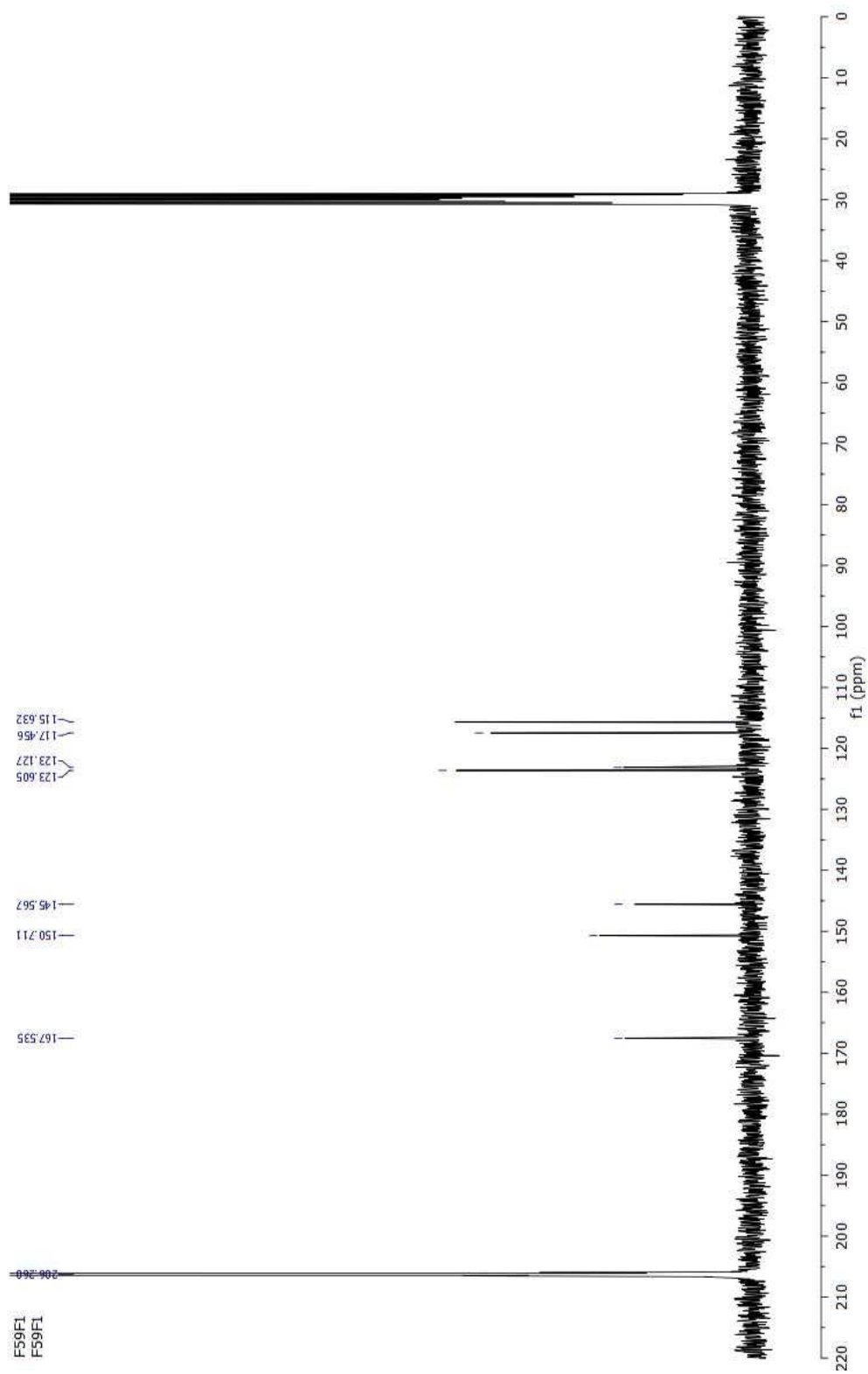
**Figure 72** The $^{13}$C-NMR spectrum of 3,4-dihydroxyflavanone (**IV**)
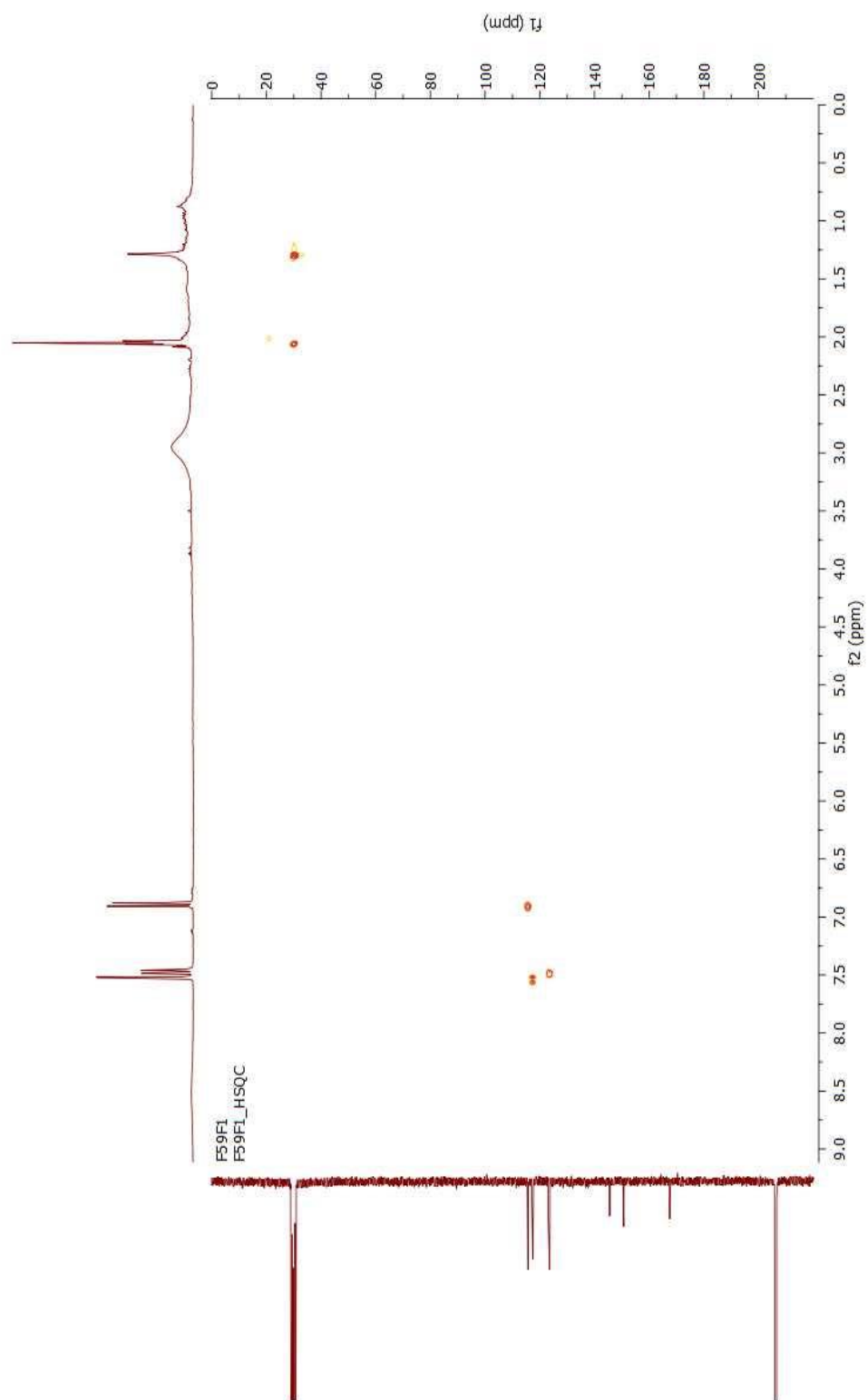
**Figure 73** The HSQC spectrum of 3,4-dihydroxyflavanone (**IV**)
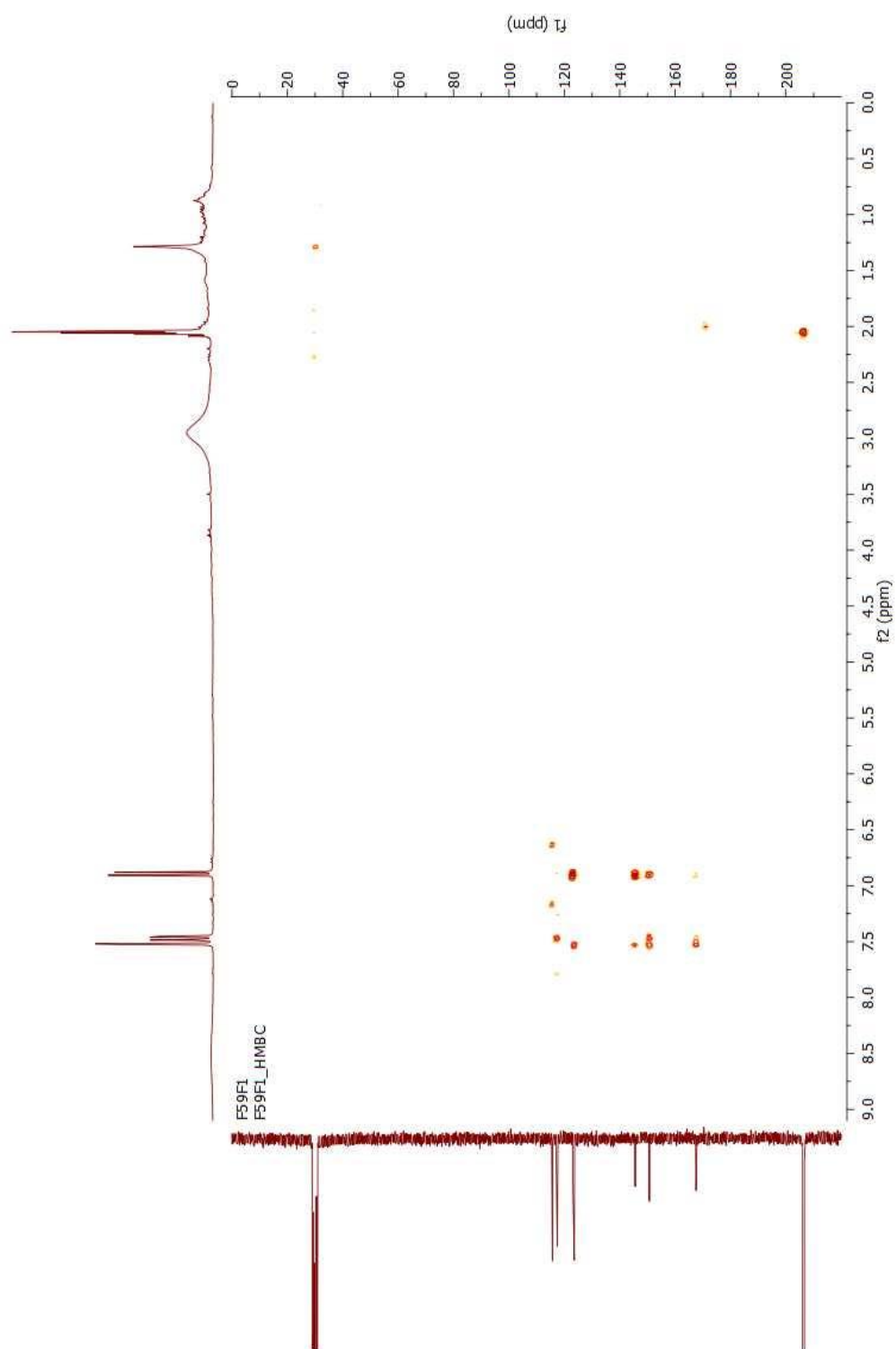
**Figure 74** The HMBC spectrum of 3,4-dihydroxyflavanone (**IV**)
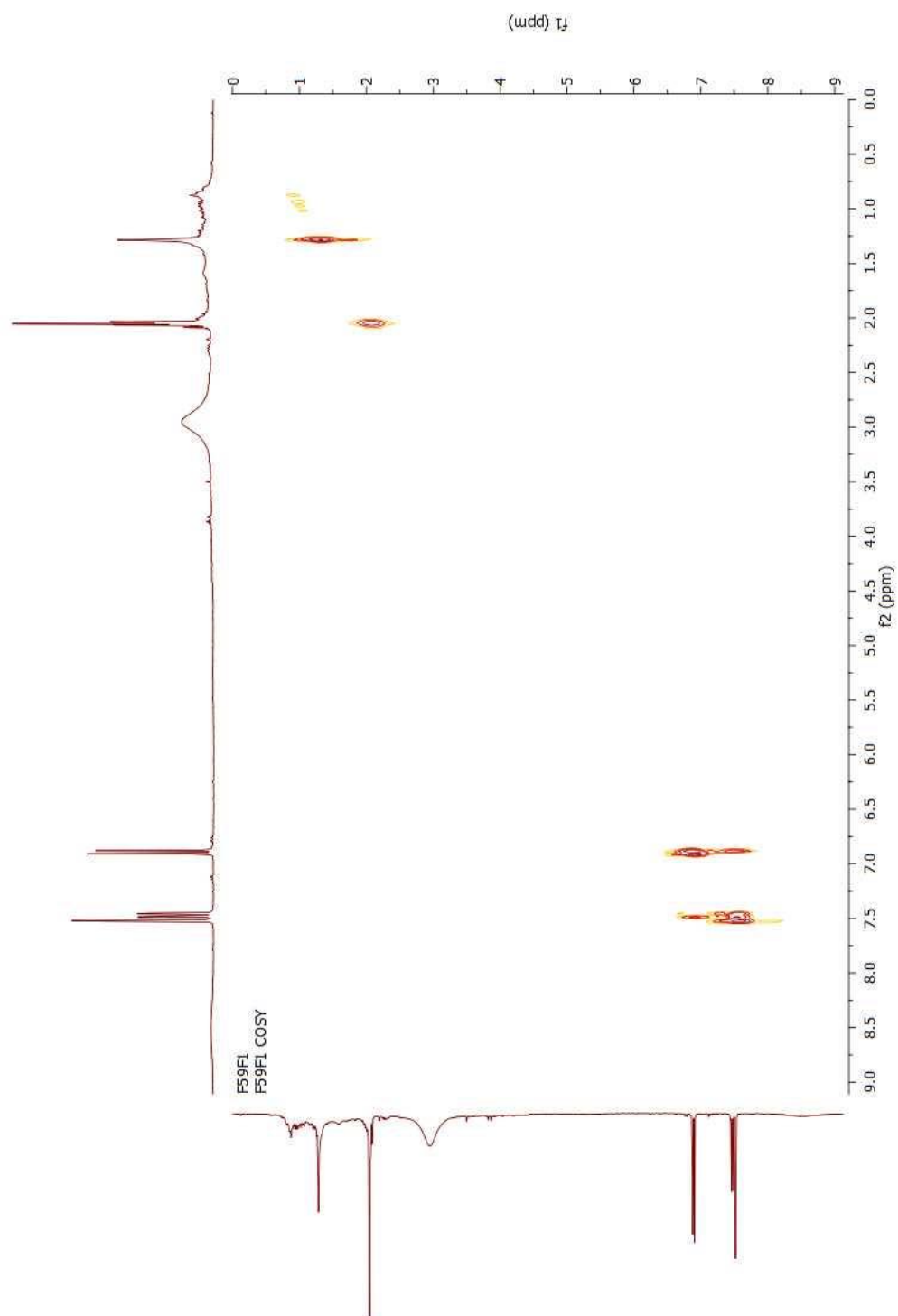
**Figure 75** The COSY spectrum of 3,4-dihydroxyflavanone (**IV**)
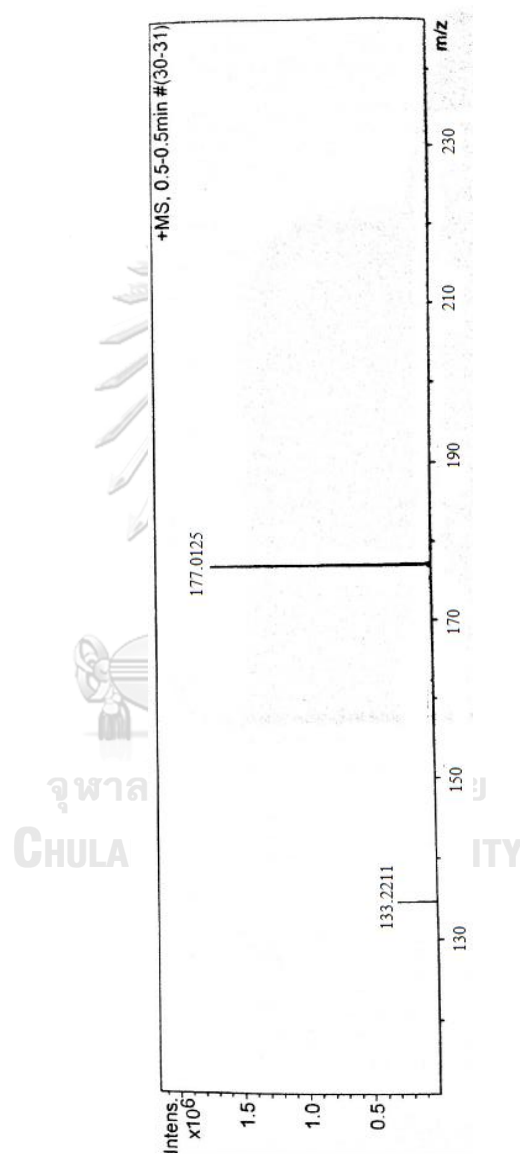
**Figure 76** The HR-EI-MS spectrum of 3,4-dihydroxyflavanone (**IV**)
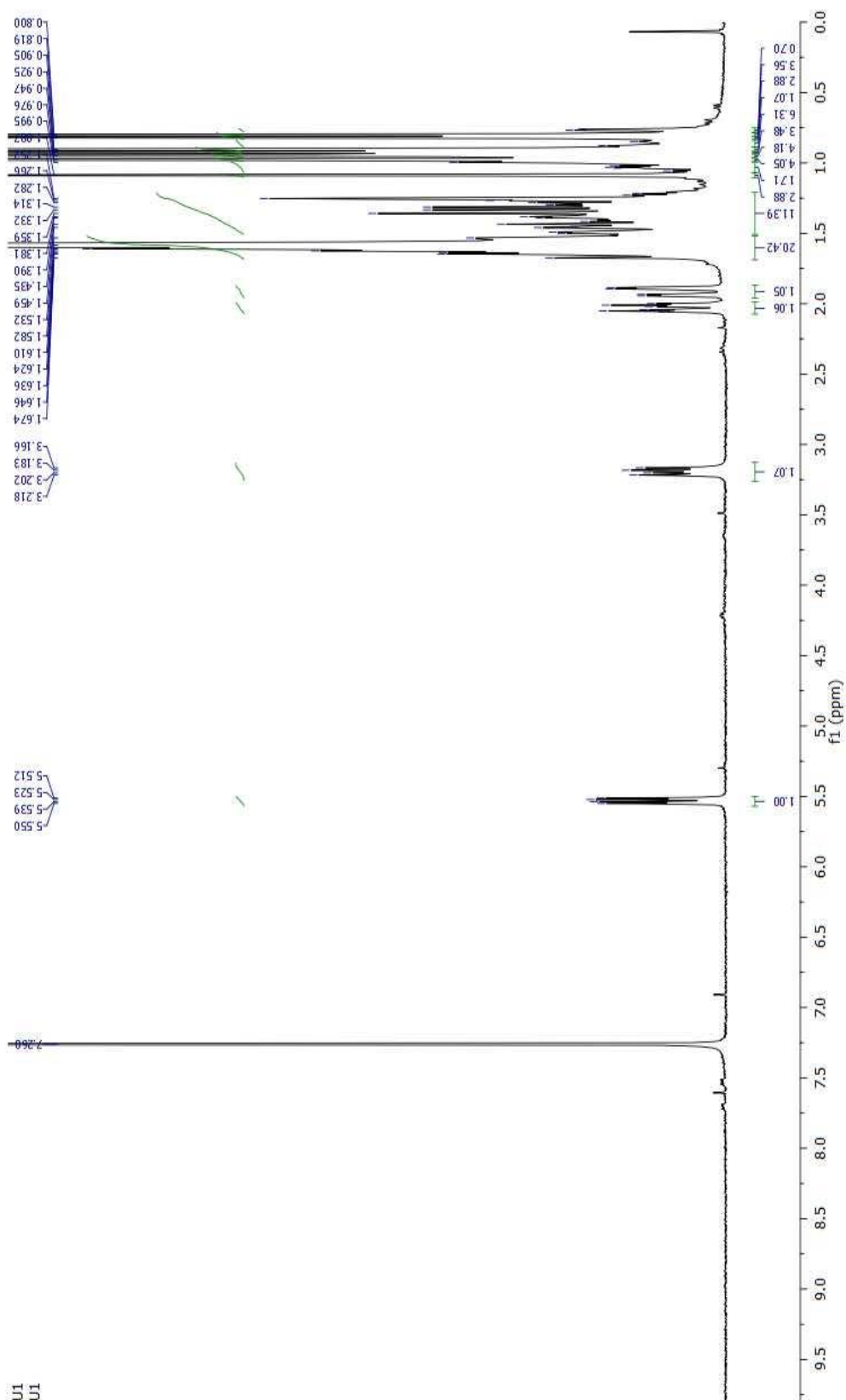
**Figure 77** The $^1$H-NMR spectrum of taraxerol (**V**)

15.430
17.496
18.788
21.308
25.897
27.140
27.989
28.796
29.820
29.918
33.090
33.344
33.692
35.112
35.785
36.672
37.562
37.719
41.320

48.744
49.273

55.581

77.004
77.203
79.078

117.110

158.163

U1
U1 13C
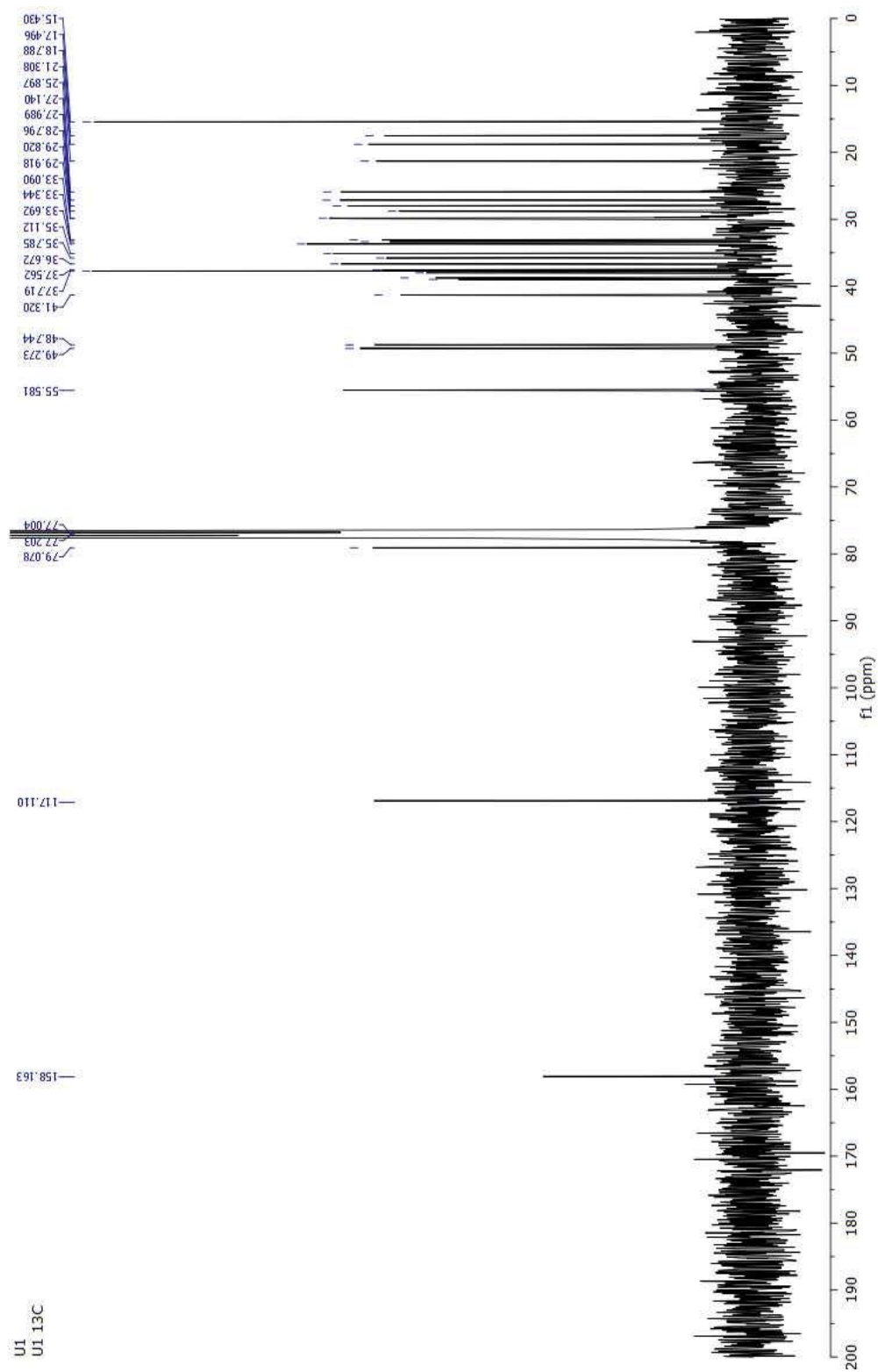
**Figure 78** The $^{13}$C-NMR spectrum of taraxerol (**V**)
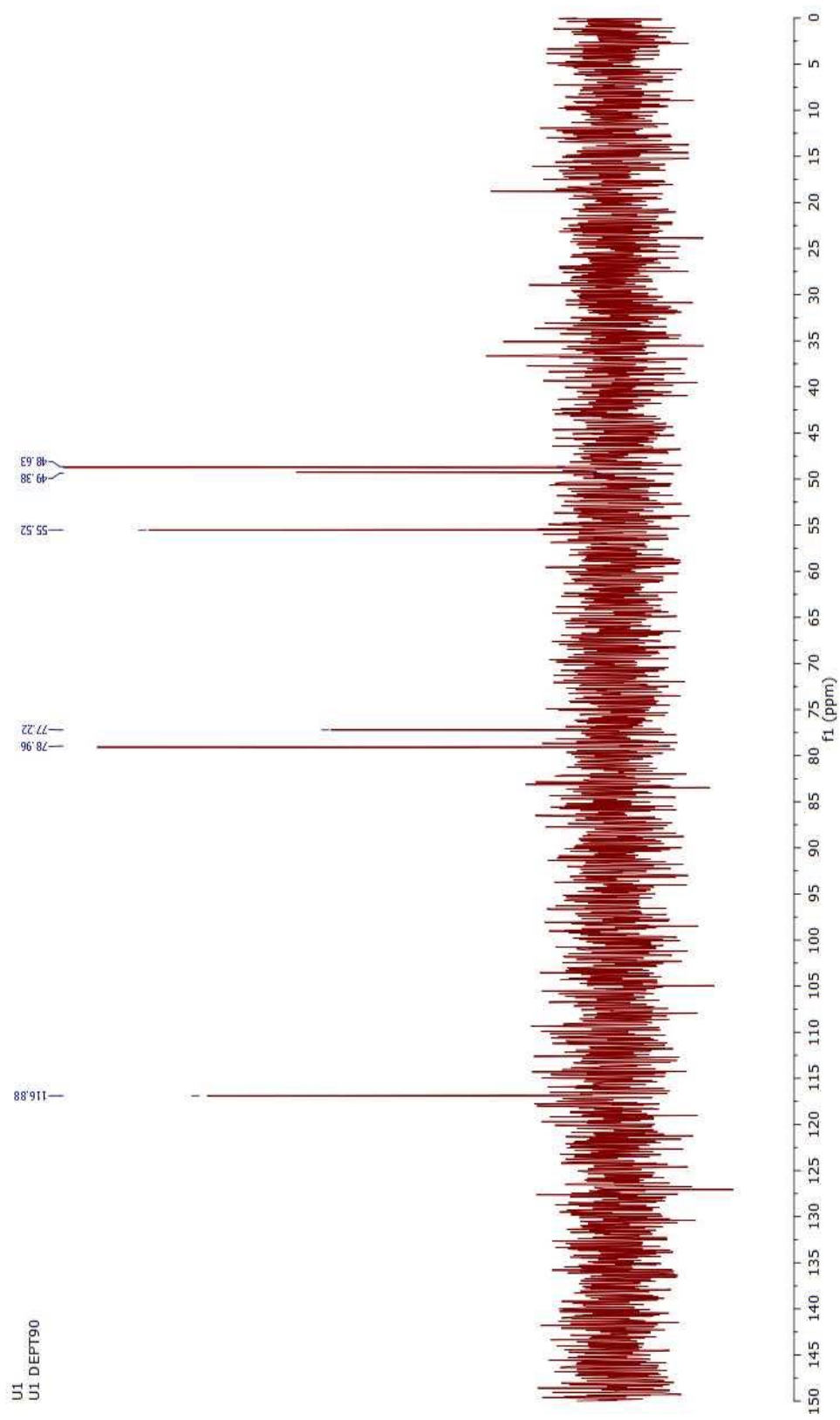
**Figure 79** The DEPT90 spectrum of taraxerol (**V**)
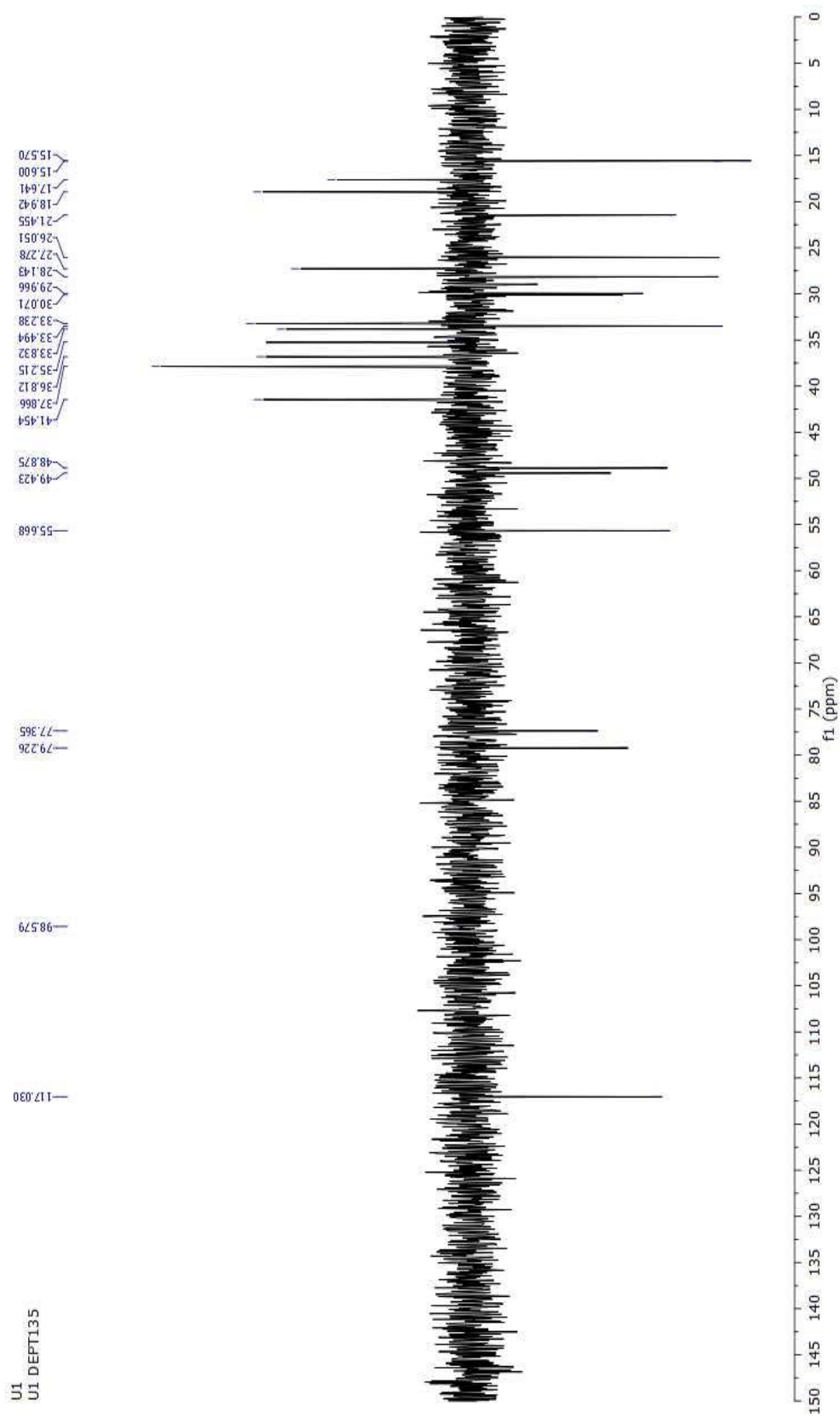
**Figure 80** The DEPT135 spectrum of taraxerol (**V**)
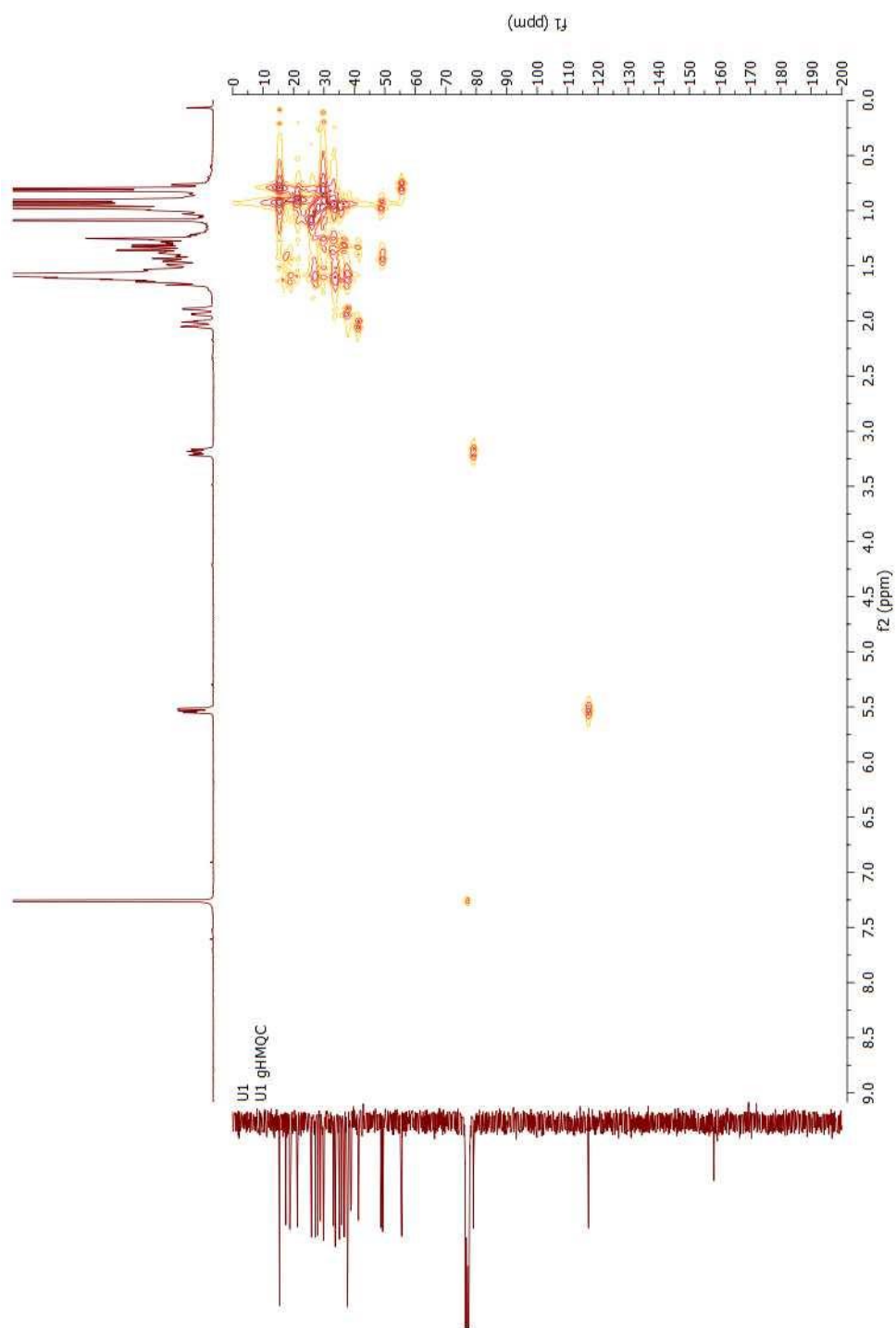
**Figure 81** The HMQC spectrum of taraxerol (**V**)
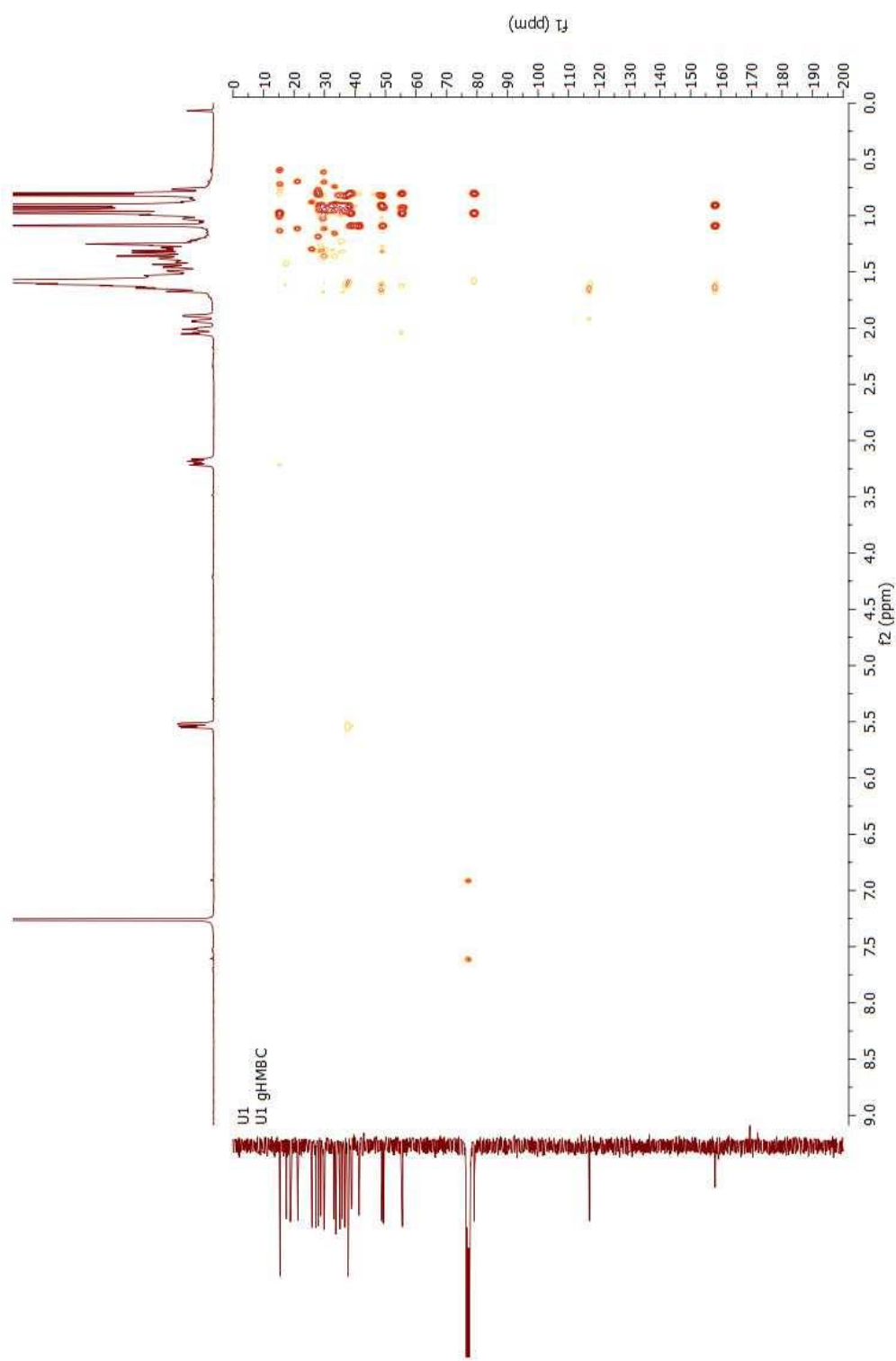
**Figure 82** The HMBC spectrum of taraxerol (**V**)
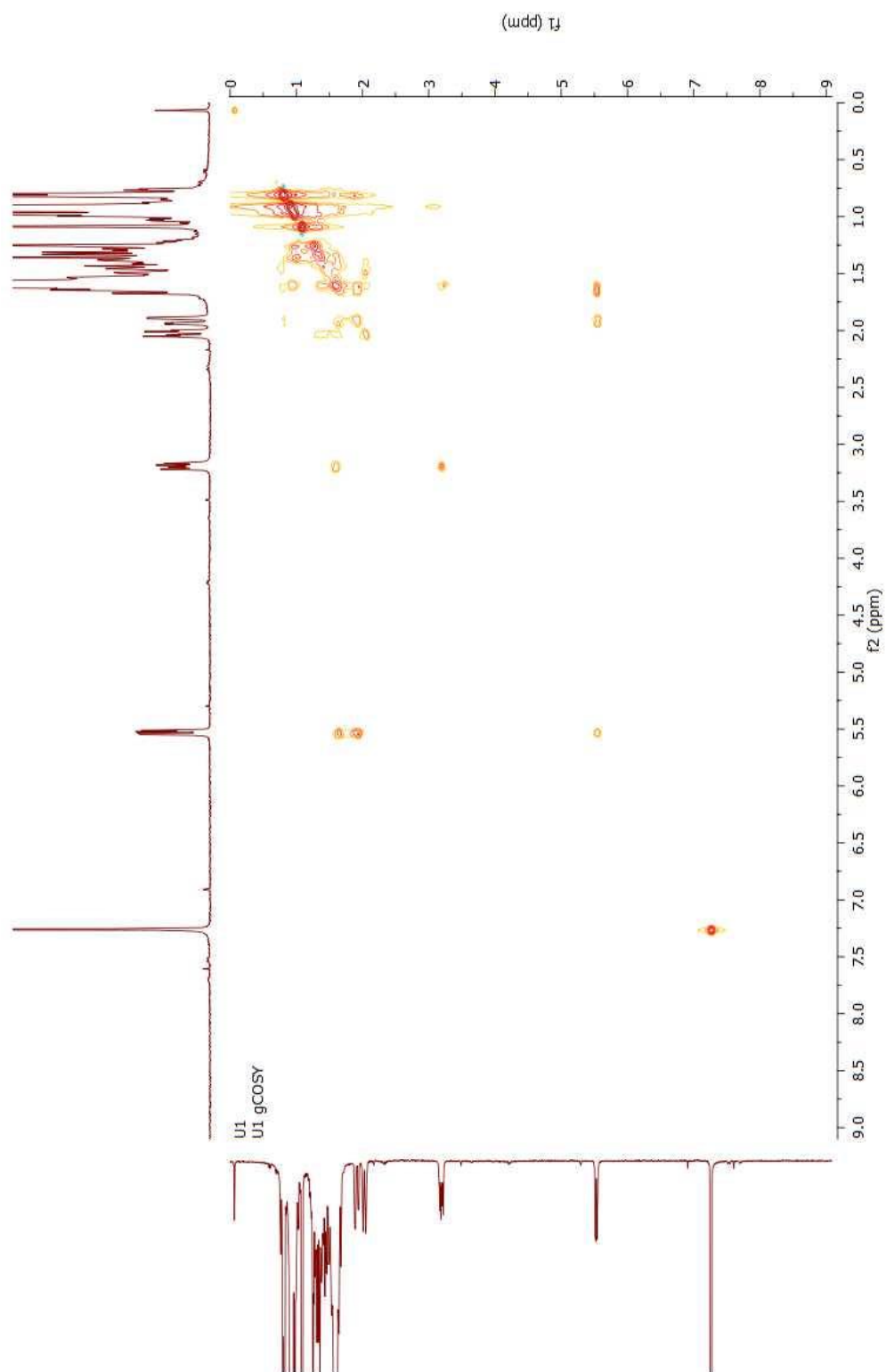
**Figure 83** The COSY spectrum of taraxerol (**V**)
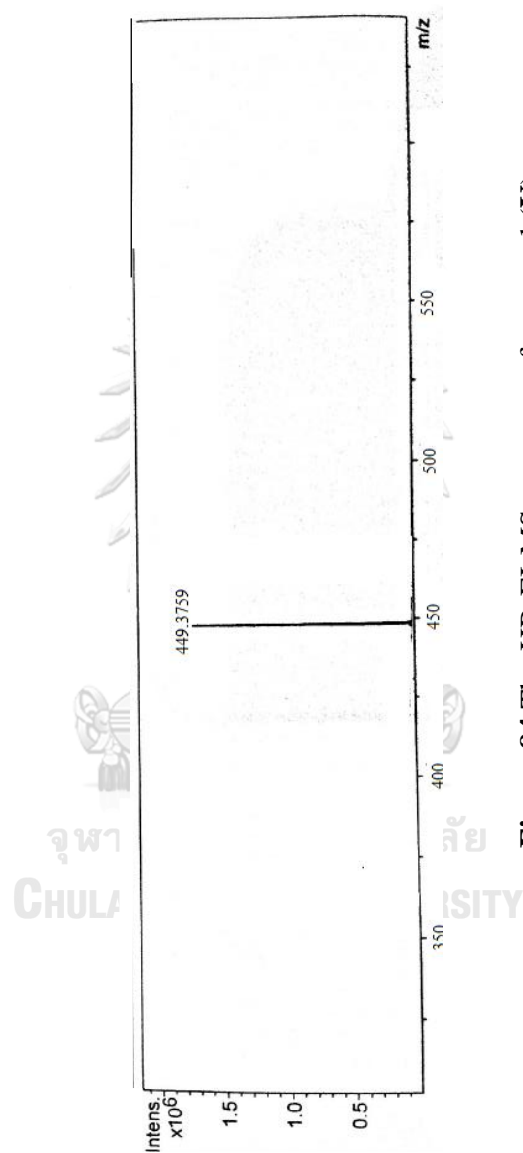
**Figure 84** The HR-EI-MS spectrum of taraxerol (**V**)
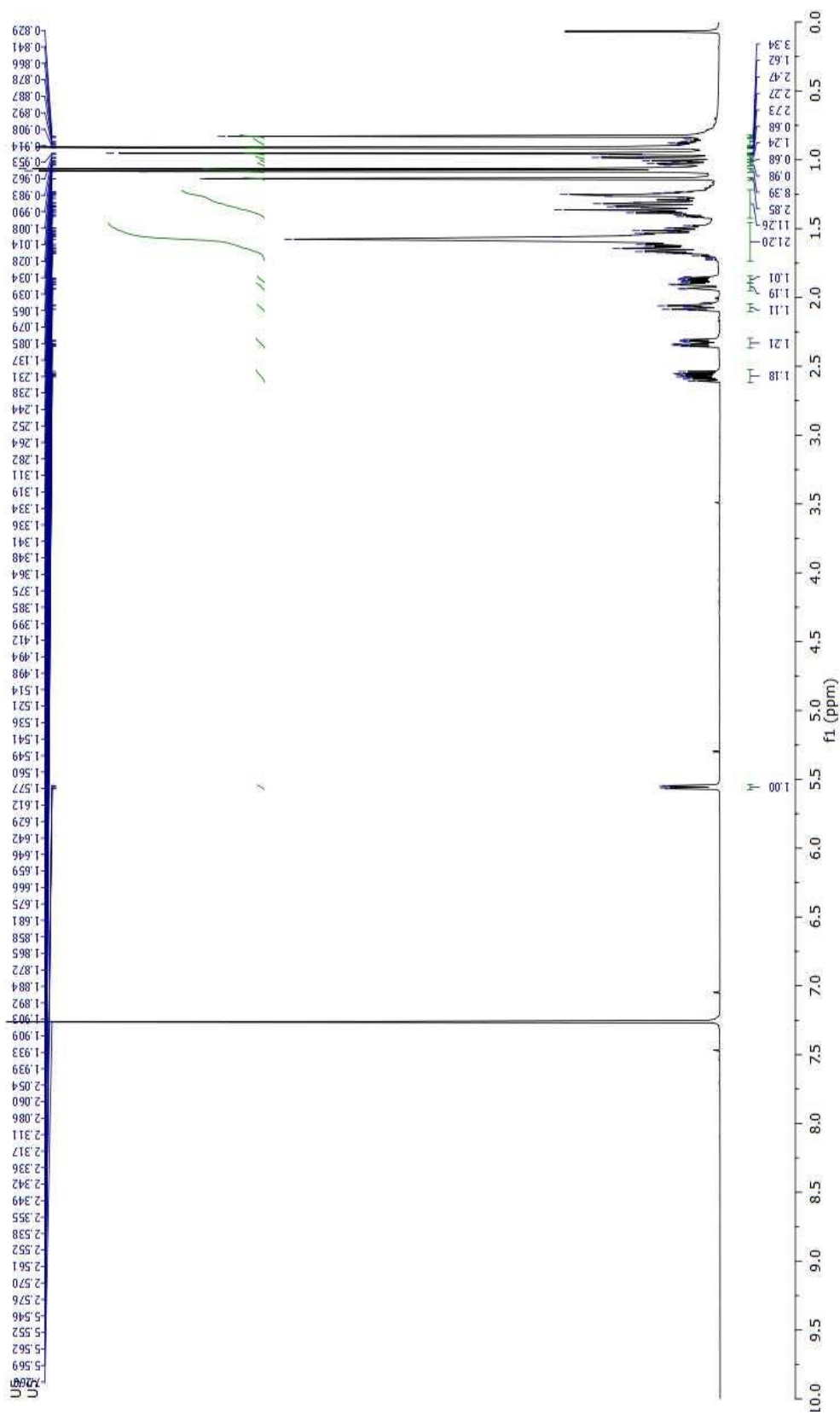
**Figure 85** The $^1$H-NMR spectrum of taraxerone (**VI**)
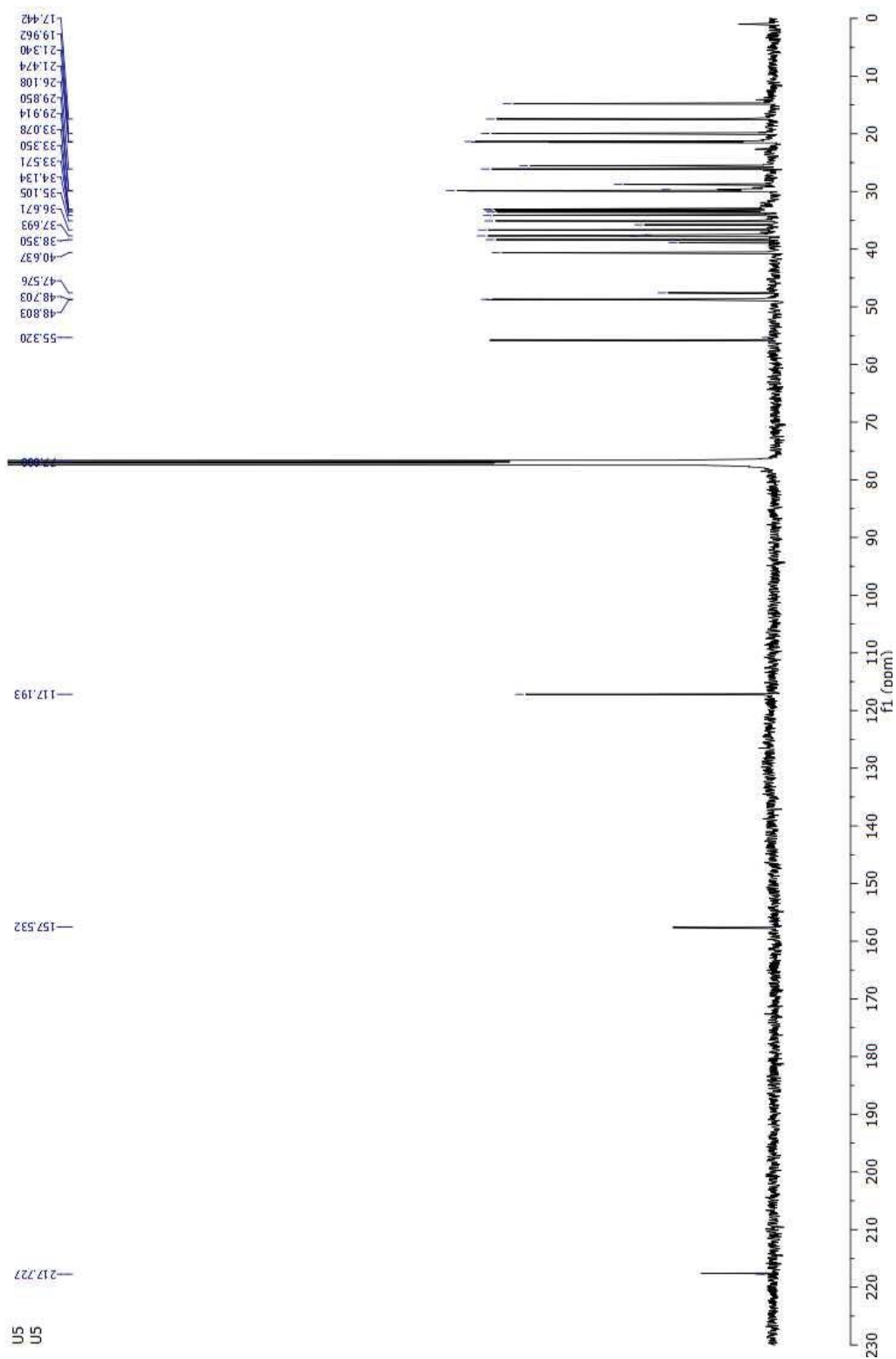
**Figure 86** The $^{13}$C-NMR spectrum of taraxerone (**VI**)

Y313
Y313_DEPT90

48.84

55.77

112.20

150 145 140 135 130 125 120 115 110 105 100 95 90 85 80 75 70 65 60 55 50 45 40 35 30 25 20 15 10 5 0
f1 (ppm)
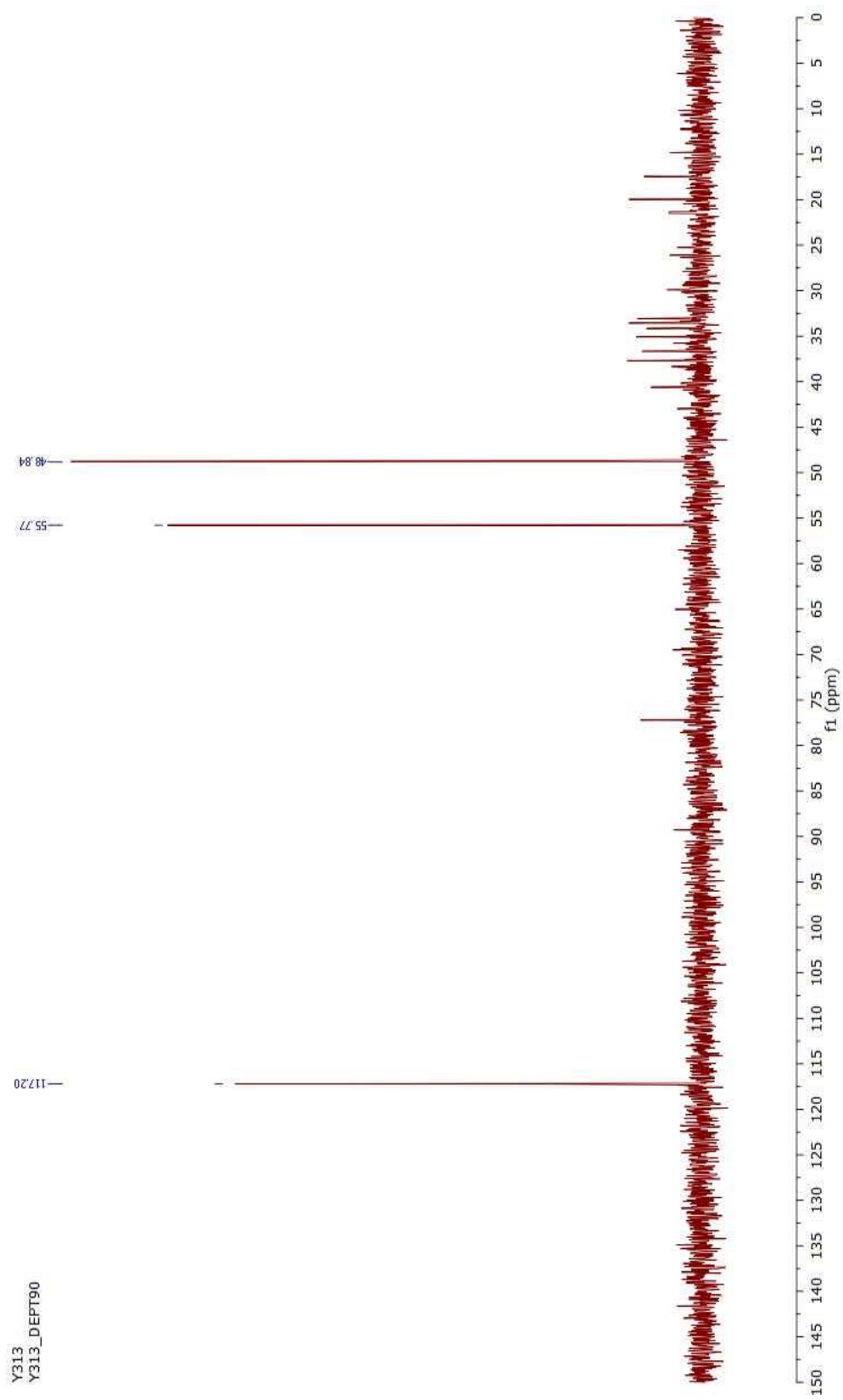
**Figure 87** The DEPT90 spectrum of taraxerone (**VI**)
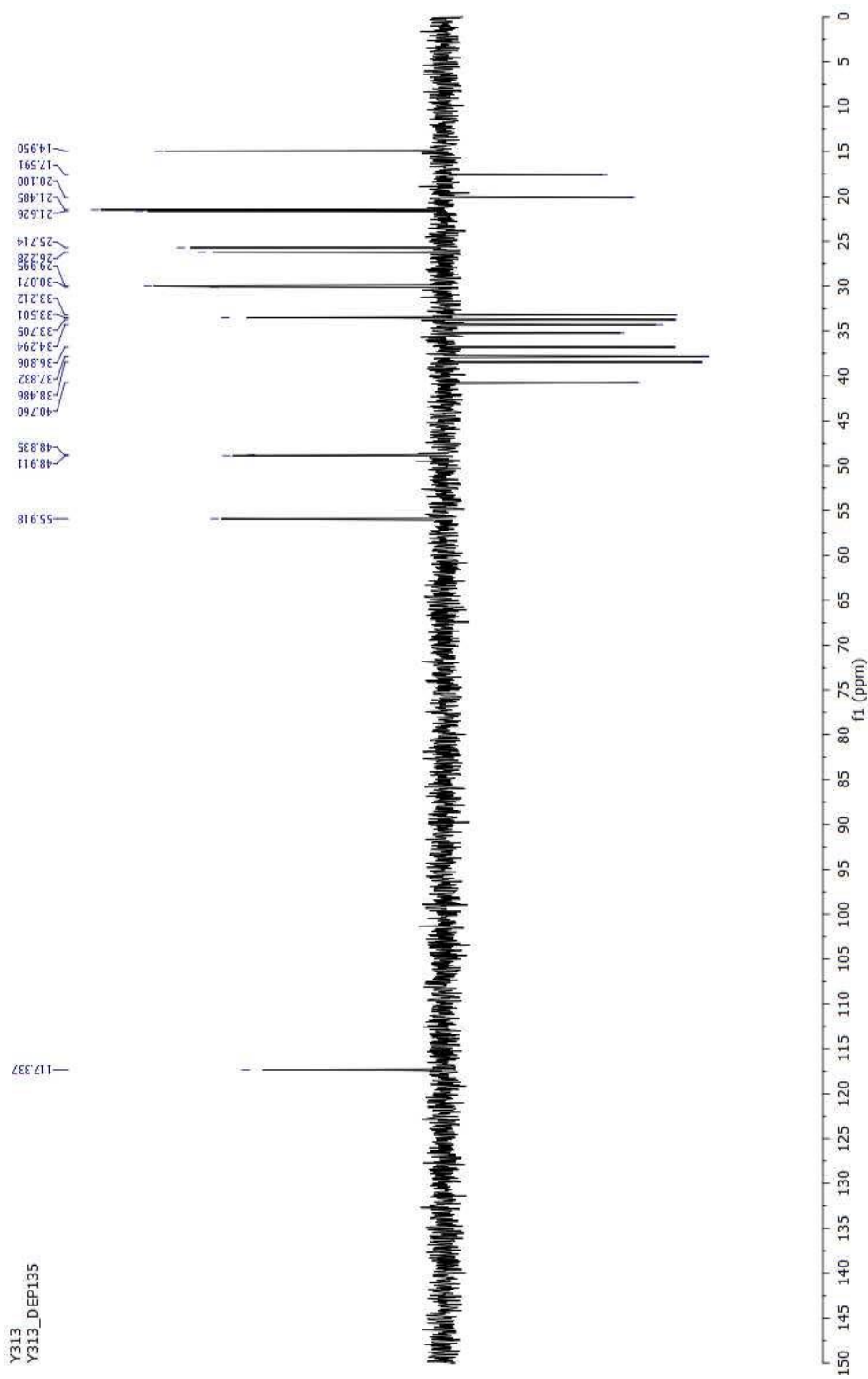
**Figure 88** The DEPT135 spectrum of taraxerone (**VI**)
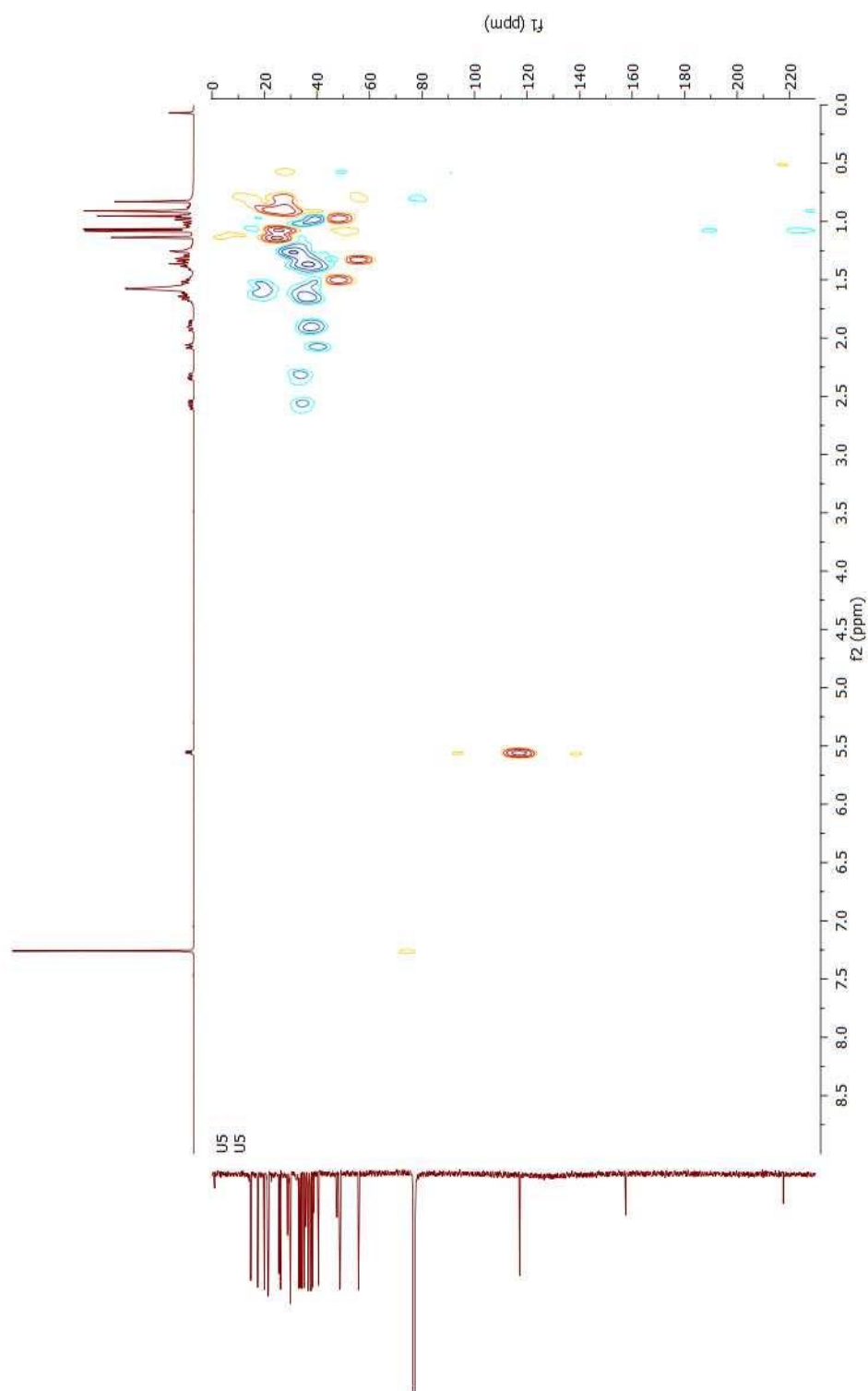
**Figure 89** The HMQC spectrum of taraxerone (**VI**)
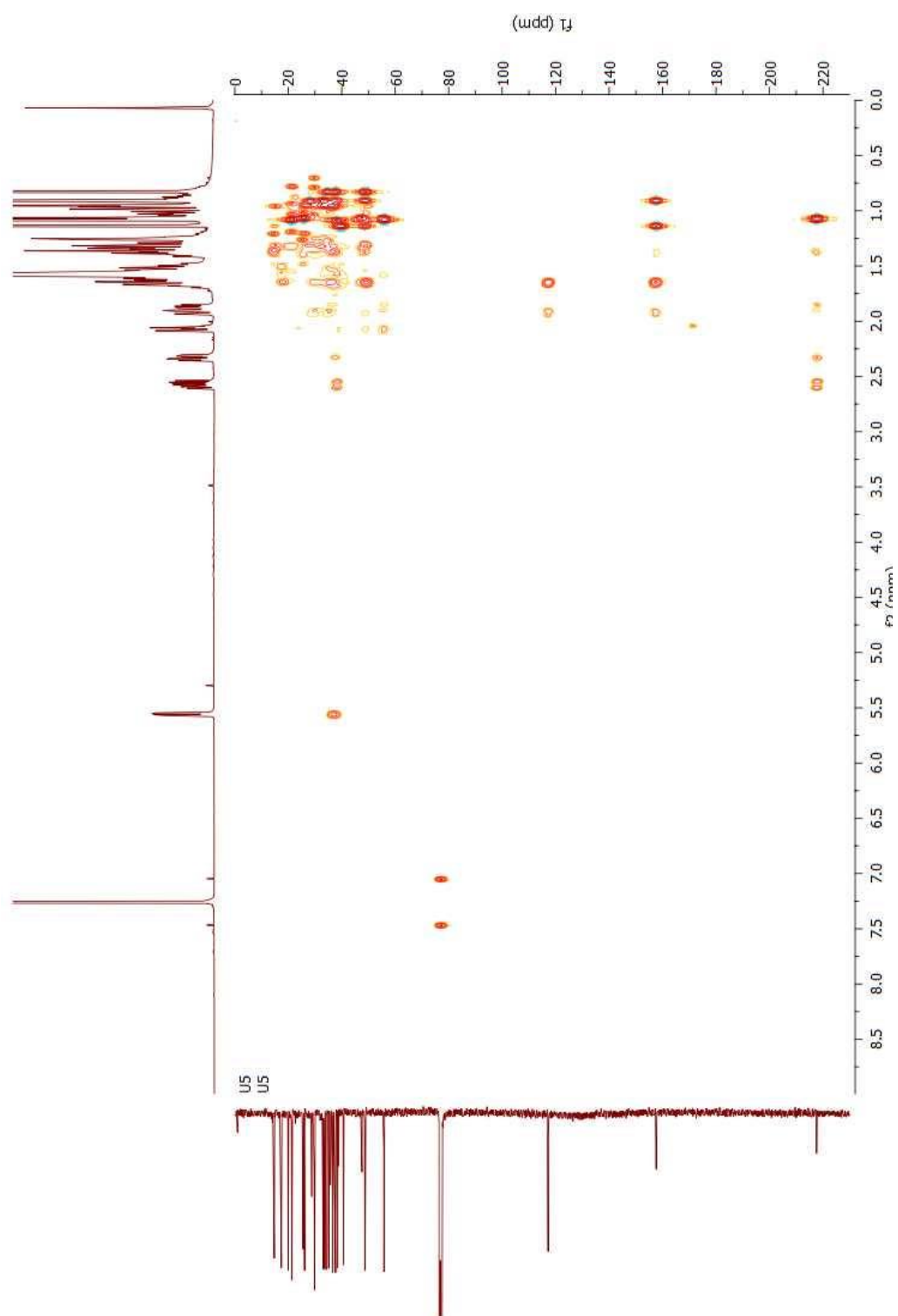
**Figure 90** The HMBC spectrum of taraxerone (**VI**)
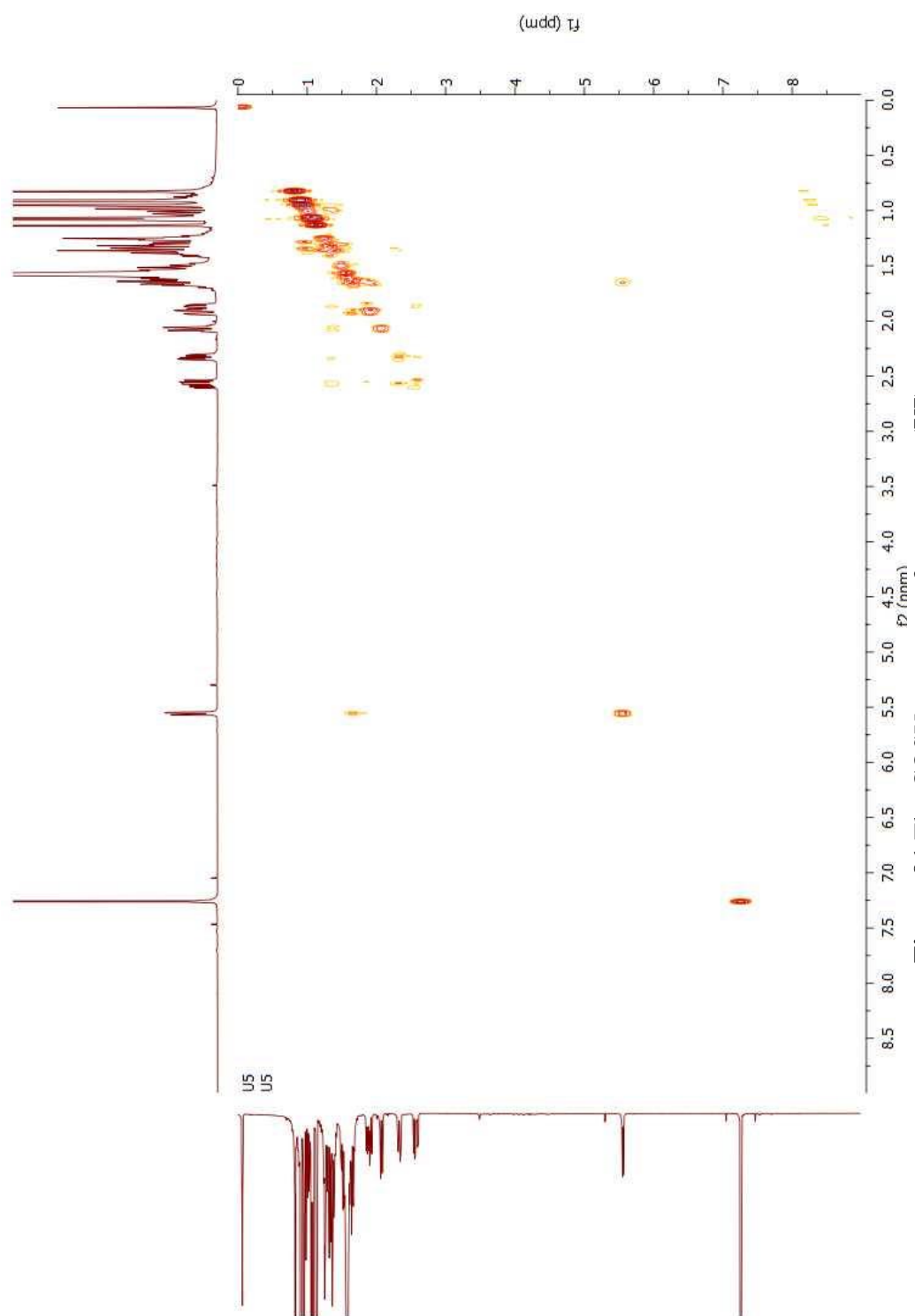
**Figure 91** The COSY spectrum of taraxerone (**VI**)
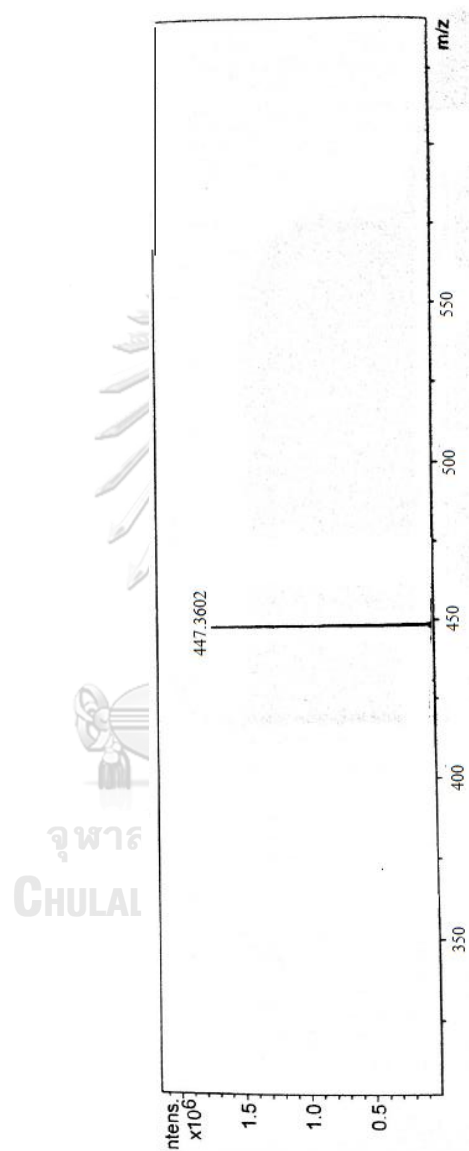
**Figure 92** The HR-EI-MS spectrum of taraxerone (**VI**)

**Figure 93** The $^1$H-NMR spectrum of lupeol acetate (**VII**)
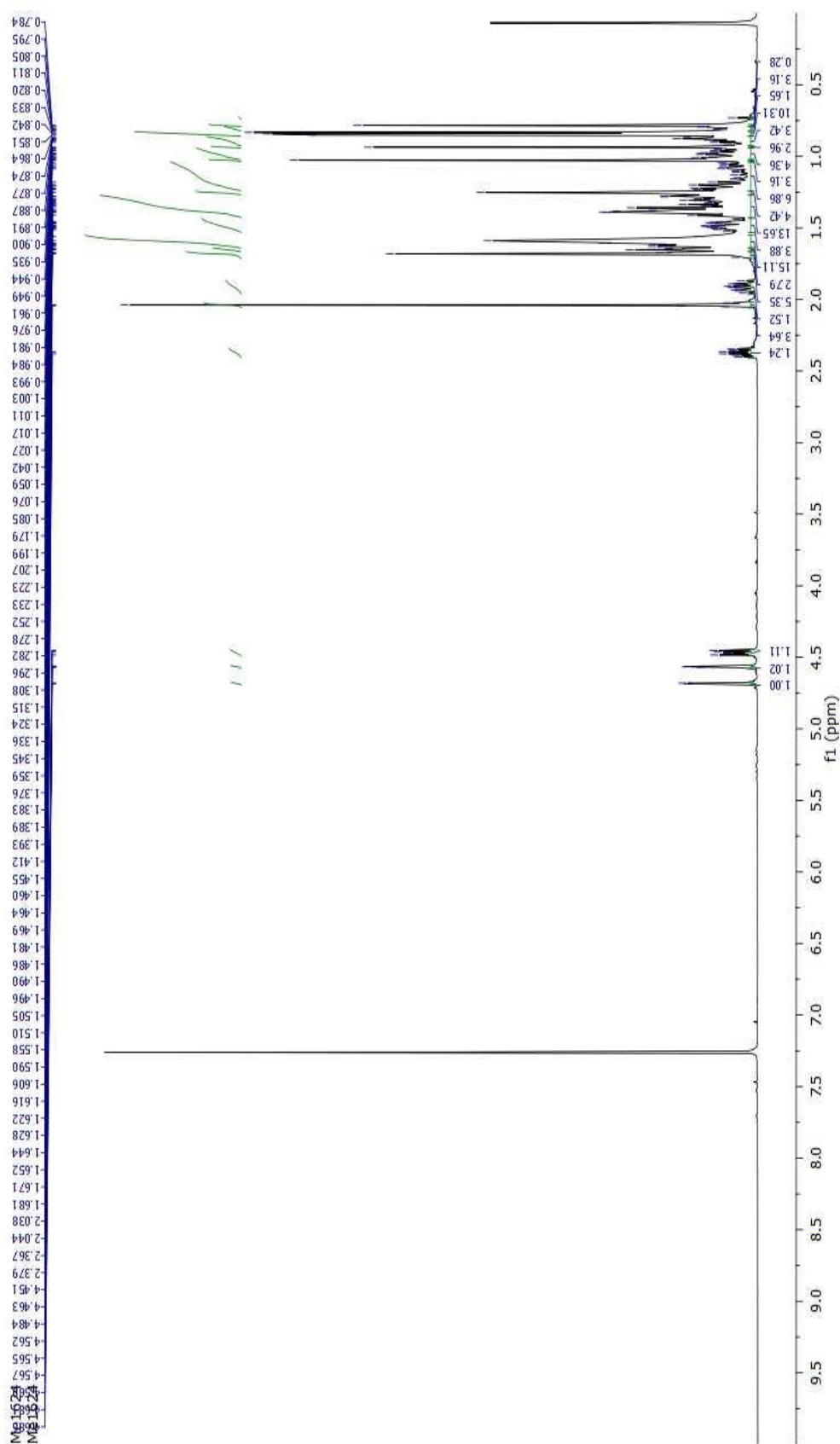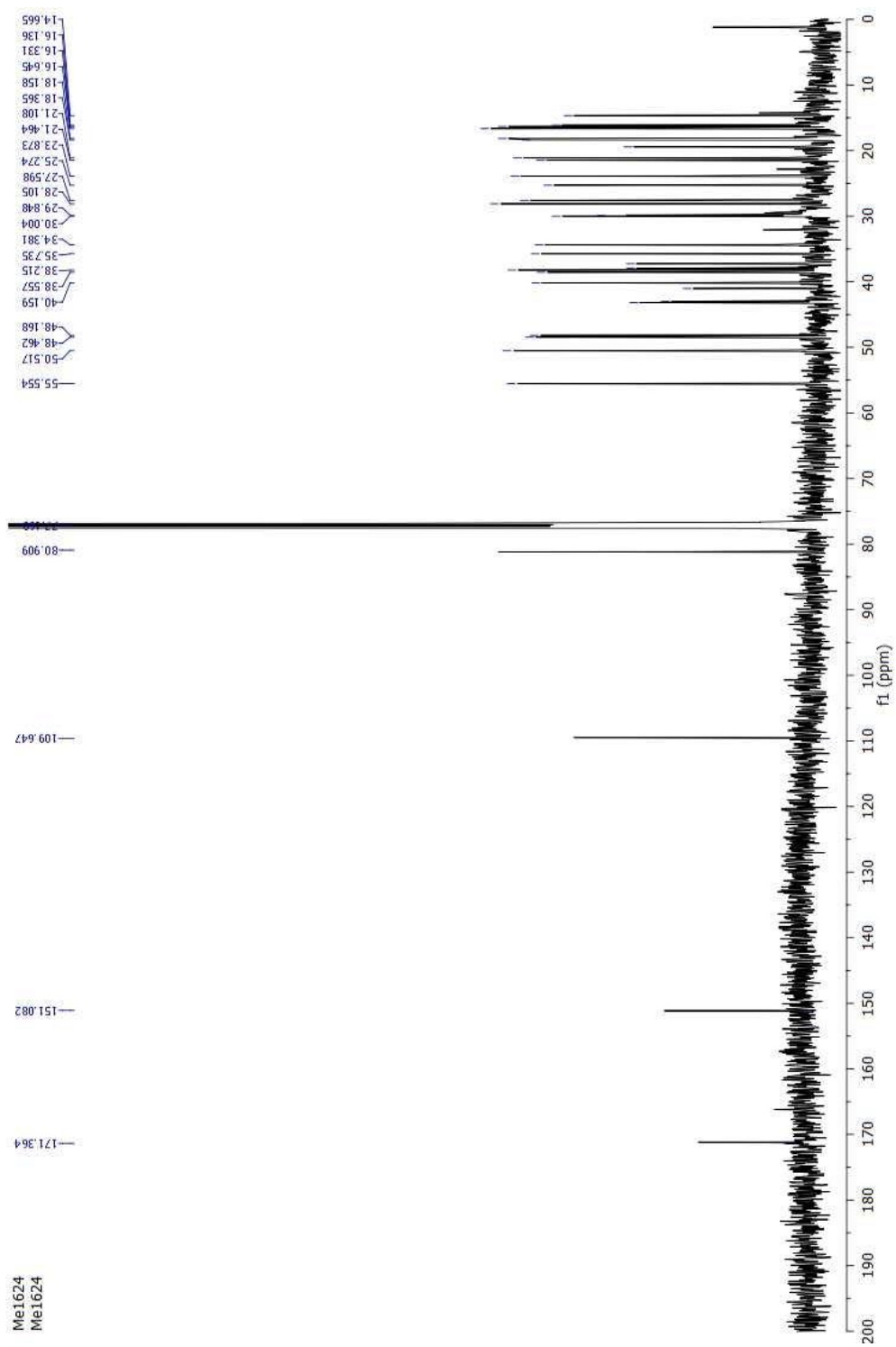
**Figure 94** The $^{13}$C-NMR spectrum of lupeol acetate (**VII**)
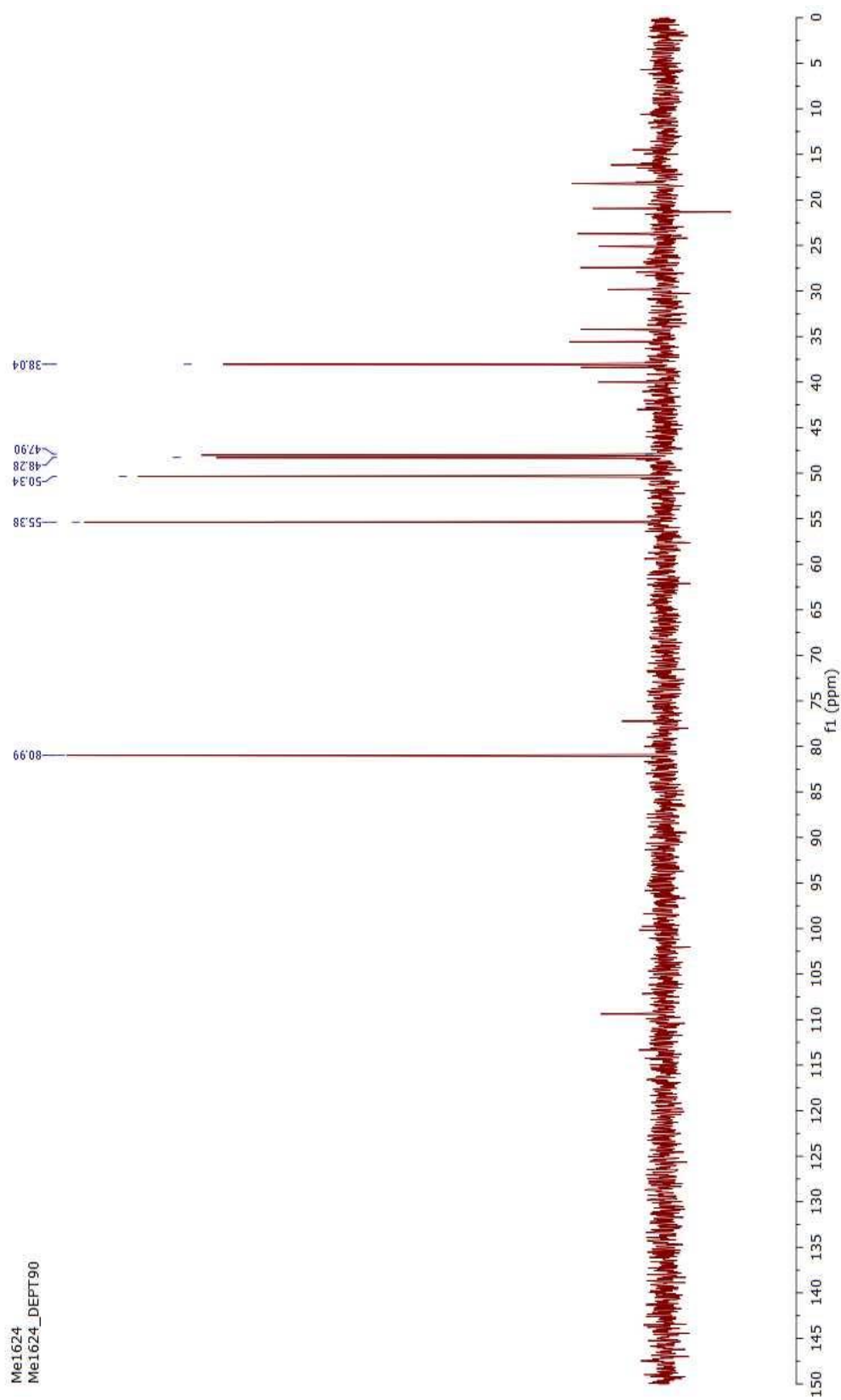
**Figure 95** The DEPT90 spectrum of lupeol acetate (**VII**)
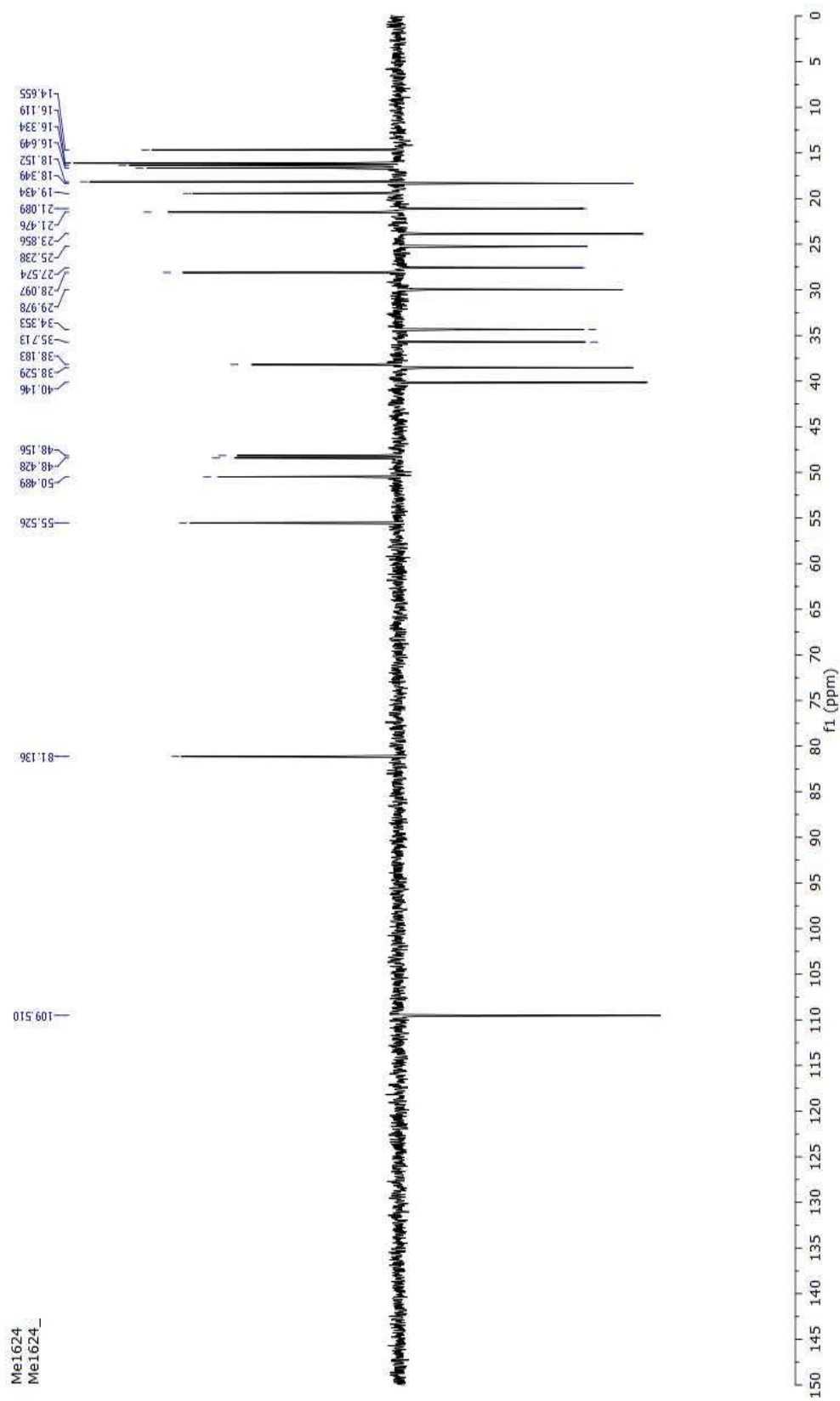
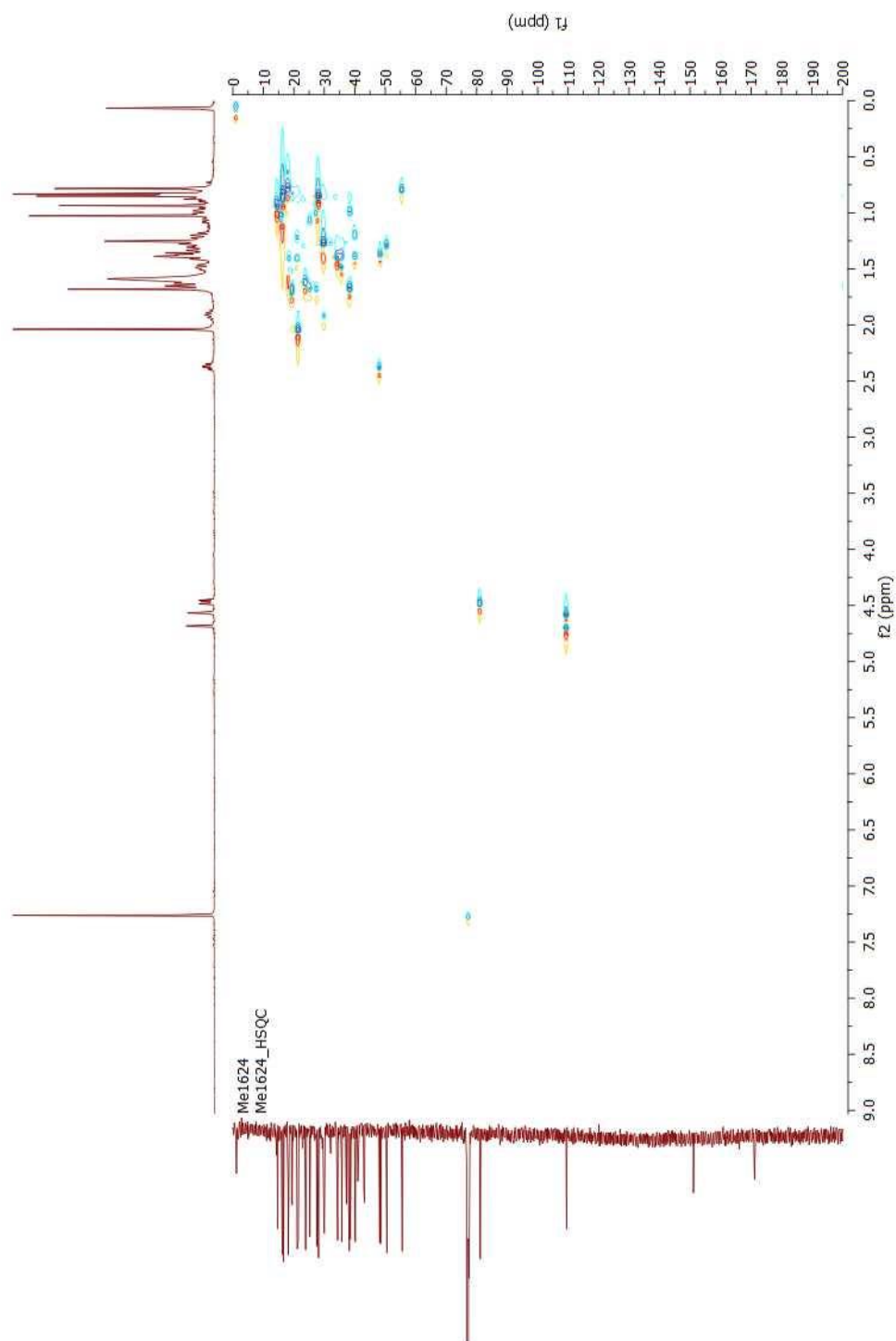**Figure 96** The DEPT135 spectrum of lupeol acetate (**VII**)
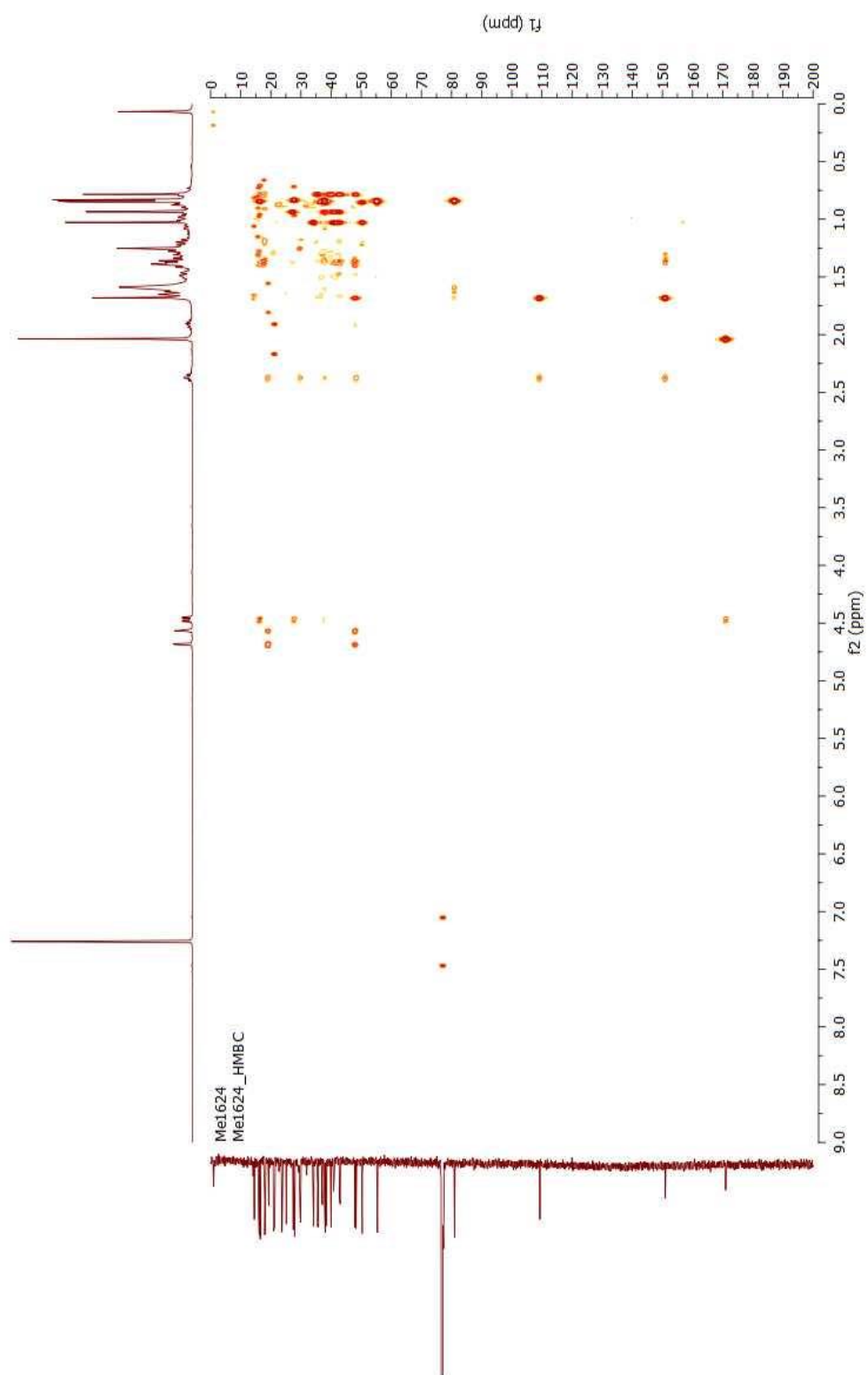
**Figure 97** The HSQC spectrum of lupeol acetate (**VII**)
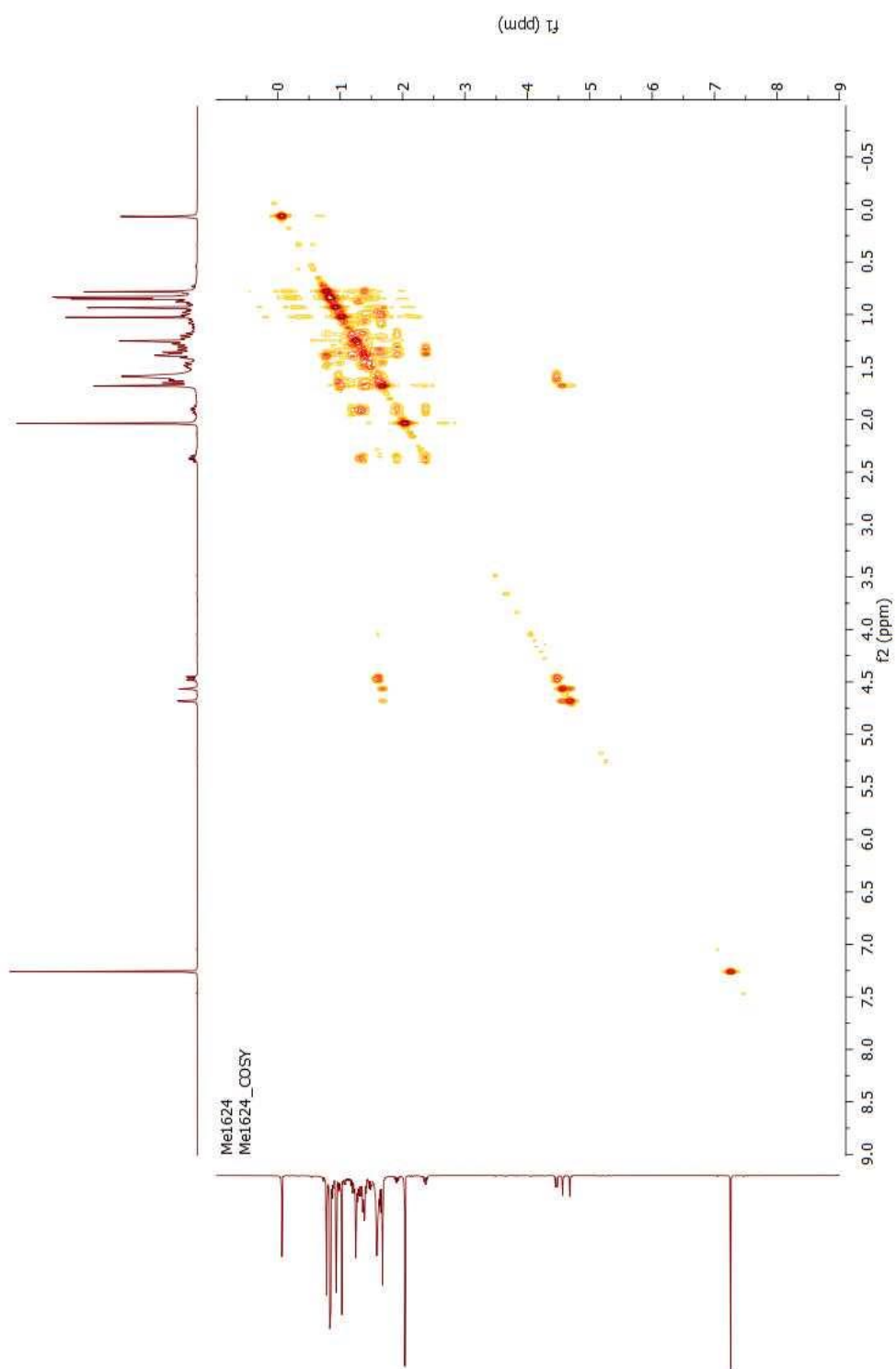
**Figure 98** The HMBC spectrum of lupeol acetate (**VII**)

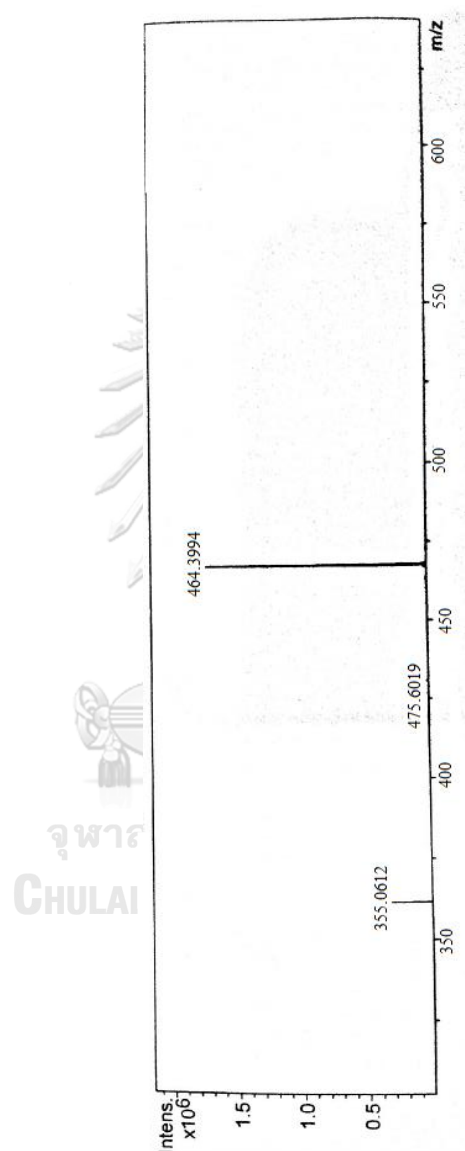**Figure 99** The COSY spectrum of lupeol acetate (**VII**)

**Figure 100** The HR-EI-MS spectrum of lupeol acetate (**VII**)

# VITA

Miss Sutthiduean Chunhakant was born on November 22, 1979 in Pare Province, Thailand. She graduated with a bachelor degree of Science in Chemistry from Department of Chemistry, Phranakorn Rajabhat University, Bangkok, Thailand in 2002. Then, she graduated with master of Science in Biotechnology from Faculty of Science, Chulalongkorn University, Bangkok, Thailand in 2007. After that, she was a lecturer at Department of Chemistry, Phranakorn Rajabhat University, Bangkok, Thailand in 2007. She was admitted doctoral's degree of Program in Biotechnology, Faculty of Science, Chulalongkorn University in 2012. She obtained financial support on her study from Pranakorn Rajabhat University. During the course of study, she obtained financial support on her research from Graduate School Chulalongkorn University. She received poster presentation award at the 9th International Symposium in Science and Technology, Cheng Shiu University, Taiwan in 2014. The title was "Antityrosinase activity of some Thai medicinal plants". She has attended at 10th International Symposium in Science and Technology at Chulalongkorn University, Thailand in 2015 in title "Antityrosinase activity of lupeol acetate from barks of Manilkara zapota". She participated the 11st International Symposium in Science and Technology, Kansai University, Japan in 2016 in title "Tyrosinase inhibitory activity of ethyl acetate extract of Manilkara zapota".

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