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นางสาวนภัสวรรณ ภูรีจารุโรจน์

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CHEMICAL CONSTITUENTS AND CYTOTOXICITY OF *PAPHIOPEDILUM EXUL* ROOTS

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การศึกษาองค์ประกอบทางเคมีของรากรองเท้านารีเหลืองกระบี่ [*Paphiopedilum exul* (Ridl.) Rolfe] วงศ์ Orchidaceae สามารถแยกสารใหม่ในกลุ่มสติลบีนได้ 1 ชนิด คือ (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene และสารที่เคยมีรายงานมาแล้ว 8 ชนิด โดยเป็นสติลบีน 5 ชนิด คือ (*E*)-5-hydroxy-3-methoxystilbene, (*E*)-5-hydroxy-3,2′-dimethoxystilbene, (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3,5′-dimethoxystilbene, (E)-5,2′-dihydroxy-3,5′-dimethoxystilbene และ (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene กับสารในกลุ่มเฟลโวนอยด์ 3 ชนิด คือ galangin, pinocembrin และ alpinetin พิสูจน์โครงสร้างทางเคมีของสารเหล่านี้ด้วยเทคนิคทางสเปกโทรสโกปี ได้แก่ UV, IR, MS และ NMR ร่วมกับการเปรียบเทียบข้อมูลที่เคยมีรายงานมาก่อนแล้ว จากนั้นศึกษาความเป็นพิษของสารเคมีที่แยกได้ต่อเซลล์มะเร็งช่องปาก (KB) เซลล์มะเร็งปอด (NCI-H187) และเซลล์มะเร็งเต้านม (MCF-7) ด้วยวิธี rezasurin microplate assay (REMA) รวมทั้งศึกษากลไกการยับยั้งเอนไซม์โทโพไอโซเมอเรสชนิดที่หนึ่งโดยใช้เซลล์ยีสต์

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NAPHATSAWAN POOREECHARUROT: CHEMICAL CONSTITUENTS AND CYTOTOXICITY OF *PAPHIOPEDILUM EXUL* ROOTS. ADVISOR: ASSOC. PROF. SUCHADA SUKRONG, Ph.D., CO-ADVISOR: ASSOC. PROF. RUTT SUTTISRI, Ph.D., 136 pp.

Chemical investigation of the roots of *Paphiopedilum exul* (Ridl.) Rolfe (family Orchidaceae) yielded one new stilbene, (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene, and eight known compounds including five stilbenes i.e. (*E*)-5-hydroxy-3-methoxystilbene, (*E*)-5-hydroxy-3,2′-dimethoxystilbene, (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3,5′-dimethoxystilbene, (E)-5,2′-dihydroxy-3,5′-dimethoxystilbene and (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene, and three flavonoids i.e. galangin, pinocembrin and alpinetin. The chemical structures of these constituents were identified and elucidated by spectroscopic techniques including UV, IR, MS and NMR, as well as comparison with previously reported data. Cytotoxicity of isolated compounds against KB, NCI-H187 and MCF-7 cell lines was then evaluated by the rezasurin microplate assay (REMA). Their topoisomerase I inhibitory activity was also examined using the yeast cell-based assay.

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LIST OF ABBREVIATIONS AND SYMBOLS

acetone-d_6	=	Deuterated acetone
br s	=	Broad singlet (for NMR spectra)
br d	=	Broad doublet (for NMR spectra)
°C	=	Degree Celsius
Calc	=	Calculated
CC	=	Column chromatography
$CDCl_3$	=	Deuterated chloroform
CD_3OD	=	Deuterated methanol
CH_2Cl_2	=	Dichloromethane
cm	=	Centimeter
cm^{-1}	=	Reciprocal centimeter (unit of wave number)
^{13}C NMR	=	Carbon-13 Nuclear Magnetic Resonance
CPT	=	Camptothecin
d	=	Doublet (for NMR spectra)
dd	=	Doublet of Doublets (for NMR spectra)
DMSO	=	Dimethyl sulfoxide
DNA	=	Deoxyribonucleic acid
δ	=	Chemical shift
ε	=	Molar absorptivity
EtOAc	=	Ethyl acetate
et al.	=	et alibi (and others)
FUC	=	Fluorescent unit from untreated condition
FUT	=	Fluorescent unit from treated condition
g	=	Gram
h	=	Hour
^1H NMR	=	Proton Nuclear Magnetic Resonance
HMBC	=	^1H-detected Heteronuclear Multiple Bond Correlation

HR-ESIMS	=	High Resolution Electrospray Ionization Mass Spectrometry
HSQC	=	Heteronuclear Single Quantum Coherence
Hz	=	Hertz
IC_{50}	=	Median Inhibitory Concentration
IR	=	Infrared
J	=	Coupling constant
Kg	=	Kilogram
L	=	Liter
λ_{max}	=	Wavelength at maximal absorption
µg	=	Microgram
µl	=	Microliter
µM	=	Micromolar
$[M+Na]^+$	=	Sodium adduct molecular ion
$[M+NH_4]^+$	=	Ammonium adduct molecular ion
m	=	Multiplet (for NMR spectra)
MeOH	=	Methanol
mg	=	Milligram
MHz	=	Megahertz
ml	=	Milliliter
mM	=	Millimolar
MS	=	Mass Spectrometry
m/z	=	Mass to charge ratio
NaOH	=	Sodium Hydroxide
n.d.	=	no data
ν_{max}	=	Wave number at maximal absorption
nm	=	Nanometer
NMR	=	Nuclear Magnetic Resonance
OD	=	Optical Density
ppm	=	Part-per-million
s	=	Singlet (for NMR spectra)

S.C. ura$^-$	=	Synthetic complete media lacking uracil
t	=	Triplet (for NMR spectra)
td	=	Triplet of doublets (for NMR spectra)
TLC	=	Thin Layer Chromatography
Top	=	Topoisomerase
UV	=	Ultraviolet
YPD	=	Yeast Peptone Dextrose

CHAPTER 1
INTRODUCTION

Orchids are highly valued for their beautiful and characteristic flowers. Moreover, orchids were also used medicinally for centuries. China has the oldest history of using orchid in medical treatment for as long as 4,800 years ago. Medicinal use of orchids was first reported in 'Materia Medica' of Chinese emperor 'Shen-nung'. In India and several countries in other continents there are also long history of therapeutic uses for orchids (Hossain, 2011).

Paphiopedilum is an Asian genus of slipper orchids within the subfamily Cypripedioideae of the family Orchidaceae and *Paphiopedilum exul* (Ridl.) Rolfe (Thai name: Rongthao nari lueang krabi) is a terrestrial lady's slipper orchid endemic to southern Thailand. The flower of this orchid species is single. Its large, pure white dorsal outer perianth has several maroon spots upon central lime green-veined patch. The inner perianth consists of two wavy, green ones and a glossy, yellow green pouch (labellum). There was no previous report of its medicinal use and chemical constituents. Although several molecular researches for the phylogenetic study of subfamily Cypripedioideae have been performed, its phytochemicals have rarely been investigated. There was only one previous report on the chemical constituents of a member of this genus i.e. *P. godefroyae* (Lertnitikul *et al.*, 2016). Therefore, phytochemical study of *Paphiopedilum exul* is a very enticing topic for phytochemists aiming to explore and gather detailed chemical data on these slipper orchids.

Cancer is a leading cause of death in all countries. In Thailand, statistics from Bureau of Policy and Strategy, Ministry of Public Health revealed that the ratio of mortality from cancer per 100,000 population was 87.6 in 2008 and increased to 98.5 in 2012. Moreover, data from World Health Organization (WHO) indicated that the number of new cases tend to increase from 14 to 22 million in the next 20 years. Major

aim of cancer treatment is to eradicate the malignant cells and extend the survival of patients. Chemotherapy is one of the potential treatments of cancer and plants are an alternative source of chemotherapeutic agents. Several well-known anticancer drugs currently used are derived from phytochemicals or their derivatives including *Vinca* alkaloids from *Catharanthus roseus*, taxanes from *Taxus brevifolia*, podophyllotoxin from *Podophyllum peltatum*, and camptothecin and its semisynthetic derivatives from *Camptotheca acuminata* (Fabricant and Farnsworth, 2001).

Preliminary evaluation the methanolic extract of *P. exul* roots demonstrated that it had potential to inhibit growth of cancer cells *in vitro*. The extract was thus subjected to the process of isolation and evaluation of cytotoxicity to identify the bioactive compounds which might have potential to be developed as anticancer agents.

The purposes of this research were as follows:

1. Isolation of compounds from the roots of *P. exul*
2. Identification of the chemical structures of isolated compounds
3. Evaluation of cytotoxicity of the isolated compounds

CHAPTER 2
LITERATURE REVIEW

2.1. Historical review of *Paphiopedilum exul*

2.1.1. Family Orchidaceae

Orchidaceae, commonly known as the orchid family, is a family of monocotyledonous flowering plants belonging to the order Asparagales (Bremer *et al.*, 2009). It is one of the two largest families of angiosperms, along with Asteraceae, and consists of about 900 genera and more than 27,000 accepted species (The Plant List, 2013). Because of its diversity, orchid family has been divided into five subfamilies, i.e. subfamily Apostasioideae, Cypripedioideae, Epidendroideae, Orchidoideae and Vanilloideae (Lin *et al*., 2015). Orchids are perennial herbs which can be found growing as terrestrial, epiphytic or lithophytic plants all over the earth especially in tropical areas. Their stems grow in two patterns: monopodial and sympodial, which have different directions of new leaf growth. The monopodial growth provides new leaf added to the apex, growing upward from a single bud, while sympodial plants make new leaf adjacent to an old one, so they often grow laterally. The roots of terrestrial orchids may be rhizome, corm or tuber, whereas epiphytic ones have modified aerial roots and some can form pseudobulbs. The leaves of orchids are simple leaves with leaf sheath, but with no stipule. The leaf venation is parallel-veined, except for some orchids in the subfamily Vanilloideae. The leaf shape can be ovate, lanceolate or orbiculate. The phyllotaxy is alternate. The flowers usually are zygomorphic and can occur as simple or inflorescence. They have two whorls of perianth, 3 inner and 3 outer ones. The medial inner perianth was modified to be a lip-like element called ‘labellum’, which is the important characteristic of orchid flower distinguishing them from other plants. Orchid flowers usually have 1 stamen, but those in subfamily Cypripedioideae and genus *Apostasia* have 2 stamens, whereas 3 stamens are found

only in genus *Neuwiedia*. The stamens are fused together with pistil to form a cylindrical structure called column or gynostegium. The ovaries are inferior, with parietal placentation (or axile in the subfamily Apostasioideae). The fruits are capsules containing numerous, very small seeds. Its unique flower shape, the size and number of its seeds and its pollination mechanism are the reasons why Orchidaceae family is diverse, widespread and comprised of enormous number of species (Dressler, 1981).

Orchids have high ornamental value because of their magnificent, characteristic flowers. In addition to decorative purpose, orchids were used as medicines for centuries. Medicinal use of orchid was first reported in China about 2,800 years B.C. in 'Materia Medica' of Chinese emperor 'Shen-nung'. *Bletilla striata*, *Gastrodia elata* and *Dendrobium* species are well known orchids in Chinese medicine. There are also long history of orchids for therapeutic uses in India, Europe, America, Australia and Africa (Hossain, 2011).

2.1.2. Subfamily Cypripedioideae

The subfamily Cypripedioideae is monophyletically classed as a group of slipper orchids consisting of five genera including *Cypripedium*, *Mexipedium*, *Phragmipedium*, *Selenipedium* and *Paphiopedilum*. The members of this subfamily are unique because of their distinct morphological characteristics. The most obvious one is the slipper-shaped, pouch-like labellum. Their flowers have two fertile stamens and a shield-like staminode. The lateral, outer perianths are fused to be synsepal (Lindley, 1840).

A small number of reports have mentioned folk medicinal uses of some members of this subfamily. In Indian traditional medicine, the roots of *Cypripedium pubescens (Cypripedium parviflorum* var. *pubescens)* was used to treat diabetes, diarrhea, dysentery, paralysis, impotence and malnutrition (Khory, 1982). *C. pubescens* was also employed as an antispasmodic and a sedative in both Indian and American folk medicine. Interestingly, its dried, powdered roots or fluidextract was official listed in United States Pharmacopoeia (USP) and used in the treatment of many ailments

(Singh and Duggal, 2009). *C. parviflorum*, or yellow lady's slipper, is another important medicinal herb in North America. Its root powder was used as drinking preparation for the treatment of insomnia, anxiety, fever, headache, neuralgia and many other diseases (Sievers, 1930). Roots of *C. elegans*, an Asian *Cypripedium* species found in China and Nepal, was used as nerve tonic in psychological disorders, epilepsy and rheumatism. Powdered roots of *C. calceolus*, a slipper orchid widely distributed from Europe to Asia, were boiled in hot water, sweetened with sugar and drunk as pain reducing agent (Singh and Dey, 2005). Moreover, seed capsules of *Selenipedium chica*, another slipper orchid of a different genus, are occasionally used as substitute of *Vanilla* (Duggal, 1971).

2.1.3. Genus *Paphiopedilum*

Paphiopedilum Pfitzer is a genus of lady's slipper orchids native to subtropical and tropical Asia (Cox *et al.*, 1997). The name of this genus comes from two Greek words: 'paphos' which means Venus and 'pedilon' which means pouch, thus it is often called Venus slipper orchid. There are about 130 accepted species belonging to this genus. Most of them are terrestrials, some are epiphytes and lithophytes. They grow in sympodial pattern lacking pseudobulbs. The new shoot takes over when the old one dies. Each plant has several leaves which are fleshy and glossy. The leaf shape can be short and rounded, or long and narrow. Some species have mottled leaves. Their roots are thick and fleshy. The flowers can be simple ones or in inflorescences (Braem *et al.*, 1999).

There are 14 valid species, including 17 taxa, found growing in Thailand. They have been recorded as follows (Office of the Forest Herbarium, 2014):

1. *Paphiopedilum appletonianum* (Gower) Rolfe
2. *Paphiopedilum bellatulum* (Rchb. f.) Stein
3. *Paphiopedilum callosum* (Rchb. f.) Stein var. *callosum*
4. *Paphiopedilum callosum* (Rchb. f.) Stein var. *potentianum* (O. Gruss & Roeth) P. J. Cribb

5. *Paphiopedilum callosum* (Rchb. f.) Stein var. *sublaeve* (Rchb. f.) P. J. Cribb
6. *Paphiopedilum charlesworthii* (Rolfe) Pfitzer
7. *Paphiopedilum concolor* (Lindl. ex Bateman) Pfitzer
8. *Paphiopedilum esquirolei* Schltr. or *Paphiopedilum hirsutissimum* (Lindl. ex Hook.) Stein var. *esquirolei* (Schltr.) K. Karas. & K. Saito
9. *Paphiopedilum godefroyae* (God.-Leb.) Stein
10. *Paphiopedilum godefroyae* (God.-Leb.) Stein var. *ang-thong* (Fowlie) Braem
11. *Paphiopedilum exul* (Ridl.) Rolfe
12. *Paphiopedilum niveum* (Rchb. f.) Stein
13. *Paphiopedilum parishii* (Rchb. f.) Stein
14. *Paphiopedilum sukhakulii* Schoser & Senghas
15. *Paphiopedilum thaianum* Iamwir.
16. *Paphiopedilum vejvarutianum* O. Gruss & Roellke
17. *Paphiopedilum villosum* (Lindl.) Stein

2.1.4. *Paphiopedilum exul*

Paphiopedilum exul (Ridl.) Rolfe was first described in the Gardeners' Chronicle as *Cypripedium insigne* var. *exul* by Ridley in 1891 and transferred to the genus *Paphiopedilum* in 1896 (Cribb and Robbins, 1993). It is a terrestrial lady's slipper orchid endemic to Peninsular Thailand. It was found in rock crevices at sea level up to 50 meters. Its leaves are oblong, 1.8-3 cm in width and 25-30 cm in length, suberect, clear green in colour, fleshy and glossy. The flower is single, with hairy peduncle, 15-20 cm long. The large dorsal outer perianth is cuspidate, pure white with central lime green-veined and maroon spotted. The lateral inner perianths are wavy green and the central one is a glossy, yellow green pouch (Nanakorn and Watthana, 2008).

Figure 1 *Paphiopedilum exul* (Ridl.) Rolfe (Nanakorn and Watthana, 2008)

2.2. Chemical constituents of orchids in the subfamily Cypripedioideae

The Orchidaceae has been reported as sources of many bioactive secondary metabolites including alkaloids, flavonoids, lignans, terpenoids and stilbenoids. However, phytochemical and pharmaceutical studies of slipper orchids have rarely been performed. The chemical constituents found in orchids of the subfamily Cypripedioideae are shown in **Table 1** and **Figure 4**.

2.2.1. Stilbenes

Stilbenes are a subtype of stilbenoids which consist of five categories: stilbenes, oligostilbenes, bibenzyls, bisbibenzyls and phenanthrenes. The skeletal structure of stilbenes is 1,2-diphenylethylene, that is, two phenyl rings conjugated by a double bond (**Figure 2**). They have two possible isomers, *trans*-and *cis*-configuration, and are called *(E)*- and *(Z)*-stilbenes, respectively. Biosynthetically, they are originated from 4-coumaryl-CoA or cinnamoyl-CoA in the phenylpropanoid pathway. From 1995 to 2008, about 125 stilbenes had been reported in plant kingdom including marchantiophytes (Hepaticae and Lejeuneaceae), pteridophytes (Ophioglossaceae), gymnosperms (Gnetaceae), monocots (Iridaceae, Liliaceae, Orchidaceae, Stemonaceae and Zingiberaceae) and dicots (Aceraceae, Asteraceae, Burseraceae, Combretaceae, Cyperaceae, Dipterocarpaceae, Euphorbiaceae, Leguminosae, Meliaceae, Moraceae, Polygonaceae, Rosaceae and Vitaceae) (Shen *et al.*, 2013).

Although there are limited reports of stilbenes in family Orchidaceae, some studies showed that slipper orchids are rich sources of this type of compounds. Twenty-four stilbenes have been isolated from orchid species in the subfamily Cypripedioideae. Thirteen stilbenes (**1-2**, **7-11**, **13-15**, **17-19**) were isolated from *Phragmipedium calurum*. Some of them exhibited antiproliferative activity against several human cancer cell lines. Ten stilbenoid constituents (**1-3**, **10-11**, **13-14**, **17-18**, **21**) were present in *Phragmipedium longifolium*. *Phragmipedium hybrid*, a hybrid of *P. longifolium* and *P. lindleyanum*, afforded nine stilbenoid compounds (**2**, **6**, **10-12**, **16**, **18**, **20-21**); some of them are substituted with one or two, rather unusual 4-hydroxybenzyl moieties (Garo *et al.*, 2007; Starks *et al.*, 2012). In addition, nine stilbenes (**2**, **4-8**, **22-24**) were found in *Paphiopedilum godefroyae* and were shown to be cytotoxicity against NCI-H187 cell line (Lertnitikul *et al.*, 2016).

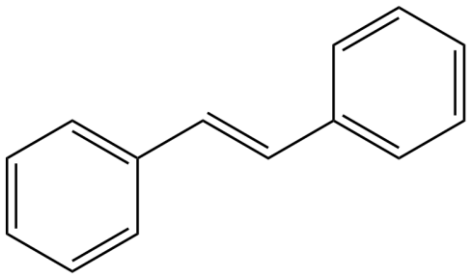
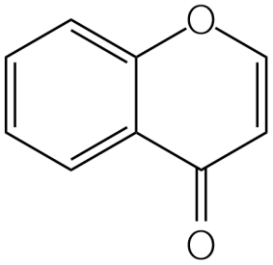


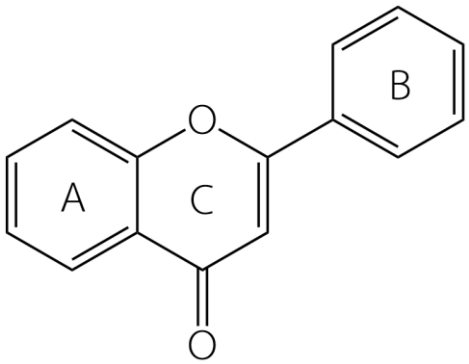
Figure 2 Structure of 1,2-diphenylethylene

2.2.2. Flavonoids

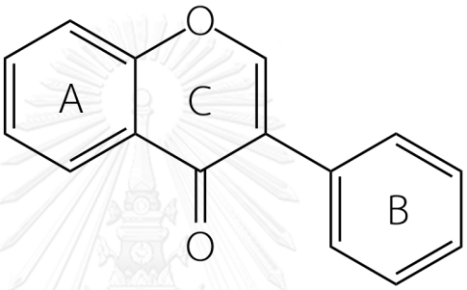
Flavonoids are an important group of plant secondary metabolites derived from phenylpropanoid pathway. Characteristic structure of flavonoids is γ-benzopyrone nucleus carrying a phenyl B ring attached to 2-position, or 3-position for isoflavonoids (**Figure 3**). Flavonoids have been found abundantly in plant kingdom, but only two of them have previously been reported from slipper orchids. Chrysin (**25**) was found in *Cypripedium macranthos* var. *rebunense* and was demonstrated to possess antifungal activity (Shimura *et al.*, 2007). Another flavonoid, pinocembrin, has been isolated from the roots of *Paphiopedilum godefroyae* from Thailand (Lertnitikul *et al.*, 2016).



(a) γ-benzopyrone nucleus



(b) flavonoid skeleton



(c) isoflavonoid skeleton

Figure 3 Skeletal structures of flavonoids

2.2.3. Miscellaneous

In addition to stilbenes and flavonoids, other classes of phytochemicals have also been reported as constituents of the orchids in subfamily Cypripedioideae. Lusianthrin (**27**), a phenanthrene, was found in *Cypripedium macranthos* var. *rebunense* with antifungal activity (Shimura *et al.*, 2007). A dihydrophenanthrene, orchinol (**28**), and a skin sensitizing quinone, cypripedin (**29**), have been isolated from the leaves of *Cypripedium calceolus* (Schmalle and Hausen, 1979). An alkylresorcinol, 5-(2-acetoxynonyl) resorcinol (**30**), was isolated from *Phragmipedium calurum* during an anticancer screening (Starks *et al.*, 2012).

2.3. Anticancer and topoisomerase-targeted drug development

2.3.1. Cancer and drug discovery

Cancer is a group of diseases with uncontrolled cell growth as an important pathological feature. The abnormal cells grow in unknown direction and have potential to spread to other parts of the body (De Martel *et al.*, 2012). Currently, over 100 types of cancer have been found (National Cancer Institute, 2016). Several treatments have been used to cure cancer including surgery, chemotherapy, radiation therapy, hormonal therapy, targeted therapy and palliative care, depending on the type of cancer. Chemotherapy is one of major cancer treatment in order to decrease the number of abnormal cells. Development of chemotherapeutic drugs focuses on chemical agents that have potential to kill cancerous cells. Plants are one of alternative sources of anticancer agents and many anticancer drugs used in clinical treatment are derived from plant metabolites including *Vinca* alkaloids (vincristine, vinblastine) from *Catharanthus roseus*, taxanes (paclitaxel, docetaxel) from *Taxus brevifolia*, podophyllotoxin from *Podophyllum peltatum*, and camptothecin and its semi-synthesized derivatives (topotecan, irinotecan) from *Camptotheca acuminata* (Fabricant and Farnsworth, 2001).

2.3.2. Topoisomerase-targeted anticancer agents

Research on the underlying mechanisms of anticancer drugs is one of important steps in drug development. DNA topoisomerase (Top) enzymes are important for the process of cell proliferation and play essential roles in DNA replication and transcription. They are also the targets of several currently used anticancer drugs (Forterre, 2012). Two types of DNA topoisomerase-targeted drugs are known: Top I inhibitors such as topotecan and irinotecan and Top II inhibitors such as etoposide and tenoposide. Mechanistically, both types of topoisomerase inhibitors can be distinguished, based on their mechanism of enzyme inhibition, into two distinct

categories. The first category is “poison” which stabilizes the covalent enzyme-DNA complex, known as cleavage complex, and thus preventing DNA relegation. The second one is “catalytic inhibitor” which interferes other steps in the catalytic cycle of topoisomerase enzymes. The majority of currently used topoisomerase-targeted anticancer drugs are ‘poison’ because of their potent cytotoxicity. However, application of catalytic inhibitors in cancer treatment has also been investigated. Catalytic inhibitors can be used as adjunctive treatment (Makeyev *et al.*, 2012; Mir *et al.*, 2012). An example is dexarazoxane, an approved topoisomerase II catalytic inhibitor, which is used for preventing cardiotoxicity and minimizing extravasation following tissue damage from anthracyclines treatment (Xiang *et al.*, 2009; Schulmeister, 2011; Kushwah, 2013). Furthermore, a topoisomerase I catalytic inhibitor, evodiamine, has been reported to enhance inhibiting activity in camptothecin-resistant cancer cell lines (Pan *et al.*, 2012).

Table 1 Chemical constituents of orchids in the subfamily Cypripedioideae

Compound	Source	Plant part	Biological activities	References
(*E*)-5-Hydroxy-3-methoxystilbene (**1**)	*Phragmipedium calurum*	whole plant	-	Garo *et al*., 2007; Starks *et al*., 2012
	Phragmipedium longifolium	whole plant		
(*E*)-5,2′-Dihydroxy-3-methoxy stilbene (**2**)	*Phragmipedium calurum*	whole plant	cytotoxic	Garo *et al*., 2007; Lertnitikul *et al*., 2016
	Phragmipedium longifolium	whole plant		
	Phragmipedium hybrid	whole plant		
	Paphiopedilum godefroyae	roots		
(*E*)-5,3′-Dihydroxy-3-methoxy stilbene (**3**)	*Phragmipedium longifolium*	whole plant	-	Garo *et al*., 2007
(*E*)-5,4′-Dihydroxy-3-methoxy stilbene (**4**)	*Paphiopedilum godefroyae*	roots	cytotoxic	Lertnitikul *et al*., 2016
(*E*)-5-Hydroxy-3,2′dimethoxy stilbene (**5**)	*Paphiopedilum godefroyae*	roots	cytotoxic	Lertnitikul *et al*., 2016

Table 1 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

Compound	Source	Plant part	Biological activities	References
(*E*)-5,2′-Dihydroxy-3,5′-dimethoxy stilbene (**6**)	*Phragmipedium* hybrid	whole plant	cytotoxic	Garo *et al*., 2007; Lertnitikul *et al*., 2016
	Paphiopedilum godefroyae	roots		
(*E*)-2′-Hydroxy-3,5-dimethoxy stilbene (**7**)	*Phragmipedium calurum*	whole plant	cytotoxic	Garo *et al*., 2007; Starks *et al*., 2012; Lertnitikul *et al*., 2016
	Paphiopedilum godefroyae	roots		
(*E*)-2′,3′-Dihydroxy-3,5-dimethoxy stilbene (**8**)	*Phragmipedium calurum*	whole plant	cytotoxic	Garo *et al*., 2007; Lertnitikul *et al*., 2016
	Paphiopedilum godefroyae	roots		
(*E*)-4-Hydroxy-3-methoxystilbene (**9**)	*Phragmipedium calurum*	whole plant	-	Garo *et al*., 2007
(*E*)-2-(4″-Hydroxybenzyl)-5-hydroxy-3-methoxystilbene (**10**)	*Phragmipedium calurum*	whole plant	-	Garo *et al*., 2007; Starks *et al*., 2012
	Phragmipedium longifolium	whole plant		
	Phragmipedium hybrid	whole plant		

Table 1 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

Compound	Source	Plant part	Biological activities	References
(*E*)-2-(4″-Hydroxybenzyl)-5,2′-dihydroxy-3-methoxystilbene (**11**)	*Phragmipedium calurum*	whole plant	-	Garo *et al*., 2007
	Phragmipedium longifolium	whole plant		
	Phragmipedium hybrid	whole plant		
(*E*)-2-(4″-Hydroxybenzyl)-5,2′-dihydroxy-3,5′-dimethoxystilbene (**12**)	*Phragmipedium* hybrid	whole plant	-	Garo *et al*., 2007
(*E*)-2-(4″-Hydroxybenzyl) -3-hydroxy-5-methoxystilbene (**13**)	*Phragmipedium calurum*	whole plant	cytotoxic	Garo *et al*., 2007; Starks *et al*., 2012
	Phragmipedium longifolium	whole plant		
(*E*)-2-(4″-Hydroxybenzyl)-3,2′-dihydroxy-5-methoxystilbene (**14**)	*Phragmipedium calurum*	whole plant	-	Garo *et al*., 2007
	Phragmipedium longifolium	whole plant		
(*E*)-2-(4″-Hydroxybenzyl)-2′-hydroxy-3,5-dimethoxystilbene (**15**)	*Phragmipedium calurum*	whole plant	cytotoxic	Starks *et al*., 2012

Table 1 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

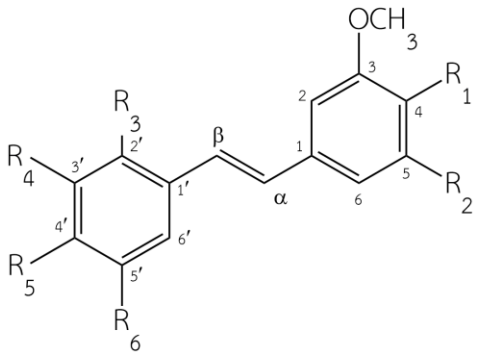
Compound	Source	Plant part	Biological activities	References
(*E*)-2-(4″-Hydroxybenzyl)-2′,3′-dihydroxy-3,5-dimethoxystilbene (**16**)	*Phragmipedium* hybrid	whole plant	-	Garo *et al*., 2007
(*E*)-2,6-Bis(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene (**17**)	*Phragmipedium calurum*	whole plant	cytotoxic	Garo *et al*., 2007; Starks *et al*., 2012
	Phragmipedium longifolium	whole plant		
(*E*)-2,6-Bis(4″-hydroxybenzyl)-5,2′-hydroxy-3-methoxystilbene (**18**)	*Phragmipedium calurum*	whole plant	-	Garo *et al*., 2007
	Phragmipedium longifolium	whole plant		
	Phragmipedium hybrid	whole plant		
(*E*)-4-(4″-Hydroxybenzyl)-5-hydroxy-3-methoxystilbene (**19**)	*Phragmipedium calurum*	whole plant	cytotoxic	Starks *et al*., 2012
Phragmidimer A (**20**)	*Phragmipedium* hybrid	whole plant	-	Garo *et al*., 2007

Table 1 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

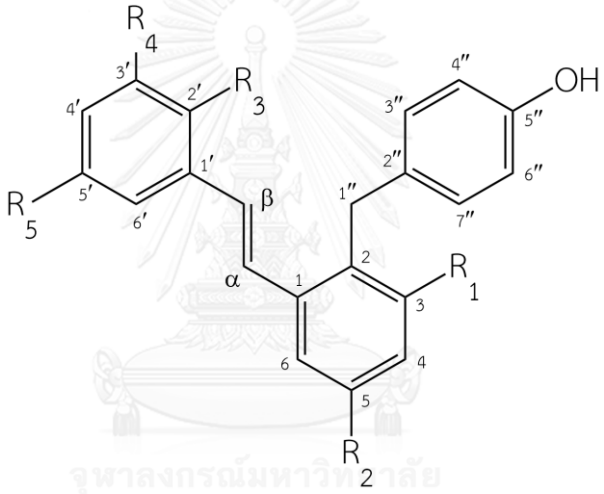
Compound	Source	Plant part	Biological activities	References
Phragmidimer B (**21**)	*Phragmipedium longifolium*	whole plant	-	Garo *et al*., 2007
	Phragmipedium hybrid	whole plant		
2-(5′-Hydroxy-3′-methoxyphenyl)-6-hydroxy-5-methoxybenzofuran (**22**)	*Paphiopedilum godefroyae*	roots	cytotoxic	Lertnitikul *et al*., 2016
2-(5′-Hydroxy-3′-methoxyphenyl)-5,6-dimethoxybenzofuran (**23**)	*Paphiopedilum godefroyae*	roots	cytotoxic	Lertnitikul *et al*., 2016
2-(3′,5′-Dimethoxyphenyl)-6-hydroxy-5-methoxybenzofuran (**24**)	*Paphiopedilum godefroyae*	roots	cytotoxic	Lertnitikul *et al*., 2016
Chrysin (**25**)	*Cypripedium macranthos* var. *rebunense*	plantlets	antifungal	Shimura *et al*., 2007
Pinocembrin (**26**)	*Paphiopedilum godefroyae*	roots	anticancer, antimicrobial, antifungal, anti-inflammatory, neuroprotective	Lertnitikul *et al*., 2016; Rasul *et al*., 2013

Table 1 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

Compound	Source	Plant part	Biological activities	References
Lusianthrin (**27**)	*Cypripedium macranthos* var. *rebunense*	plantlets	antifungal	Shimura *et al*., 2007
Orchinol (**28**)	*Cypripedium calceolus*	leaves	-	Schmalle and Hausen, 1979
Cypripedin (**29**)	*Cypripedium calceolus*	leaves	skin sensiting	Schmalle and Hausen, 1979
5-(2-Acetoxynonyl) resorcinol (**30**)	*Phragmipedium calurum*	whole plant	-	Starks *et al*., 2012

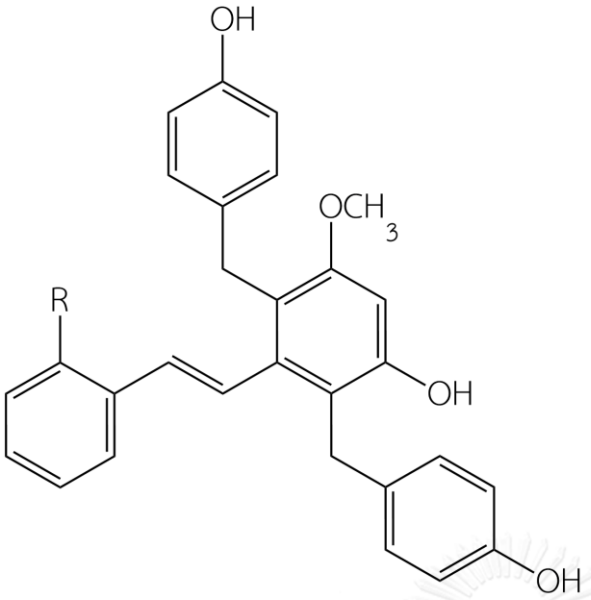


	R_1	R_2	R_3	R_4	R_5	R_6
1	H	OH	H	H	H	H
2	H	OH	OH	H	H	H
3	H	OH	H	OH	H	H
4	H	OH	H	H	OH	H
5	H	OH	OCH_3	H	H	H
6	H	OH	OH	H	H	OCH_3
7	H	OCH_3	OH	H	H	H
8	H	OCH_3	OH	OH	H	H
9	OH	OH	H	H	H	H



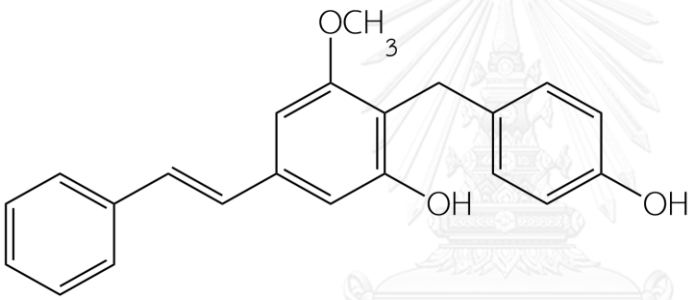
	R_1	R_2	R_3	R_4	R_5
10	CH_3	H	H	H	H
11	CH_3	H	OH	H	H
12	CH_3	H	OH	H	OCH_3
13	H	CH_3	H	H	H
14	H	CH_3	OH	H	H
15	CH_3	CH_3	OH	H	H
16	CH_3	CH_3	OH	OH	H

Figure 4 Chemical constituents of orchids in the subfamily Cypripedioideae



17: R = H

18: R = OH



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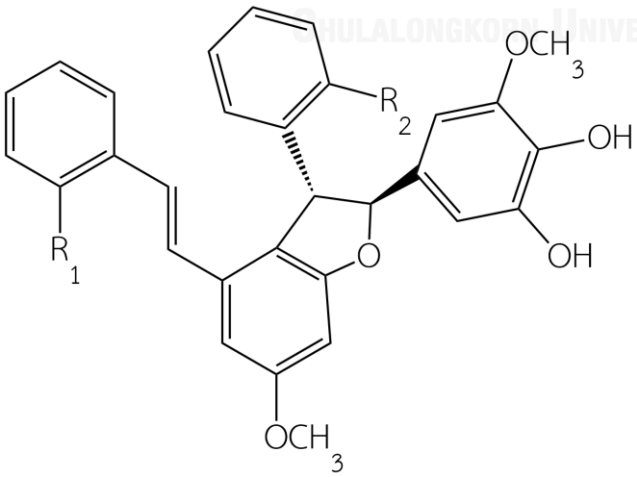


	R_1	R_2
20	H	OH
21	OH	H

Figure 4 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

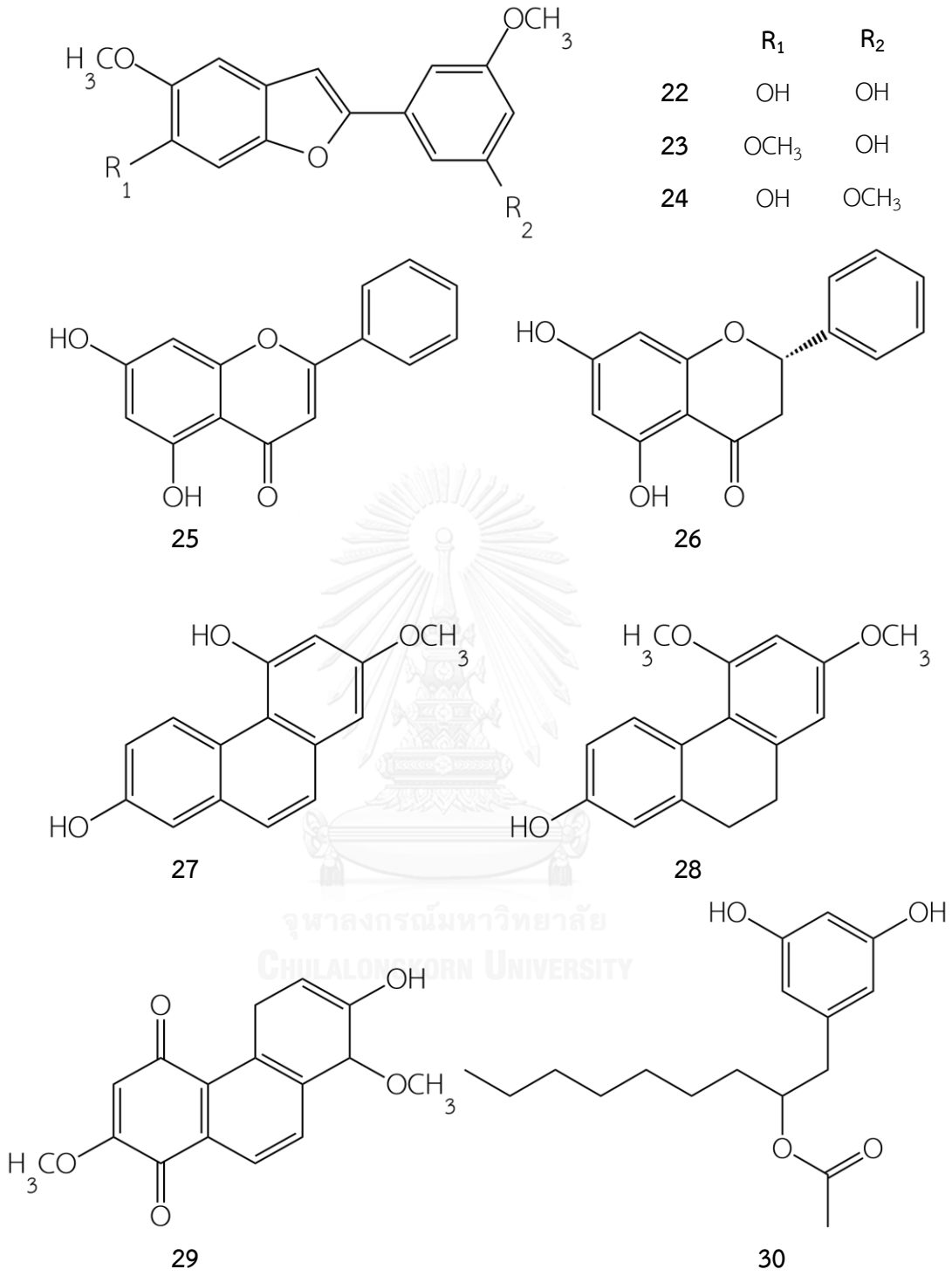


Figure 4 Chemical constituents of orchids in the subfamily Cypripedioideae (continued)

CHAPTER 3
EXPERIMENTAL

3.1. Source of Plant Materials

The whole plants of *Paphiopedilum exul* (Ridl.) Rolfe were purchased from Chatuchak market, Bangkok, in January 2015. It was identified by comparison with authentic specimen (QBG No. 13143) at the herbarium of the Botanical Garden Organization, Ministry of Natural Resources and Environment, Thailand. The fresh roots were separated from the plants, cleaned and dried at temperature not more than 50 °C.

3.2. General Techniques

3.2.1. Analytical Thin Layer Chromatography (TLC)

Technique:	One dimension, ascending
Adsorbent:	Silica gel 60 F254 (E. Merck) pre-coated plates
Layer thickness:	0.2 mm
Distance:	5 cm
Temperature:	Laboratory temperature (30-35 °C)
Detection:	1. Ultraviolet light (254 and 365 nm)
	2. Anisaldehyde reagent and heating at 105 °C for 10 minutes

3.2.2. Column Chromatography

3.2.2.1. Conventional Column Chromatography

Adsorbent: Silica gel 60 number 7734 (particle size 0.063-0.200 mm) and number 9385 (particle size 0.040-0.063 mm) (E. Merck)

Packing method: Wet packing: The adsorbent was mixed with the eluent into slurry, then poured into a column and allowed to settle.

Sample loading: The sample was dissolved in a small amount of the eluent, and then applied gently on top of the column.

Detection: Fractions were examined by TLC technique, as described in 3.2.1.

3.2.2.2. Size-Exclusion Column Chromatography

Gel filter: Sephadex LH-20 (Pharmacia Biotech AB)

Packing method: Gel filter was suspended in the eluent and left standing to swell for 24 hours before it was poured into the column and allowed to set tightly.

Sample loading: The sample was dissolved in a small amount of the eluent, and then applied gently on top of the column.

Detection: Fractions were examined by TLC technique, as described in 3.2.1.

3.2.3. Spectroscopic Techniques

3.2.3.1. Ultraviolet (UV) Spectra

UV spectra were obtained on a Shimadzu UV-160A spectrophotometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University). Methanol was used as solvent.

3.2.3.2. Infrared (IR) Spectra

IR spectra (KBr disc) were recorded on a Perkin Elmer FT-IR 1760X spectrometer (Scientific and Technological Research Equipment Center, Chulalongkorn University).

3.2.3.3. Mass Spectra

Electrospray Ionization (ESI) mass spectra were obtained on a Bruker micrOTOF mass spectrometer (Department of Medical Sciences, Ministry of Public Health).

3.2.3.4. Proton and Carbon-13 Nuclear Magnetic Resonance (^{1}H and ^{13}C NMR) Spectra

^{1}H (300 MHz) and ^{13}C (75 MHz) NMR spectra were recorded on a Bruker DPX-300 FT-NMR spectrometer (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

The solvents used were $CDCl_3$, acetone-d_6 or DMSO-d_6. The solvent signals were used as reference for the calibration of chemical shifts.

3.2.4. Optical Rotation

Optical rotation was measured on a Perkin-Elmer 314 polarimeter using a sodium lamp operating at 589 nm (Pharmaceutical Research Instrument Center, Faculty of Pharmaceutical Sciences, Chulalongkorn University).

3.3. Extraction and Isolation of Compounds from *Paphiopedilum exul* Roots

Dried roots of *P. exul* (380 g) were ground and macerated three times with MeOH 3 L at room temperature. The combined MeOH extract was concentrated under reduced pressure to give 100 g of dried MeOH extract. A portion of the extract (50 g) was separated on a silica gel column (1.25 kg, 10 × 40 cm), washed down with *n*-hexane/acetone (3:1). Eighty three fractions (200 ml each) were collected and washed down with MeOH, and combined into eight major fractions (A-H) after comparison of their TLC profiles, as shown in **Table 2**.

Table 2 Combined fractions from MeOH extract

Fraction code	from fraction number	Weight (g)
A	1-4	0.40
B	5-16	1.12
C	17-30	2.91
D	31-38	0.56
E	39-68	9.90
F	69-74	1.53
G	75-83	1.28
H	Wash down with MeOH	31.59

3.3.1. Isolation of compounds PE01 and PE02

An amount (16.7 mg) of compound PE01 was obtained from fraction C when it crystallized from the eluated solution of this fraction. The remaining fraction C (2.91 g) was chromatographed on a silica gel column (150 g, 4.5 × 19 cm), eluted with CH_2Cl_2/acetone (40:1), to provide one hundred and seventy two subfractions (20 ml each). All subfractions were combined according to their TLC profiles to yield six major subfractions (C1-C6), as shown in **Table 3**. Crystals of compound PE01 (87.5 mg) was obtained from subfraction C6, making its total yield to be 104.2 mg. (0.00208% yield). Another compound, PE02 (90.7 mg, 0.00181% yield) crystallized from subfraction C5.

Table 3 Combined fractions from fraction C

Subfraction code	from fraction number	Weight (g)
C1	1-16	0.20
C2	17-40	0.90
C3	41-50	0.04
C4	51-57	0.02
C5	58-72	0.58
C6	73-172	1.23

3.3.2. Isolation of compounds PE03 and PE04

Subfraction C2 (890 mg) was subjected to a Sephadex LH-20 CC using MeOH as the eluent, to yield three subfractions (C21-C23). Subfraction C23 (300 mg) was separated by sephadex LH-20, using MeOH as the eluent, gave two subfractions (C231-C232). Subfraction C22 and C232 (649 mg) were pooled and loaded on silica gel column (30 g, 2.5 x 14 cm), eluted with CH_2Cl_2, to provide three subfractions (C221-C223). Silica gel CC of subfraction C221 using CH_2Cl_2 as an eluent yielded four subfractions (C2211-C2214). Separation of subfraction C2211 on a silica gel column (15 g, 1.5 x 17 cm) eluting with hexane/ CH_2Cl_2 (1:1) provided five subfractions (C22111-C22115). Compounds PE03 (9.0 mg) and PE04 (6.7 mg) were obtained from subfractions C22111 and C22115 after evaporation of eluting solvent, respectively. Subsequent purification of subfraction C22112 over a silica gel column (10 g, 1.5 x 11 cm), using hexane/ CH_2Cl_2 (1:2) as the mobile phase, afforded compound PE03 (19.8 mg) from subfraction C221121. Subfraction C22113 was subjected to silica gel CC (10 g, 1.5 x 11

cm), washed down with hexane/ CH_2Cl_2 (1:2), to yield five subfractions (C221131-C221135). Compound PE03 (24.9 mg) was obtained from subfraction C221131, providing a total amount of 53.7 mg (0.00107% yield). The other compound, PE04 (7.7 mg) was obtained from subfraction C221135. Silica gel CC (10 g, 1.5 x 11 cm) of subfraction C22114, using CH_2Cl_2 as an eluent, gave four subfractions (C221141-C221144), and compound PE04 (7.0 mg) was obtained from subfraction C221143. Subfractions C222, C2212, C221134 and C221142 were combined and chromatographed on a silica gel column (10 g, 1.5 x 11 cm), eluted with CH_2Cl_2, to give two subfractions (C2221-C2222), and compound PE04 (18.4 mg) was obtained from subfraction C2222. Purification 0f subfraction C2221 over a silica gel column (10 g, 1.5 x 11 cm), eluting with hexane/ CH_2Cl_2 (1:2), afforded compound PE04 (40.0 mg). The total yield of compound PE04 was 79.8 mg (0.00160% yield).

3.3.3. Isolation of compound PE05

Fraction E (9.90 g) was separated on a silica gel column (300 g, 4.5 × 40 cm), washed down with *n*-hexane/acetone (2:1), to provide one hundred and twenty subfractions (30 ml each). After verifying their TLC profiles, these collected subfractions were combined into six subfractions (E1-E6), as shown in **Table 4**. Size exclusion chromatography of fraction E5 (780 mg) on a Sephadex LH-20 column eluted with MeOH yielded four subfractions (E51-E54). Subfraction E53 (290 mg) was chromatographed on a silica gel column (15 g, 2 × 14 cm), eluted with CH_2Cl_2/acetone (20:1), to give five subfractions (E531-E535). Compound PE05 (58.0 mg, 0.00116% yield) was obtained from subfraction E534.

Table 4 Combined fractions from fraction E

Subfraction code	from fraction number	Weight (g)
E1	1-30	0.31
E2	31-42	0.67
E3	43-66	3.75
E4	67-82	0.49
E5	83-102	0.78
E6	103-120 and wash down with MeOH	2.31

3.3.4. Isolation of compound PE06

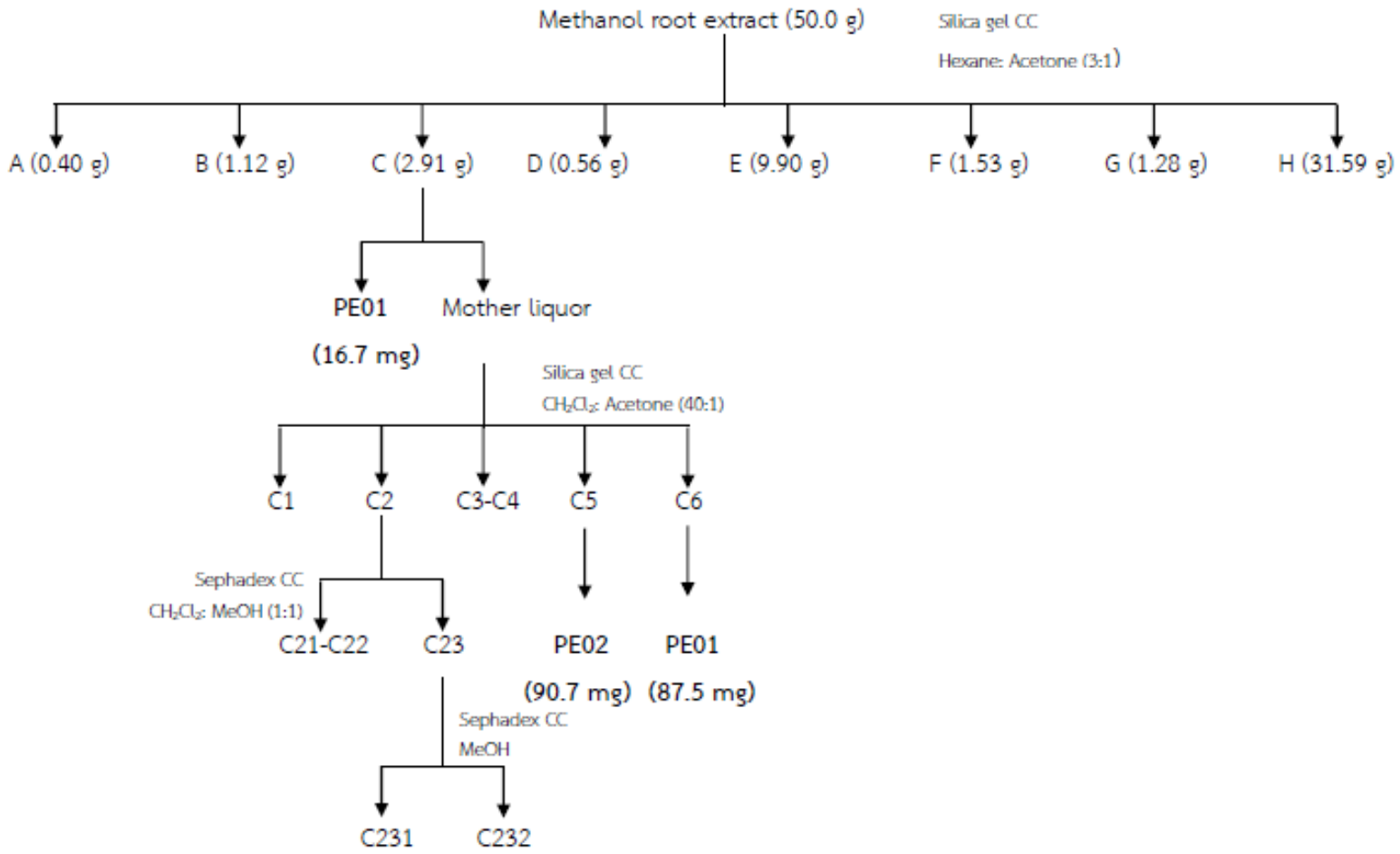
Subfraction E532 (102 mg) was subjected to silica gel CC (10 g, 2 × 10 cm) eluted with CH_2Cl_2/EtOAc (9:1) to give three subfractions (E5321-E5323). Compound PE06 (1.1 mg, 0.00002% yield) was obtained as colorless needle crystals from subfraction E5323.

3.3.5. Isolation of compound PE07

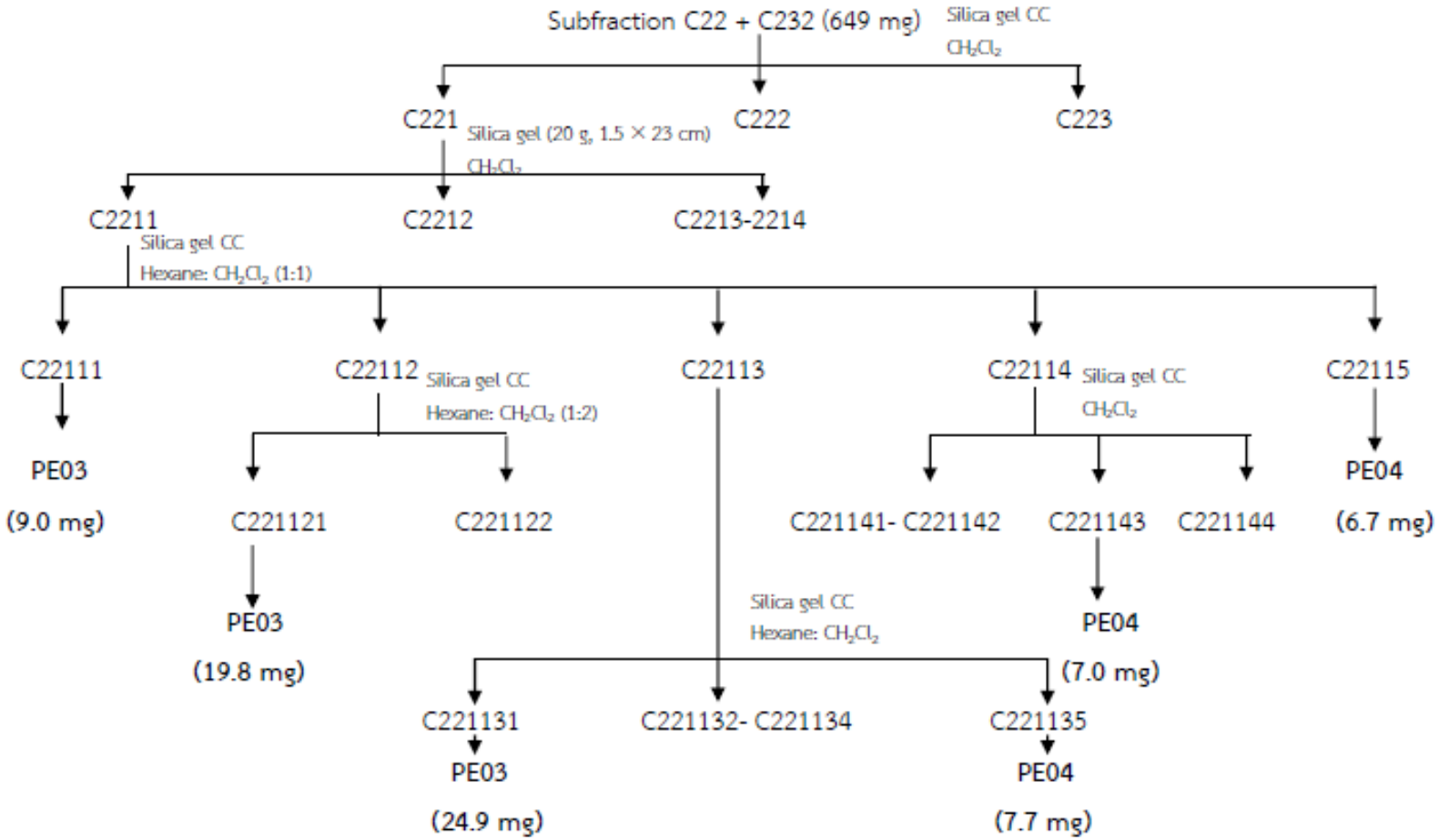
Subfraction E3 (3.4 g) was chromatograph on a silica gel column (175 g, 4.5 × 26 cm) eluted with CH_2Cl_2/acetone (30:1) to give nine subfractions (E31-E39). Subfraction E38 yielded compound PE07 (32.0 mg, 0.00064% yield) after evaporation of the eluting solvent.

3.3.6. Isolation of compounds PE08 and PE09

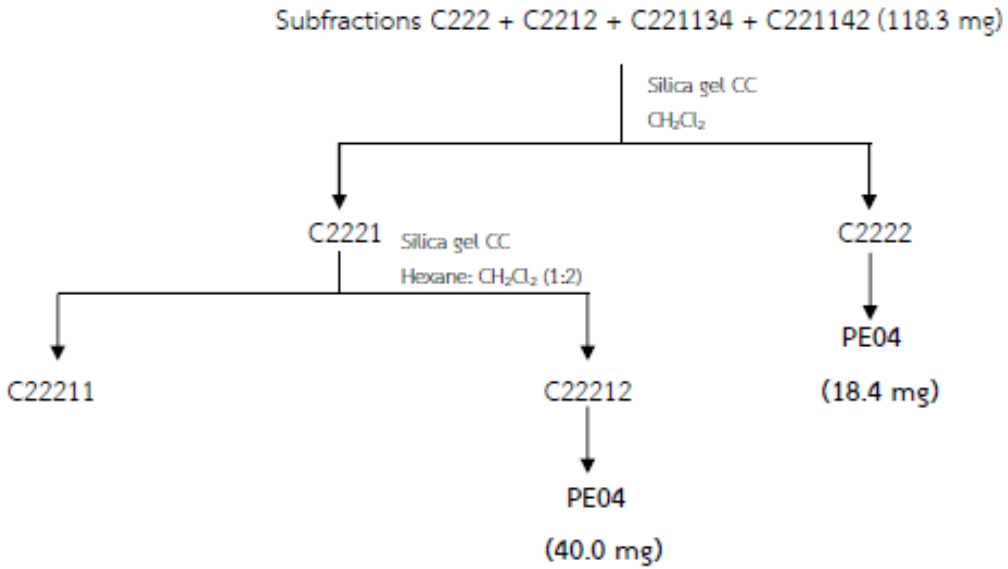
Further purification of subfraction E34 (491 mg) on a silica gel column (175 g, 4.5 × 26 cm), eluted with CH_2Cl_2/acetone (30:1), afforded five subfractions (E341-E344). Compound PE08 (38.4 mg) was obtained from subfraction E344 after solvent evaporation. Silica gel CC (5 g, 1 × 14 cm) of subfraction E343 yielded three subfractions (E3431-E3433), and compound PE08 (5.2 mg) was also obtained from subfraction E3433. Thus, the total amount of compound PE08 was 43.6 mg (0.00087% yield). The other compound, PE09 (2.0 mg) was obtained from subfraction E3431. Subfraction E342 (15.3) was further chromatographed on silica gel (6.5 g, 1 × 19 cm) to give three subfractions (E3421-E3423), and compound PE09 (13.3 mg) was obtained from subfraction E3423. Therefore, the total yield of compound PE09 was 15.3 mg (0.00031% yield).



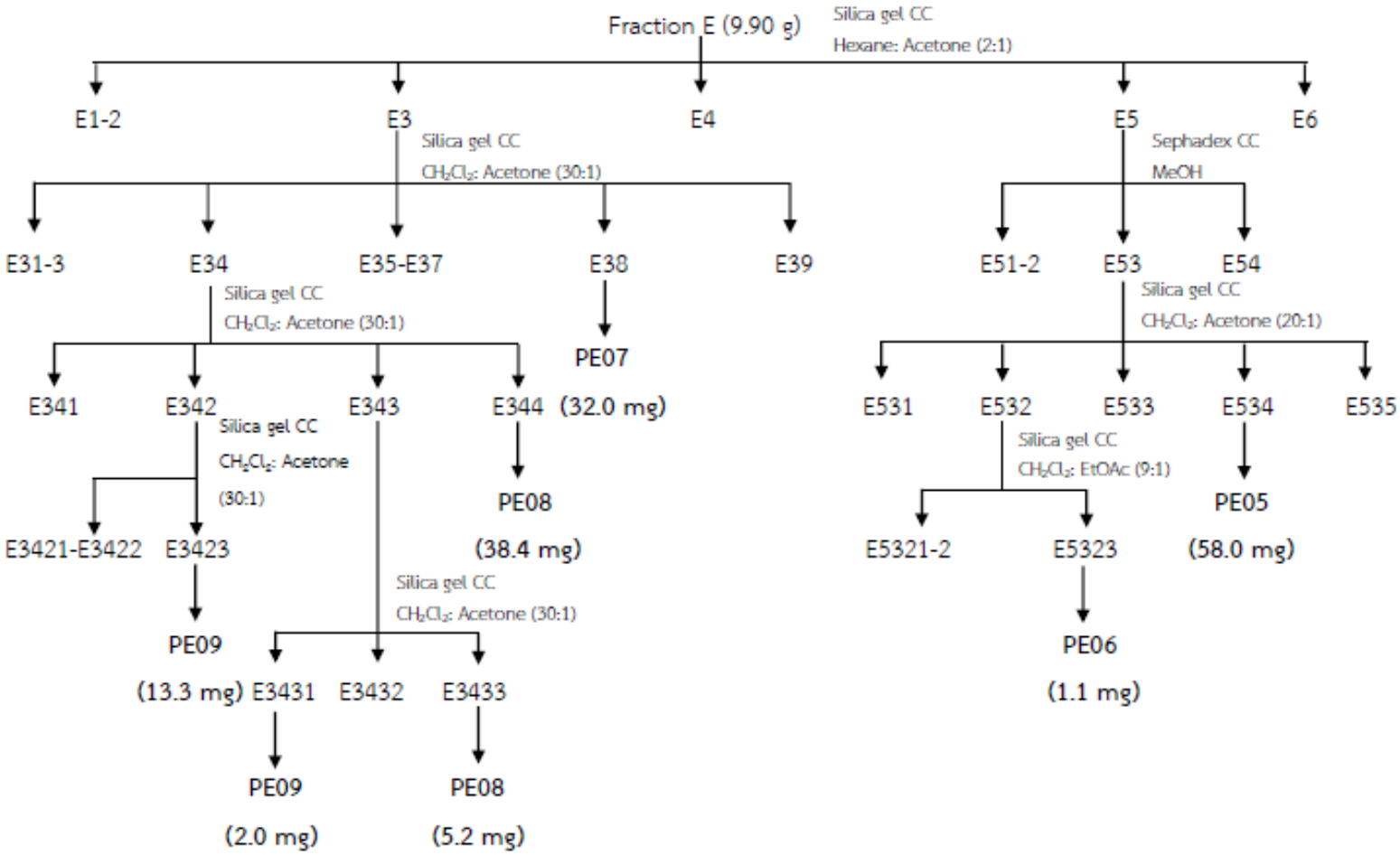
Scheme 1 Isolation of compounds from methanolic extracts of Paphiopedilum exul roots



Scheme 1 Isolation of compounds from methanolic extracts of *Paphiopedilum exul* roots (continued)



Scheme 1 Isolation of compounds from methanolic extracts of *Paphiopedilum exul* roots (continued)



Scheme 1 Isolation of compounds from methanolic extracts of *Paphiopedilum exul* roots (continued)

3.4. Physical and spectral data of isolated compounds

3.4.1. Compound PE01

Compound PE01 was obtained as yellow needle crystals, soluble in methanol.

UV : λ_{max} (MeOH) nm (log ε): 228 (4.26), 273 (4.24), 359 (4.23); **Figure 14**.

IR: ν_{max} cm^{-1} : 3141, 1655, 1606, 1306; **Figure 15**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 293.0478; **Figure 16**.

^{1}H NMR : δ ppm, 300 MHz, in DMSO-d_6; 6.21 (1H, *d*, *J* = 2.1 Hz), 6.46 (1H, *d*, *J* = 2.1 Hz), 7.52 (1H, *m*), 7.55 (2H, *m*), 8.14 (2H, *dd*, *J* = 2.1, 1.5 Hz), 9.69 (1H, *br s*), 10.87 (1H, *br s*), 12.36 (1H, *s*); **Figure 17**.

^{13}C NMR : δ ppm, 75 MHz, in DMSO-d_6; 93.7, 98.4, 103.3, 127.6, 127.6, 128.6, 128.6, 130.0, 131.0, 137.2, 145.8, 156.5, 160.9, 164.3, 176.4; **Figure 18**.

3.4.2. Compound PE02

Compound PE02 was obtained as orange crystals, soluble in acetone.

UV : λ_{max} (MeOH) nm (log ε): 228 (4.16), 257 (3.36), 286 (4.25); **Figure 19**.

IR: ν_{max} cm^{-1} : 3012, 1628, 1600, 1298; **Figure 20**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 279.0664; **Figure 21**.

$[\alpha]^{20}_D$: -0.036

^{1}H NMR : δ ppm, 300 MHz, in acetone-d_6; 2.80 (1H, *dd*, *J* = 16.8, 3.0 Hz), 3.17 (1H, *dd*, *J* = 16.8, 12.6 Hz), 5.57 (1H, *dd*, *J* = 12.6, 3.0 Hz), 5.96 (1H, *d*, *J* = 2.1 Hz), 5.99 (1H, d, *J* = 2.1 Hz), 7.42 (1H, m), 7.45 (2H, m), 7.55 (1H, *br s*), 7.58 (1H, *br s*), 9.63 (1H, *s*), 12.16 (1H, *s*); **Figure 22**.

^{13}C NMR : δ ppm, 75 MHz, in acetone-d_6; 43.3, 79.6, 95.6, 96.6, 102.9, 127.0, 127.0, 129.2, 129.2, 129.2, 139.7, 163.9, 165.0, 167.0, 196.5; **Figure 23**.

3.4.3. Compound PE03

Compound PE03 was obtained as a brown semisolid, soluble in dichloromethane.

UV : λ_{max} (MeOH) nm (log **ε**): 229 (4.29), 302 (4.47); **Figure 24**.

IR: ν_{max} cm^{-1} : 3376, 1605, 1587, 1144, 957, 825, 749, 690; **Figure 25**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 249.0927; **Figure 26**.

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; 3.80 (3H, *s*), 4.96 (1H, *br s*), 6.32 (1H, *t*, *J* = 2.1 Hz), 6.59 (1H, *br s*), 6.63 (1H, *br s*), 6.97 (1H, *d*, *J* = 16.3 Hz), 7.05 (1H, *d*, *J* = 16.3 Hz), 7.24 (1H, *m*), 7.34 (2H, *t*, *J* = 7.5 Hz), 7.48 (2H, *d*, *J* = 7.5 Hz); **Figure 27**.

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; 55.4, 101.0, 104.9, 105.9, 126.6, 126.6, 127.8, 128.3, 128.7, 128.7, 129.4, 137.0, 139.7, 156.9, 161.1; **Figure 28**.

3.4.4. Compound PE04

Compound PE04 was obtained as a brown semisolid, soluble in dichloromethane.

UV : λ_{max} (MeOH) nm (log **ε**): 230 (4.21), 314 (4.29); **Figure 29**.

IR: ν_{max} cm^{-1} : 3384, 1605, 1589, 1144, 962, 826, 748, 667; **Figure 30**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 279.1004; **Figure 31**.

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; 3.80 (3H, *s*), 3.87 (3H, *s*), 5.22 (1H, *br s*), 6.31 (1H, *br s*), 6.62 (1H, *br s*), 6.65 (1H, *br s*), 6.88 (1H, *d*, *J* = 8.3 Hz), 6.95 (1H, *t*, *J* = 7.6, Hz), 6.98 (1H, *d*, J = 16.4 Hz), 7.21 (1H, *dd*, *J* = 8.3, 7.6 Hz), 7.42 (1H, *d*, *J* = 16.4 Hz), 7.55 (1H, *d*, 7.6 Hz); **Figure 32**.

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; 55.4, 55.5, 100.7, 105.2, 105.9, 110.9, 120.7, 124.2, 126.2, 126.5, 128.7, 128.8, 140.3, 156.8, 156.9, 161.1; **Figure 33**.

3.4.5. Compound PE05

Compound PE05 was obtained as a white amorphous powder, soluble in acetone.

UV : λ_{max} (MeOH) nm (log ε): 227.5 (4.43), 322 (4.29); **Figure 34**.

IR: ν_{max} cm^{-1} : 3253, 1604, 1575, 1228, 1176, 1140, 962; **Figure 35**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 371.1301; **Figure 36**.

^{1}H NMR : δ ppm, 300 MHz, in acetone-d_6; 3.78 (3H, *s*), 4.00 (2H, *s*), 6.45 (1H, *d*, *J* = 2.1 Hz), 6.66 (2H, *d*, *J* = 8.4 Hz), 6.79 (1H, *br d*, *J* = 7.6 Hz), 6.80 (1H, *d*, *J* = 2.1 Hz), 6.86 (1H, *t*, *J* = 7.8 Hz), 7.01 (2H, *d*, *J* = 8.4 Hz), 7.07 (1H, *td*, *J* = 7.6, 1.5 Hz), 7.29 (1H, *d*, *J* = 16.2 Hz), 7.46 (1H, *br d*, *J* = 7.8 Hz), 7.48 (1H, *d*, *J* = 16.2 Hz), 7.98 (1H, *s*), 8.19 (1H, *s*), 8.63 (1H, *s*); **Figure 37**.

^{13}C NMR : δ ppm, 75 MHz, in acetone-d_6; 30.0, 55.6, 99.0, 104.4, 115.5, 115.5, 116.4, 119.7, 120.4, 125.3, 125.7, 127.0, 127.3, 129.1, 129.7, 129.7, 133.3, 139.3, 155.5, 155.8, 157.3, 159.5; **Figure 38**.

3.4.6. Compound PE06

Compound PE06 was obtained as colorless needle crystals, soluble in methanol.

UV : λ_{max} (MeOH) nm (log ε): 229.5 (3.21), 282.5 (3.25); **Figure 40**.

IR: ν_{max} cm^{-1} : 3228, 1655, 1604, 1592, 1203; **Figure 41**.

HR-ESIMS : $[M+NH_4]^+$ at *m/z* 276.1164; **Figure 42**.

$[\alpha]^{20}_D$: -0.039

^{1}H NMR : δ ppm, 300 MHz, in DMSO-d_6; 2.60 (1H, *dd*, *J* = 16.5, 3.0 Hz), 2.96 (1H, *dd*, *J* = 16.5, 12.3 Hz), 3.72 (3H, *s*), 5.46 (1H, *dd*, *J* = 12.3, 3.0 Hz), 5.97 (1H, *d*, *J* = 2.1 Hz), 6.04 (1H, *d*, *J* = 2.1 Hz), 7.37 (1H, *m*), 7.40 (2H, *m*), 7.47 (2H, *m*); **Figure 43**.

^{13}C NMR : δ ppm, 75 MHz, in DMSO-d_6; 44.9, 55.7, 78.1, 93.5, 95.7, 104.5, 126.5, 126.5, 128.4, 128.6, 128.6, 139.3, 163.3, 164.1, 164.7, 187.4; **Figure 44**.

3.4.7. Compound PE07

Compound PE07 was obtained as a brown semisolid, soluble in dichloromethane.

UV : λ_{max} (MeOH) nm (log **ε**): 227 (4.25), 295 (4.23); **Figure 45**.

IR: ν_{max} cm^{-1} : 3351, 1590, 1194, 1145, 962, 806, 732, 679; **Figure 46**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 295.0973; **Figure 47**.

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; 3.78 (6H, *s*), 6.32 (1H, *t*, *J* = 2.0 Hz), 6.60 (1H, *d*, *J* = 2.0 Hz), 6.62 (1H, *d*, *J* = 2.0 Hz), 6.69 (1H, *dd*, *J* = 8.7, 2.4 Hz), 6.72 (1H, *d*, *J* = 8.7 Hz), 6.95 (1H, *d*, *J* = 16.2 Hz), 7.02 (1H, *d*, *J* = 2.4 Hz), 7.28 (1H, *d*, *J* = 16.2 Hz); **Figure 48**.

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; 55.4, 55.8, 101.1, 105.1, 105.9, 111.6, 114.7, 116.9, 123.6, 125.1, 129.7, 139.8, 147.3, 153.9, 156.9, 161.1; **Figure 49**.

3.4.8. Compound PE08

Compound PE08 was obtained as a brown semisolid, soluble in dichloromethane.

UV : λ_{max} (MeOH) nm (log **ε**): 227.5 (4.35), 304.5 (4.42); **Figure 50**.

IR: ν_{max} cm^{-1} : 3364, 1584, 1144, 972, 826, 775, 681; **Figure 51**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 295.0975; **Figure 52**.

^{1}H NMR : δ ppm, 300 MHz, in $CDCl_3$; 3.79 (3H, *s*), 3.83 (3H, *s*), 6.32 (1H, *t*, *J* = 2.1 Hz), 6.48 (1H, *d*, *J* = 8.1 Hz), 6.52 (1H, *d*, *J* = 8.1 Hz), 6.60 (1H, *t*, *J* = 2.1 Hz), 6.62 (1H, *t*, *J* = 2.1 Hz), 7.08 (1H, *t*, J = 8.1 Hz), 7.09 (1H, *d*, *J* = 17.0 Hz), 7.23 (1H, *d*, *J* = 17.0 Hz); **Figure 53**.

^{13}C NMR : δ ppm, 75 MHz, in $CDCl_3$; 55.4, 55.8, 100.9, 103.1, 104.9, 105.8, 108.8, 113.2, 121.3, 128.6, 132.2, 140.2, 154.2, 156.8, 158.5, 161.1; **Figure 54**.

3.4.9. Compound PE09

Compound PE09 was obtained as brown amorphous powder, soluble in acetone.

UV : λ_{max} (MeOH) nm (log **ε**): 228.5 (4.23), 299.5 (4.27); **Figure 57**.

IR: ν_{max} cm^{-1} : 3276, 1596, 1140, 957, 827, 747, 687; **Figure 58**.

HR-ESIMS : $[M+Na]^+$ at *m/z* 355.1369; **Figure 59**.

^{1}H NMR : δ ppm, 300 MHz, in acetone-d_6; 3.76 (3H, *s*), 4.00 (2H, *s*), 4.63 (1H, *s*), 4.83 (1H, *s*) 6.39 (1H, *d*, *J* = 2.1 Hz), 6.68 (2H, *d*, *J* = 8.6 Hz), 6.70 (1H, *d*, *J* = 2.1 Hz), 6.90 (1H, *d*, *J* = 16.2 Hz), 7.01 (2H, *d*, *J* = 8.6 Hz), 7.24 (1H, *t*, *J* = 7.2 Hz), 7.30 (1H, *d*, *J* = 16.2 Hz), 7.30 (2H, *t*, *J* = 7.2 Hz), 7.38 (2H, *d*, *J* = 7.2 Hz); **Figure 60**.

^{13}C NMR : δ ppm, 75 MHz, in acetone-d_6; 29.9, 55.7, 98.5, 104.2, 115.0, 115.1, 115.1, 120.1, 126.4, 126.6, 126.6, 127.7, 128.7, 128.7, 129.2, 129.2, 130.9, 133.6, 137.4, 138.5, 153.4, 154.8, 159.0; **Figure 61**.

3.5. Evaluation of Cytotoxicity

3.5.1. Cytotoxicity against cancer cell lines

The cytotoxic activity of the isolated compounds against human cancer cell lines was evaluated by the Bioassay laboratory of the National Center for Genetic Engineering and Biotechnology (BIOTEC). In this study, small cell lung carcinoma (NCI-H187, ATCC CRL-5804), epidermoid carcinoma of oral cavity (KB, ATCC CCL-17) and breast adenocarcinoma (MCF-7, ATCC HTB-22) cell lines were selected as representative cancer cell lines. The assay was performed according to the method described by O'Brien *et al*. (2000).

In brief, cell suspensions at appropriate concentrations (9 x 10^5 cells/ml for NCI-H187 and MCF-7 cells and 7 x 10^5 cells/ml for KB cells) were plated and incubated at 37°C supplemented with 5% CO_2 overnight. Then, the samples were

added. After the incubation period (5 days for NCI-H187 cells and 3 days for KB and MCF-7 cells) 12.5 µl of 62.5 µg/ml resazurin solution was added to each well, and the cells were further incubated at 37°C for 4 hours. Fluorescence signal was then measured at excitation and emission wavelengths of 530 nm and 590 nm, respectively. Percent inhibition of cell growth was calculated as follows.

% Inhibition = [1-(FUT/FUC)] x 100

Whereas FUT and FUC are the mean fluorescent unit from treated and untreated conditions, respectively.

Dose response curves were plotted from six concentrations of two-fold serially diluted test compound and the sample concentrations that inhibited cell growth by 50% (IC_{50} values) were derived using the SOFTMax Pro software (Molecular Devices, USA).

3.5.2. Topoisomerase I inhibition using yeast cell-based assay

3.5.2.1. Construction of yeast

Saccharomyces cerevisiae strain RS190 (ATCC 208354, *MAT*a, *top1*Δ), genotype a *top1-8 [top1 ::LEU2] ade2-1 ura3-1 his3-11 trp1-1 leu2-3, 112 can1-100*, was purchased from American Type Culture Collection (ATCC). The gene of *Arabidopsis thaliana* topoisomerase I was cloned into yeast vector by the Gateway Cloning Technology (Invitrogen), and the integrity of the constructs was verified by DNA sequencing. The Gateway™ expression vector pYES-DEST52 was used for the expression for *S. cerevisiae*. This plasmid also contains *URA3*, selectable markers for the selection and maintenance of plasmid bone sequences in auxotrophic yeast strain. Furthermore, this plasmid has strong inducible promoter (*pGAL1*) which enables the study of topoisomerase enzyme function.

3.5.2.2. Media for yeast culture

3.5.2.2.1. Growth media (YPD media) (DifcoTM)

YPD agar and YPD broth were used for maintaining and propagating yeasts in molecular microbiology procedures. The compositions of these culture media are described in **Table 20**.

3.5.2.2.2. Synthetic complete media lacking uracil (S.C. ura- media)

S.C. ura^- media was used for selecting and maintaining transgenic yeast. The composition of this culture media is described in **Table 20**.

3.5.2.3. Yeast cell-based assay of topoisomerase I inhibition

Topoisomerase I inhibitory activitiy of *P. exul* root extract and isolated compounds was evaluated using transgenic yeast. The samples were solubilized in dimethylsufoxide (DMSO), sonicated, and filtered through 0.25 µm Millipore filter. Then, samples were diluted with S.C. ura^- media to give several concentrations (400, 200, and 100 µg/ml of extract or 100 and 50 µM of isolated compounds). Two different condition media were prepared, i.e. glucose and galactose containing S.C. ura^- media. Positive controls were 2.5, 5 and 10 µg/ml of camptothecin (CPT), and vehicle control was 1% DMSO.

The suspension of yeasts in liquid S.C. ura^- media containing glucose was incubated at 30°C for 18 hours with shaking (200 rpm). The culture was adjusted to 0.3 of OD_{600} and serial ten-fold ten-fold to 10^0, 10^{-1}, 10^{-2} and 10^{-3} fold of starting suspension. Aliquots (5 µl each) of each concentration of yeast suspension were spotted on prepared plates. Spotted plates were incubated at 30°C for 48 hours, and cell yeast viability was observed. The yeast survival was determined by comparing

the viability of colonies in the vehicle control culture (DMSO plate) and positive control culture (CPT plate) on glucose and galactose agar media.

CHAPTER 4
RESULT AND DISCUSSION

The methanolic root extract of *Paphiopedilum exul* showed cytotoxicity against several cancer cell lines, and topoisomerase I inhibitory activity. Then, its chemical constituents were investigated. Twelve compounds (PE01-PE12) were isolated using chromatographic techniques as described in Chapter 3. Identification and structure elucidation of these compounds were achieved using spectroscopic techniques, including UV, IR, MS and NMR. Isolated compounds were evaluated for their biological activities, including cytotoxicity against three human cancer cell lines i.e. small cell lung carcinoma (NCI-H187), epidermoid carcinoma of oral cavity (KB) and breast adenocarcinoma (MCF-7). Their topoisomerase I inhibitory activity was also investigated.

4.1. Identification of compound PE01 (galangin)

Compound PE01 was obtained as yellow needle crystals (104.2 mg, 0.00208 % yield). It appeared as a yellow spot on TLC plate upon spraying with anisaldehyde reagent and heating at 105 °C for 10 minutes. According to the $[M+Na]^+$ peak at *m/z* 293.0478 in the mass spectrum (**Figure 16**), its molecular formula could be determined as $C_{15}H_{10}O_5$.

The IR spectrum of compound PE01 (**Figure 15**) showed absorption bands of hydroxyl groups at 3141 cm^{-1}, conjugated carbonyl at 1655 cm^{-1}, C=C stretching at 1606 cm^{-1} and C-C bending at 1306 cm^{-1}. These bands are commonly found in flavonoid compounds.

The ^1H NMR spectrum of compound PE01 (**Figure 17** and **Table 5**) showed seven aromatic proton signals and three hydroxyl singlets. The most downfield signal at δ 12.36 could be assigned to a hydroxyl substituent at position 5, which intermolecularly hydrogen-bonded to the C-4 carbonyl group. Two doublet signals at δ 6.21 (1H, *d*, *J* = 2.1 Hz) and 6.46 (1H, *d*, *J* = 2.1 Hz) represented *meta*-coupled

aromatic protons of positions 6 and 8, respectively, on ring A of the flavonoid structure. No singlet signal of proton at position 3 was observed, suggesting that PE01 might be a flavonol. The proton signals at δ 7.52 (1H, *m*, H-4′), 7.55 (2H, *m*, H-3′/H-5′) and 8.14 (2H, *dd*, *J* = 6.9, 1.5 Hz, H-2′/H-6′) represented aromatic protons of the unsubstituted B ring. The broad hydroxyl proton signals at δ 9.69 and 10.87 could be assigned to 3-OH and 7-OH, respectively.

The ^{13}C NMR spectrum (**Figure 18** and **Table 5**) exhibited thirteen peaks including two double peaks in agreement with fifteen carbons of a basic flavonol structure. The most downfield peak at δ 176.4 was assigned to C-4 carbonyl group.

Therefore, compound PE01 was identified as 3,5,7-trihydroxyflavone (galangin) and its structure was confirmed by comparison of its spectroscopic data with previous reports (Wawer and Zielinska, 2001; De Souza and De Giovani, 2005).

Galangin was commonly found in honey and propolis (Cheng and Wong, 1996). In addition, several plants, such as *Alpinia officinarum* (Zingiberaceae) (Zhang *et al*., 2014), *Piper aleyreanum* (Piperaceae) (Facundo *et al.*, 2012) and *Helichrysum aureonitens* (Asteraceae) (Meyer *et al.*, 1997), also contain this flavonoid. Galangin exhibited various biological activities including anticancer, antimicrobial, antioxidant, anti-inflammatory, enzyme modulating and effect on metabolic process (Patel *et al.*, 2012).

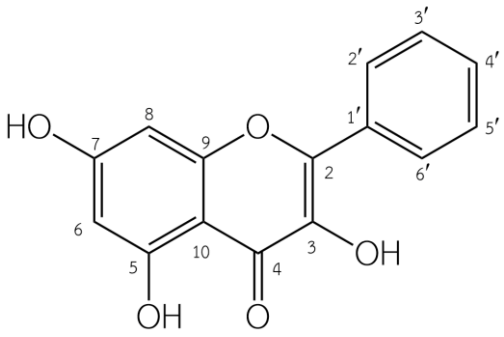


Figure 5 Chemical structure of galangin

Table 5 ^{1}H and ^{13}C NMR spectral data of compound PE01 (in DMSO-d_6) and galangin (^{1}H NMR in CD_3OD and ^{13}C NMR in DMSO-d_6)

Position	Compound PE01		Galangin	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)[a]	δ_C[b]
2	-	145.8	-	146.5
3	-	137.2	-	138.0
4	-	176.4	-	177.1
5	-	160.9	-	161.6
6	6.21 (d, 2.1)	98.4	6.21 (d)	99.2
7	-	164.3	-	165.1
8	6.46 (d, 2.1)	93.7	6.43 (d)	94.4
9	-	156.5	-	157.3
10	-	103.3	-	104.1
1′	-	130.0	-	130.7
2′	8.14 (dd, 6.9, 1.5)	127.6	8.20 (dd)	128.4
3′	7.55 (m)	128.6	7.62 (dd)	129.3
4′	7.52 (m)	131.0	7.53 (m)	131.8
5′	7.55 (m)	128.6	7.62 (dd)	129.3
6′	8.14 (dd, 6.9, 1.5)	127.6	8.20 (dd)	128.4
3-OH	9.69 (br s)	-	9.59 (s)	-
5-OH	12.36 (s)	-	12.43 (s)	-
7-OH	10.87 (br s)	-	10.74 (s)	-

[a] De Souza and De Giovani, 2005

[b] Wawer and Zielinska, 2001

4.2. Identification of compound PE02 (pinocembrin)

Compound PE02 was obtained as orange crystals (90.7 mg, 0.00181% yield). It appeared as an orange spot on TLC plate upon spraying with anisaldehyde reagent and heating at 105 °C for 10 minutes. Its specific optical rotation $[\alpha]^{20}_{D}$ was found to be -0.036° (c = 0.06, MeOH). According to the $[M+Na]^+$ peak at *m/z* 279.0664 in the mass spectrum (**Figure 21**), its molecular formula could be deduced as $C_{15}H_{12}O_4$. Its IR spectrum (**Figure 20**) showed hydroxyl absorption bands at 3012 cm^{-1}, conjugated carbonyl band at 1628 cm^{-1}, C=C stretching band at 1600 cm^{-1} and C-C bending band at 1298 cm^{-1}. These data suggested that PE02 might be a flavonoid derivative.

The ^{1}H NMR spectrum of compound PE02 (**Figure 22** and **Table 6**) was partially similar to that of compound PE01. The *meta*-coupled H-6 and H-8 of ring A could be observed as a pair of doublets at δ 5.96 (1H, *d*, *J* = 2.1 Hz) and 5.99 (1H, *d*, *J* = 2.1 Hz), respectively. Five protons of the unsubstituted B ring resonated at δ 7.42 (1H, *m*, H-4′), 7.45 (2H, *m*, H-3′/H-5′) and 7.56 (2H, *d*, *J* = 6.6 Hz, H-2′/H-6′). The hydrogen-bonded 5-OH appeared as the most downfield singlet at δ 12.16. The major difference was the presence of additional aliphatic proton signals at δ 2.80 (1H, *dd*, *J* = 16.8, 3.0 Hz, H-3α), 3.17 (1H, *dd*, *J* = 16.8, 12.6 Hz, H-3β), 5.57 (1H, *dd*, *J* = 12.6, 3.0 Hz, H-2), representing positions 2 and 3 of a flavanone skeleton. The other hydroxyl proton signal at δ 9.63 could be assigned to 7-OH.

The ^{13}C NMR spectrum (**Figure 23** and **Table 6**) showed thirteen peaks including two double peaks from fifteen carbons of a flavanone derivative. The most downfield peak at δ 196.5 corresponded to the keto-carbonyl C-4. Two aliphatic carbon signals at δ 43.3 and 79.6 represented C-3 and C-2, respectively.

Therefore, compound PE02 was identified as 5,7-dihydroxyflavanone (pinocembrin), and its structure was confirmed by comparison of its spectroscopic data with a previous report (Neacsu *et al.*, 2007).

Pinocembrin has been found in many flowering plant families including Zingiberaceae, Piperaceae, Lauraceae and Asteraceae (Rasul *et al.*, 2013) and also reported in some gymnosperms, i.e. *Pinus massoniana* (Pinaceae) (Zhang *et al.*, 2013)

and *Ginkgo biloba* (Ginkgoaceae) (López-Gutiérrez *et al.*, 2016). The flavanone exhibited several interesting biological activities including anticancer, antimicrobial, antifungal, anti-inflammatory and neuroprotective effects (Rasul *et al*., 2013).

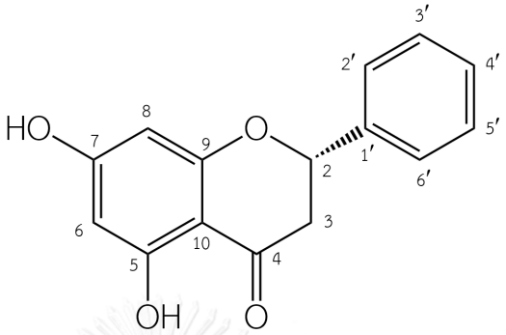


Figure 6 Chemical structure of pinocembrin

Table 6 ^{1}H and ^{13}C NMR spectral data of compound PE02 (in acetone-d_6) and pinocembrin (in CD_3OD)

Position	Compound PE02		Pinocembrin[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
2	5.57 (dd, 12.6, 3.0)	79.6	5.44 (12.8, 3.1)	80.5
3α	2.80 (dd, 16.8, 3.0)	43.3	2.76 (dd, 17.0, 3.1)	41.2
3β	3.17 (dd, 16.8, 12.6)		3.07 (dd, 17.0, 12.8)	
4	-	196.5	-	197.3
5	-	165.0	-	165.5
6	5.96 (d, 2.1)	96.6	5.90 (d, 2.2)	97.2
7	-	167.0	-	168.4
8	5.99 (d, 2.1)	95.6	5.93 (d, 2.2)	96.2
9	-	163.9	-	164.7
10	-	102.9	-	103.4
1′	-	139.7	-	140.4
2′	7.56 (d, 6.6)	127.0	7.48 (m)	127.4
3′	7.45 (m)	129.2	7.41 (m)	129.7
4′	7.42 (m)	129.2	7.36 (m)	129.6
5′	7.45 (m)	129.2	7.41 (m)	129.7
6′	7.56 (d, 6.6)	127.0	7.48 (m)	127.4
5-OH	12.16 (s)	-	-	-
7-OH	9.63 (s)	-	-	-

[a] Neacsu *et al*., 2006

4.3. Identification of compound PE03

Compound PE03 was obtained as a brown semisolid (44.0 mg, 0.00088% yield). It appeared as a reddish purple spot upon spraying with anisaldehyde reagent and heating at 105 °C for 10 minutes. Its molecular formula could be determined as $C_{15}H_{14}O_2$ based on the $[M+Na]^+$ peak at *m/z* 249.0927 in the ESI mass spectrum (**Figure 26**). Its IR spectrum (**Figure 25**) displayed absorption bands of hydroxyl groups at 3376 cm^{-1}, C=C stretching at 1605 and 1587 cm^{-1}, C-O stretching at 1144 cm^{-1}, *trans*-double bond C-H bending at 957 cm^{-1} and C-H out-of-plane bending of *meta*-substituted benzenoid compound at 825, 749 and 690 cm^{-1}. The number of carbon atoms in the molecule, which could be deduced as 14 from its molecular formula and NMR data, suggested that this compound is a stilbene derivative.

The ^{1}H NMR spectrum of compound PE03 (**Figure 27** and **Table 7**) revealed signals of eight aromatic protons, two olefinic protons and one methoxy group. Two one-proton doublets (*J* = 16.3 Hz) occurring at δ 6.79 (H-α) and 7.05 (H-β), represented olefinic protons of the *trans*-double bond between two benzene rings of a stilbenoid. A group of *meta*-coupled aromatic proton signals appearing at δ 6.32 (1H, *t*, *J* = 2.1 Hz, H-4), 6.59 (1H, *br s*, H-2) and 6.63 (1H, *br s*, H-6) were indicative of the 1,3,5-trisubstituted ring A. A methoxy signal at δ 3.80 (3H, *s*, 3-OCH_3) and a hydroxyl broad singlet at δ 4.96 (1H, *s*, 5-OH) represented those substituents at positions 3 and 5, respectively. The rest of the aromatic proton signals at δ 7.24 (1H, *m*, H-4′), 7.34 (2H, *t*, *J* = 7.5 Hz, H-3′/H-5′) and 7.48 (2H, *d*, *J* = 7.5 Hz, H-2′/H-6′) could be assigned to the unsubstituted ring B of the stilbene PE03.

The ^{13}C NMR spectrum (**Figure 28** and **Table 7**), which showed fifteen carbon signals representing twelve aromatic carbons (including two oxygen-substituted ones at δ 161.1 and 156.9), two olefinic carbons (at δ 128.3 and 129.4) and a methoxy carbon (at δ 55.4), suggested a *trans*-stilbene structure substituted with one methoxy group and one hydroxyl group consistent with the ^{1}H NMR spectral data. Therefore, compound PE03 was identified as (*E*)-5-hydroxy-3-methoxystilbene (pinosylvin

monomethylether), and its structure was confirmed by comparison of the spectroscopic data with literature values (Ngo and Brown, 1998).

(*E*)-5-Hydroxy-3-methoxystilbene has been found as a constituent in pteridophytes, gymnosperms and flowering plants including Orchidaceae (Starks *et al.*, 2012) and has been reported to possess various activities including anti-inflammatory (Laavola *et al.*, 2015), antimicrobial (Plumed-Ferrer *et al.*, 2013), antifungal (Araujo *et al.*, 2009), antiproliferation, inhibition of NF-**K**B and NO production (Sobolev *et al.*, 2011).

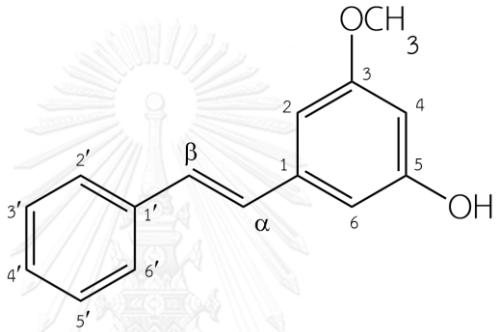


Figure 7 Chemical structure of (*E*)-5-hydroxy-3-methoxystilbene

Table 7 ^{1}H and ^{13}C NMR spectral data of compound PE03 and (*E*)-5-hydroxy3-methoxystilbene (in $CDCl_3$)

Position	PE03 in $CDCl_3$		(*E*)-3-methoxy-5-hydroxystilbene[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	-	139.7	-	139.6
2	6.59 (br s)	104.9	6.60 (s)	104.7
3	-	161.1	-	160.9
4	6.32 (t, 2.1)	101.0	6.37 (s)	101.2
5	-	156.9	-	157.3
6	6.63 (br s)	105.9	6.62 (s)	106.3
α	6.97 (d, 16.3)	128.3	6.93 (d, 16.3)	128.4
β	7.05 (d, 16.3)	129.4	6.99 (d, 16.3)	129.2
1′	-	137.0	-	137.1
2′	7.48 (d, 7.5)	126.6	7.42 (d, 7.5)	126.6
3′	7.34 (t, 7.5)	128.7	7.30 (t, 7.5)	128.6
4′	7.24 (m)	127.8	7.21 (t, 7.5)	127.7
5′	7.34 (t, 7.5)	128.7	7.30 (t, 7.5)	128.6
6′	7.48 (d, 7.5)	126.6	7.42 (d, 7.5)	126.6
5-OH	4.96 (br s)	-	-	-
3-OMe	3.80 (s)	55.4	3.74 (s)	55.3

[a] Ngo and Brown, 1998

4.4. Identification of compound PE04

Compound PE04 was obtained as a brown semisolid (79.8 mg, 0.0016% yield). It appeared as a violet spot on TLC plate when sprayed with anisaldehyde reagent and heated. Its molecular formula was determined to be $C_{16}H_{16}O_3$ based on the sodium adduct molecular ion $[M+Na]^+$ peak at *m/z* 279.1004 in the mass spectrum (**Figure 31**).

The IR spectrum of compound PE04 (**Figure 30**) showed absorption bands of hydroxyl groups at 3384 cm^{-1}, C=C stretching at 1605 and 1589 cm^{-1}, C-O stretching at 1144 cm^{-1}, *trans*-double bond C-H bending at 962 cm^{-1} and C-H out-of-plane bending of *meta*-substituted benzenoid compound at 826, 748 and 677 cm^{-1} which are commonly found in stilbenoid compounds.

The ^{1}H NMR spectrum of compound PE04 (**Figure 32** and **Table 8**) exhibited signals of seven aromatic protons, two olefinic protons of a *trans*-double bond at δ 6.98 (1H, *d*, *J* = 16.4 Hz, H-α) and 7.42 (1H, *d*, *J* = 16.4 Hz, H-β) and two methoxy groups (at δ 3.80 and 3.87). The differences from that of compound PE03 were an additional methoxy signal at δ 3.87 (2′-OCH_3) and the signals for four adjacent protons on ring B of this compound at δ 6.88 (1H, *d*, *J* = 8.3 Hz, H-3′), 6.95 (1H, *t*, *J* = 7.6 Hz, H-5′), 7.21 (1H, *dd*, *J* = 8.3, 7.6 Hz, H-4′) and 7.55 (1H, *d*, *J* = 7.6 Hz, H-6′). The signals of H-3′ and H-5′ were shifted upfield from those of PE03 as a result of the electron donating effect of the 2′-methoxy substituent.

The ^{13}C NMR spectrum (**Figure 33** and **Table 8**) showed sixteen carbon signals including twelve aromatic carbons, two olefinic carbons (at δ 124.2 and 128.8) and two methoxy carbons (at δ 55.4 and 55.5), supportive of a *trans*-stilbene basic structure substituted with two methoxy groups and one hydroxyl group as determined by ^{1}H NMR spectral data. The three most downfield signals of the oxygen-substituted carbon atoms at δ 161.1, 157.0 and 156.8 were assignable to C-3, C-5 and C-2′, respectively, based on comparison with reported values (Lertnitikul *et al.*, 2016).

Therefore, compound PE04 was identified as (*E*)-5-hydroxy-3,2′-dimethoxystilbene and confirmed by comparison of its spectroscopic data with a previous study on another species of Venus slippers' orchid, *Paphiopedilum godefroyae.* The compound was shown to be cytotoxic against human small cell lung cancer cell line (NCI-H187) with an IC_{50} value of 77.30 μM (Lertnitikul *et al.*, 2016).

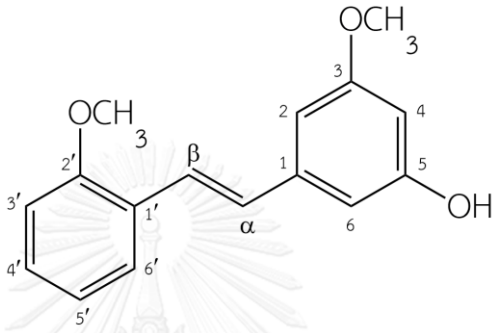


Figure 8 Chemical structure of (*E*)-5-hydroxy-3,2′-dimethoxystilbene

Table 8 ^{1}H and ^{13}C NMR spectral data of compound PE04 and (*E*)-5-hydroxy-3,2′-dimethoxystilbene (in $CDCl_3$)

Position	Compound PE04		(*E*)-3,2′-dimethoxy-5-hydroxystilbene[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	-	140.3	-	140.3
2	6.65 (br s)	105.0	6.64 (br s)	105.0
3	-	161.1	-	161.0
4	6.31 (br s)	100.7	6.31 (br s)	100.7
5	-	157.0	-	156.9
6	6.62 (br s)	105.9	6.62 (br s)	105.9
α	6.98 (d, 16.4)	128.8	6.98 (d, 16.4)	128.8
β	7.42 (d, 16.4)	124.2	7.42 (d, 16.4)	124.2
1′	-	126.2	-	126.1
2′	-	156.8	-	156.8
3′	6.88 (d, 8.3)	111.0	6.88 (d, 8.3)	110.9
4′	7.21 (dd, 8.3, 7.6)	128.7	7.24 (dd, 8.3, 7.6)	128.7
5′	6.95 (t, 7.6)	120.7	6.95 (d, 7.6)	120.7
6′	7.55 (d, 7.6)	126.5	7.55 (d, 7.6)	126.5
5-OH	5.22 (br s)	-	-	-
3-OMe	3.80 (s)	55.4	3.80 (s)	55.4
2′-OMe	3.87 (s)	55.5	3.87 (s)	55.5

[a] Lertnitikul *et al*., 2016

4.5. Identification of compound PE05

Compound PE05 was obtained as a white powder (58.0 mg, 0.00116% yield). It appeared as a reddish brown spot o TLC plate upon spraying with anisaldehyde reagent and heating at 105 °C. Based on the sodium adduct molecular ion $[M+Na]^+$ peak at *m/z* 371.1301 in the mass spectrum (**Figure 36**), its molecular formula was identified as $C_{22}H_{20}O_4$. The IR spectrum of compound PE05 (**Figure 35**) showed absorption bands of hydroxyl groups at 3253 cm^{-1}, C=C stretching at 1604 and 1575 cm^{-1}, C-O stretching at 1228, 1176 and 1140 cm^{-1} and *trans*-double bond C-H bending at 962 cm^{-1}.

The ^{13}C NMR spectrum of compound PE05 (**Figure 38** and **Table 9**) revealed twenty-two carbon resonances (including one methoxyl carbon signal), suggesting a stilbene substituted with a benzyl moiety. The ^1H NMR spectrum (**Figure 37** and **Table 9**) exhibited resonances of a *trans*-double bond at δ 7.29 (1H, *d*, *J* = 16.2 Hz, H-α) and 7.48 (1H, *d*, *J* = 16.2 Hz, H-β), a methoxyl group at δ 3.78 (3H, *s*, 3-OCH_3), four adjacent aromatic protons of a 1,2-disubstituted phenyl moiety at δ 6.79 (1H, *br d*, *J* = 7.6 Hz, H-3′), 6.86 (1H, *t*, *J* = 7.8 Hz, H-5′), 7.07 (1H, *td*, *J* = 7.6, 1.5 Hz, H-4′) and 7.46 (1H, *br d*, *J* = 7.8 Hz, H-6′), and two *meta*-coupled signals at δ 6.45 (1H, *d*, *J* = 2.1 Hz, H-4) and 6.80 (1H, *d*, *J* = 2.1 Hz, H-6). Moreover, one methylene singlet at δ 4.00 (2H, *s*, H-1″) and a pair of two-proton doublets at δ 6.66 (2H, *d*, *J* = 8.4 Hz, H-4″/H-6″) and 7.01 (2H, *d*, *J* = 8.4 Hz, H-3″/H-7″) were suggestive of a *para*-substituted benzyl group. Additionally, three hydroxy singlets were observed at δ 7.98 (5″-OH), 8.19 (5-OH) and 8.63 (2′-OH).

The long-range heteronuclear correlation, HMBC (**Figure 39** and **Table 10**), confirmed the linkage between a *para*-substituted benzyl group and the *trans*-stilbene basic structure, and substitutions on the aromatic rings. The H-1″ methylene singlet of the *para*-hydroxy benzyl moiety (at δ 4.00) showed HMBC cross-peaks with the carbon signals at δ 119.7 (C-2), 129.7 (C-3″/C-7″), 133.3 (C-2″), 139.3 (C-1) and 159.5 (C-3), giving supporting evidence for its linkage to C-2. HMBC correlation between methoxy protons

at δ 3.78 and C-3 (δ 159.5) confirmed its position at this carbon, whereas two- and three-bond correlations between 2′-OH signal at δ 8.63 and C-1′ (δ 125.3) and C-2′ (δ 155.5), between 5-OH signal at δ 8.19 and C-5 (δ 157.3) and between 5″-OH signal at δ 7.98 and C-4″/C-6″ (δ 115.5) and C-5″ (δ 155.8) helped in assigning these signals.

Therefore, compound PE05 was identified as (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3-methoxystilbene, previously found in *Phragmipedium calurum* (Garo *et al.*, 2007).

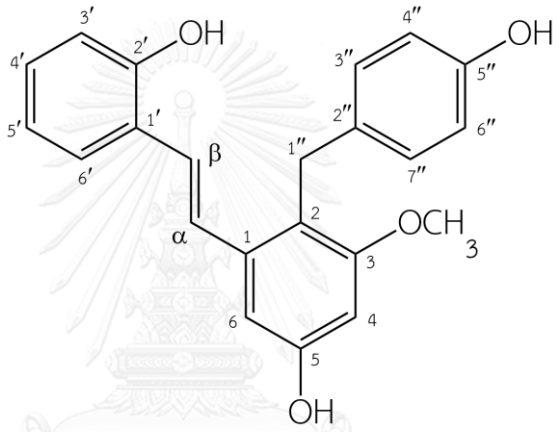


Figure 9 Chemical structure of (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3-methoxystilbene

Table 9 ^{1}H and ^{13}C NMR spectral data of compound PE05 (in acetone-d_6) and (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3-methoxystilbene (in $CDCl_3$)

Position	Compound PE05		(*E*)-2-(4″-hydroxybenzyl)-3,5′-dimethoxy-5,2′-dihydroxystilbene[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	-	139.3	-	140.4
2	-	119.7	-	120.5
3	-	159.5	-	160.3
4	6.45 (d, 2.1)	99.0	6.40 (d, 2.1)	99.4
5	-	157.3	-	157.7
6	6.80 (d, 2.1)	104.4	6.74 (d, 2.1)	105.1
α	7.29 (d, 16.2)	125.7	7.22 (d, 16.2)	126.7
β	7.48 (d, 16.2)	127.0	7.35 (d, 16.2)	127.9
1′	-	125.3	-	116.7
2′	-	155.5	-	156.3
3′	6.79 (br d, 7.6)	120.4	6.77 (m)	116.8
4′	7.07 (td, 7.6, 1.5)	129.1	7.04 (t, 7.1)	129.6
5′	6.86 (t, 7.8)	116.4	-	120.9
6′	7.46 (br d, 7.8)	127.3	7.32 (br d, 7.4)	127.7
1″	4.00 (s)	30.0	3.96 (s)	30.8
2″	-	133.3	-	134.3
3″/7″	7.01 (d, 8.4)	129.7	6.96 (d, 8.4)	130.3
4″/6″	6.66 (d, 8.4)	115.5	6.63 (d, 8.4)	116.0
5″	-	155.8	-	156.1
5-OH	8.19 (s)	-	-	-
2′-OH	8.63 (s)	-	-	-
5″-OH	7.98 (s)	-	-	-
3-OMe	3.78 (s)	55.6	3.78 (s)	56.2

[a] Garo *et al*., 2007

Table 10 HMBC spectral data of compound PE05 (in acetone-d_6)

^{1}H signal	HMBC correlations with
H-4	C-2, C-5, C-6
H-6	C-4, C-5
H-α	C-β, C-6, C-1′
H-β	C-α, C-1, C-2′, C-6′
H-3′	C-1′, C-5′
H-4′	C-2′, C-6′
H-5′	C-1′, C-3′
H-6′	C-2′, C-4′
H-1′′	C-1, C-2, C-3, C-2″, C-3″/ C-7″
H-3″/ H-7″	C-1″, C-3″/ C-7″, C-5″
H-4″/ H-6″	C-2″, C-4″/ C-6″, C-5″
5-OH	C-4, C-5, C-6
2′-OH	C-1′, C-2′
5″-OH	C-4″/ C-6″, C-5″
3-OMe	C-3

4.6. Identification of compound PE06 (alpinetin)

Compound PE06 was obtained as colorless crystals (1.1 mg, 0.00002% yield). It appeared as a yellow spot on TLC plate upon spraying with anisaldehyde reagent and heating at 105 °C for 10 minutes, suggestive of its flavonoid structure. Its specific optical rotation $[\alpha]^{20}_{D}$ was found to be -0.039 ^{o} (c = 0.07, MeOH). According to the $[M+NH_4]^+$ peak at *m/z* 276.1164 in its mass spectrum (**Figure 42**), the molecular formula of PE06 was deduced as $C_{16}H_{14}O_4$. The IR spectrum (**Figure 41**) showed absorption bands of hydroxyl groups at 3228 cm^{-1}, conjugated carbonyl at 1655 cm^{-1}, C=C stretching at 1604 and 1592 cm^{-1} and C-C bending at 1203 cm^{-1}.

The ^{1}H NMR spectrum of compound PE06 (**Figure 43** and **Table 11**) was similar to that of compound PE02, especially the upfield signals of H-2 at δ 5.46 (1H, *dd*, *J* = 12.3, 3.0 Hz) and H_2-3 at δ 2.96 (1H, *dd*, *J* = 16.5, 12.3 Hz, H-3α) and 2.60 (1H, *dd*, *J* = 16.5, 3.0 Hz, H-3β), indicating that it is a flavanone. A notable difference was the absence of the downfield singlet at about 12-13 ppm of the commonly found, hydrogen-bonded hydroxy group at position 5. The *meta*-coupled H-6 and H-8 resonated at δ 5.97 (1H, *d*, *J* = 2.1 Hz) and 6.04 (1H, *d*, *J* = 2.1 Hz), respectively, while the unsubstituted ring B was represented by a set of multiplet signals, integrated for five protons, at δ 7.37-7.47. Moreover, a methoxy peak, which appeared at δ 3.72 (3H, *s*, 5-OCH_3), suggested that position 5 was substituted by a methoxy instead of a hydroxyl group.

The ^{13}C NMR spectrum (**Figure 44** and **Table 11**) displayed fourteen peaks including two double peaks representing sixteen carbons in the molecule of PE06. Most carbon peaks were similar to those of compound PE02, with an additional methoxy signal at δ 55.7 (5-OCH_3). The most downfield signal, which is characteristic peak of carbonyl group of flavanones (C-4), occurred at a more upfield chemical shift (δ 187.4) compared to pinocembrin (PE02).

Therefore, compound PE06 was identified as 7-hydroxy-5-methoxyflavanone (alpinetin) and its spectroscopic data were in agreement with previously reported values (Itokawa *et al.*, 1981).

Alpinetin has previously been reported as a constituent of various plants, including monocots such as *Alpinia mutica* (Zingiberaceae) (Malek *et al.*, 2011), and dicots such as *Helichrysum forskahlii* (Asteraceae) (Al-Rehaily *et al.*, 2008) and *Vitex leptobotrys* (Lamiaceae) (Pan *et al.*, 2014). This flavanone exhibited various significant therapeutic activities including antiproliferation, antibacterial, and anti-inflammation (Huo *et al.*, 2012).

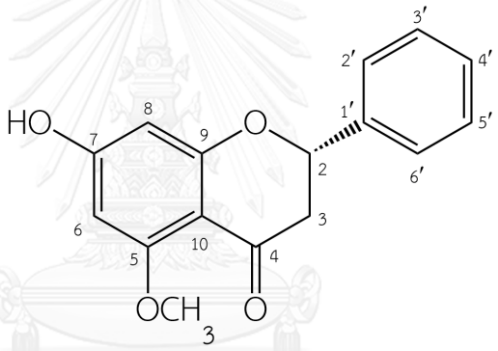


Figure 10 Chemical structure of alpinetin

Table 11 ^{1}H and ^{13}C NMR spectral data of compound PE06 and alpinetin (in DMSO-d_6)

Position	Compound PE06		Alpinetin[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
2	5.46 (dd, 12.3, 3.0)	78.1	5.44 (dd, 12, 4)	78.1
3α	2.96 (dd, 16.5, 12.3)	44.9	2.98 (dd, 14, 12)	45.0
3β	2.60 (dd, 16.5, 3.0)		2.59 (dd, 14, 4)	
4	-	187.4	-	187.4
5	-	164.1	-	164.1
6	5.97 (d, 2.1)	95.7	5.98 (d, 2)	95.8
7	-	164.6	-	164.4
8	6.04 (d, 2.1)	93.5	6.06 (d, 2)	93.5
9	-	162.3	-	162.2
10	-	104.5	-	104.6
1′	-	139.3	-	139.2
2′	7.47 (m)	126.5	7.40 (m)	126.4
3′	7.40 (m)	128.6	7.40 (m)	128.5
4′	7.37 (m)	128.4	7.40 (m)	128.3
5′	7.40 (m)	128.6	7.40 (m)	128.5
6′	7.47 (m)	126.5	7.40 (m)	126.4
5-OMe	3.72 (s)	55.7	3.72 (s)	55.7

[a] Itokawa *et al*., 1981

4.7. Identification of compound PE07

Compound PE07 was obtained as a brown semisolid (32.0 mg, 0.00064% yield). It appeared as a violet spot on TLC plate upon spraying with anisaldehyde reagent and heating at 105 °C for 10 minutes. According to the $[M+Na]^+$ peak of this compound at *m/z* 295.0973 in the mass spectrum (**Figure 47**), its molecular formula was established as $C_{16}H_{16}O_4$. Its IR spectrum (**Figure 46**) showed absorption bands of hydroxyl groups at 3351 cm^{-1}, C=C stretching at 1590 cm^{-1}, C-O stretching at 1194 and 1145 cm^{-1}, *trans*-double bond C-H bending at 962 cm^{-1} and C-H out-of-plane bending of *meta*-substituted benzenoid compound at 806, 732 and 679 cm^{-1}. From these data, together with the observation of two methoxyl functions in its NMR data, PE07 was identified as a *trans*-stilbene.

The ^1H NMR spectrum of compound PE07 (**Figure 48** and **Table 12**) exhibited signals of six aromatic protons, two olefinic protons and two methoxy groups. These were similar to those of compound PE04, in particular the signals for the 1,3,5-trisubstituted ring A which resonated at δ 3.78 (3H, *s*, 3-OCH_3), 6.32 (1H, *t*, *J* = 2.0 Hz, H-4), 6.60 (1H, *t*, *J* = 2.0 Hz, H-6) and 6.62 (1H, *t*, *J* = 2.0 Hz, H-2). The protons of the *trans*-double bond resonated at slightly different chemical shifts of δ 6.95 (1H, *d*, *J* = 16.2 Hz, H-α) and 7.28 (1H, *d*, *J* = 16.2 Hz, H-β). The major difference resulted from changes in the substitution pattern of ring B. Two *ortho*-coupled protons appeared at δ 6.72 (1H, *d*, *J* = 8.7 Hz, H-3′) and 6.69 (1H, *d*, *J* = 8.7, 2.4 Hz, H-4′), while the latter signal was also *meta*-coupled with the proton at δ 7.02 (1H, *d*, *J* = 2.4 Hz, H-6′) indicated 1,2,5-trisubstitution on this ring.

The ^{13}C NMR spectrum (**Figure 49** and **Table 12**) showed sixteen carbon signals including those of twelve aromatic carbons (six of them are quaternary carbons), two olefinic carbons and two methoxy carbons corresponding to a stilbene substituted with two methoxy groups and two hydroxy groups. Comparison of these NMR data with literature values revealed that while ring A of this compound was identical to

those of PE03 and PE04, ring B was substituted with a hydroxyl group at C-2′ and a methoxy group at C-5′ (Garo *et al*., 2007).

Therefore, compound PE07 was identified as (*E*)-5,2′-dihydroxy-3,5′-dimethoxystilbene. Previously, it was found in *Phragmipedium hybrid,* a slipper orchid hybrid of *P. longifolium* and *P. lindleyanum* (Garo *et al*., 2007).

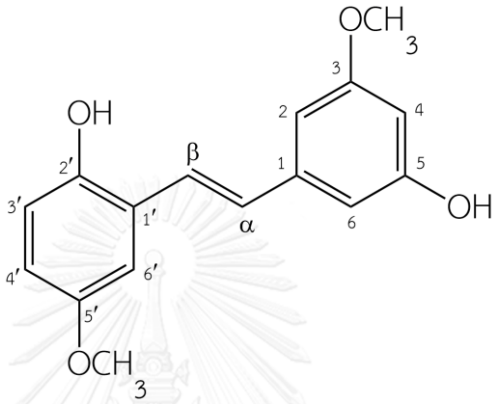


Figure 11 Chemical structure of (*E*)-5,2′-dihydroxy-3,5′-dimethoxystilbene

Table 12 ^{1}H and ^{13}C NMR spectral data of compound PE07 (in $CDCl_3$) and (*E*)-5,2′-dihydroxy-3,5′-dimethoxystilbene (in CD_3OD)

Position	PE07		(*E*)-3,5′-dimethoxy-5,2′-dihydroxystilbene[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	-	139.8	-	141.6
2	6.62 (t, 2.0)	105.1	6.59 (br d, 2.2)	107.1
3	-	161.1	-	162.8
4	6.32 (t, 2.0)	101.1	6.27 (t, 2.0)	101.9
5	-	156.9	-	159.8
6	6.60 (t, 2.0)	105.9	6.60 (br d, 2.0)	107.7
α	6.95 (d, 16.2)	129.7	7.02 (d, 16.4)	129.8
β	7.28 (d, 16.2)	123.7	7.38 (d, 16.4)	125.5
1′	-	125.1	-	126.3
2′	-	147.3	-	150.4
3′	6.72 (d, 8.7)	116.9	6.74 (d, 8.7)	117.6
4′	6.69 (dd, 8.7, 2.4)	114.7	6.68 (dd, 8.7, 2.9)	115.8
5′	-	153.9	-	154.7
6′	7.02 (d, 2.4)	111.6	7.08 (d, 2.8)	112.0
3-OMe	3.78 (s)	55.4	3.77 (s)	55.6
5′-OMe	3.78 (s)	55.8	3.78 (s)	56.0

[a] Garo *et al*., 2007

4.8. Structure elucidation of compound PE08

Compound PE08 was obtained as a brown semisolid (43.6 mg, 0.00087% yield). It appeared as a red spot on TLC plate upon spraying with anisaldehyde reagent and heating. Based on the sodium adduct molecular ion $[M+Na]^+$ peak at *m/z* 295.0975 (cald. 295.2856) in the mass spectrum (**Figure 52**), its molecular formula was established as $C_{16}H_{16}O_4$, requiring 9 degrees of unsaturation common to a *trans*-stilbene. Its IR spectrum (**Figure 51**) also exhibited absorption bands commonly found in stilbenoid compounds including those of hydroxyl groups at 3364 cm^{-1}, C=C stretching at 1584 cm^{-1}, C-O stretching at 1144 cm^{-1}, *trans*-double bond C-H bending at 972 cm^{-1} and C-H out-of-plane bending of *meta*-substituted benzenoid compound at 826, 775 and 681 cm^{-1}.

The ^{1}H NMR spectrum of compound PE08 (**Figure 53** and **Table 13**) revealed the presence of a *trans*-double bond at δ 7.09 (1H, *d*, *J* = 17.0 Hz, H-α) and 7.23 (1H, *d*, *J* = 17.0 Hz, H-β), two methoxy groups at δ 3.79 (3H, *s*, 3-OCH_3) and 3.83 (3H, *s*, 2'-OCH_3) and two sets of three protons from each aromatic ring of the stilbene. These data suggested a *trans*-stilbene structure bearing four substitutions: two hydroxy and two methoxy groups. One phenyl ring of the stilbenoid structure was identical to other stilbenes isolated from this orchid, that is, it was substituted with a hydroxyl group at C-5 and a methoxy group at C-3, as evidenced by three *meta*-coupled proton signals at δ 6.32 (1H, *t*, *J* = 2.1 Hz, H-4), 6.60 (1H, *t*, *J* = 2.1 Hz, H-6) and 6.62 (1H, *t*, *J* = 2.1 Hz, H-2). The other set of aromatic proton signals at δ 6.48 (1H, *d*, *J* = 8.1 Hz, H-3′), 6.52 (1H, *d*, *J* = 8.1 Hz, H-5′) and 7.08 (1H, *t*, J = 8.1 Hz, H-4′), suggested the 1,2,3-trisubstituted pattern of the other phenyl ring.

The ^{13}C NMR spectrum of this compound (**Figure 54** and **Table 13**) exhibited sixteen carbon signals including eight methine carbons at δ 100.9 (C-4), 103.1 (C-3′), 104.9 (C-2), 105.8 (C-6), 108.8 (C-5′), 121.3 (C-β), 128.6 (C-4′) and 132.2 (C-α), six quaternary carbons at δ 113.2 (C-1′), 140.2 (C-1), 154.2 (C-6′), 156.8 (C-5), 158.5 (C-2′) and 161.1 (C-3) and two methoxy carbons at δ 55.4 (3-OCH_3) and 55.8 (2′-OCH_3).

Confirmation of the methoxy substitutions was done through an HMBC experiment (**Figure 56**). For ring A, HMBC cross-peaks were observed between the 3-methoxy singlet at δ 3.79 and C-3 signal (δ 161.1) and between H-α signal at δ 7.09 and those of C-1 (δ 140.2), C-2 (δ 104.9) and C-6 (δ 105.8). For ring B, correlations observed between 2′-methoxy singlet at δ 3.83 and C-2′ signal (δ 158.5), and between the signals of H-β at δ 7.23 and C-1′ (δ 113.2), C-2′ (δ 158.5) and C-6′ (δ 154.2) established the 2′-OCH_3 and 6′-OH positions. Therefore, the molecular structure of compound PE08 was elucidated as a new natural product, (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene.

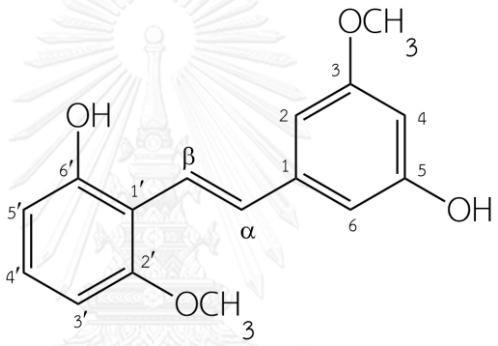


Figure 12 Chemical structure of (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene

Table 13 ^{1}H, ^{13}C NMR and HMBC spectral data of compound PE08 or (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene (in $CDCl_3$)

Position	δ_H (mult., *J* in Hz)	δ_C	HMBC
1	-	140.2	-
2	6.62 (t, 2.1)	104.9	3, 4, 6, α
3	-	161.1	-
4	6.32 (t, 2.1)	100.9	2, 3, 5
5	-	156.8	-
6	6.60 (t, 2.1)	105.8	2, 4, 5, α
α	7.09 (d, 17.0)	132.2	1, 2, 6, β, 1′
β	7.23 (d, 17.0)	121.3	1, α, 2′, 6′
1′	-	113.2	-
2′	-	158.5	-
3′	6.48 (d, 8.1)	103.1	1′, 2′, 5′
4′	7.08 (t, 8.1)	128.6	2′, 6′
5′	6.52 (d, 8.1)	108.8	1′, 3′, 6′
6′	-	154.2	-
3-OMe	3.79 (s)	55.4	3
2′-OMe	3.83 (s)	55.8	2′

4.9. Identification of compound PE09

Compound PE09 was obtained as a white powder (15.3 mg, 0.00031% yield). It appeared as a reddish brown spot on TLC plate after being sprayed with anisaldehyde reagent and heated at 105 °C. Its molecular formula was analyzed as $C_{22}H_{20}O_3$ according to the $[M+Na]^+$ peak at *m/z* 355.1369 in the mass spectrum (**Figure 59**).

The IR spectrum of compound PE09 (**Figure 58**) showed similar absorption bands to the other stilbenoids previously isolated from this orchid including those of hydroxyl groups at 3276 cm^{-1}, C=C stretching at 1596 cm^{-1}, C-O stretching at 1140 cm^{-1}, *trans*-double bond C-H bending at 957 cm^{-1} and C-H out-of-plane bending of *meta*-substituted benzenoid compound at 827, 747 and 687 cm^{-1}.

The ^{1}H NMR spectrum of compound PE09 (**Figure 60** and **Table 14**) displayed resonances similar to those observed for compound PE05, especially those of the *para*-hydroxybenzyl moiety and ring A of the *trans*-stilbene skeleton. A methylene singlet at δ 4.00 (H-1″) and a pair of two-proton doublets (*J* = 8.6 Hz) at δ 6.68 (H-4″/H-6″) and 7.01 (H-3″/H-7″) represented the hydroxybenzyl group, whereas a pair of *meta*-coupled doublets (*J* = 2.1 Hz) at δ 6.39 (H-4) and 6.70 (H-6) and a methoxyl singlet at δ 3.76 (3-OCH_3) represented ring A of PE09. Compared to those of PE05, the signals of *trans*-double bond shifted upfield to δ 6.90 (1H, *d*, *J* = 16.2 Hz, H-β) and 7.30 (1H, *d*, *J* = 16.2 Hz, H-α). Significant differences were the signal pattern of ring B aromatic protons at δ 7.24 (1H, *t*, *J* = 7.2 Hz, H-4′), 7.30 (2H, *t*, *J* = 7.2 Hz, H-3′/H-5′) and 7.38 (2H, *d*, *J* = 7.2 Hz, H-2′/H-6′) which suggested an unsubstituted phenyl moiety.

The ^{13}C NMR spectrum (**Figure 61** and **Table 14**) exhibited resonances of three quaternary carbons attached to oxygen atoms at δ 153.4 (C-5″), 154.8 (C-5), 159.0 (C-3). Four double peaks representing four pairs of symmetric carbons appeared at δ 115.1 (C-4″/C-6″), 126.6 (C-2′/C-6′), 128.7 (C-3′/C-5′) and 129.2 (C-3″/C-7″). These data were supportive of a molecular structure similar to compound PE05 but with one less hydroxyl substituent and an unsubstituted phenyl ring.

Therefore, compound PE09 was identified as (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene and its spectroscopic data were in agreement with reported values (Garo *et al*., 2007).

(*E*)-2-(4″-Hydroxybenzyl)-5-hydroxy-3-methoxystilbene was also found in other lady slipper orchids including *Phragmepedium calurum* and *P. longifolium*. Its cytotoxic activity against several cell lines such as NCI-H460 (large cell lung carcinoma), NCI-H226 (lung squamous cell carcinoma), NCI-H522 (non-small cell lung adenocarcinoma), PC-3 (prostate adenocarcinoma), M14 (amelanotic melanoma) and A549 (non-small cell lung adenocarcinoma), has been reported as moderate (Starks *et al*., 2012).

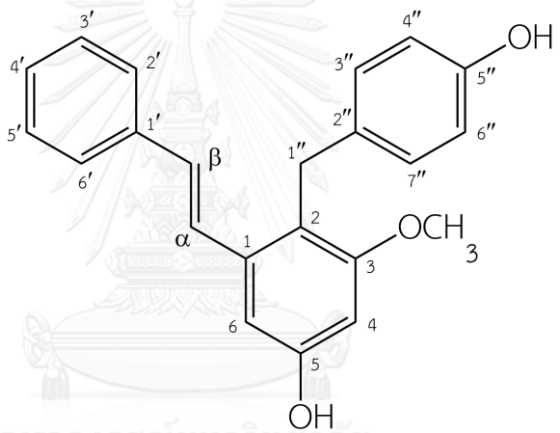


Figure 13 Chemical structure of (*E*)-2-(4″-hydroxybenzyl)-3,5′-dimethoxy-5-hydroxystilbene

Table 14 ^{1}H and ^{13}C NMR spectral data of compound PE09 (in $CDCl_3$) and (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene (in CD_3OD)

Position	Compound PE09		(*E*)-2-(4''-hydroxybenzyl)-3,5'-dimethoxy-5-hydroxystilbene[a]	
	δ_H (mult., *J* in Hz)	δ_C	δ_H (mult., *J* in Hz)	δ_C
1	-	138.5	-	140.4
2	-	120.2	-	120.5
3	-	159.0	-	160.6
4	6.39 (d, 2.1)	98.5	6.42 (d, 2.0)	99.6
5	-	154.8	-	158.2
6	6.70 (d, 2.1)	104.2	6.71 (d, 2.0)	104.9
α	7.30 (d, 16.2)	126.4	7.31 (d, 16.2)	128.4
β	6.90 (d, 16.2)	130.9	6.91 (d, 16.2)	130.7
1′	-	137.4	-	139.5
2′	7.38 (d, 7.2)	126.6	7.39 (d, 7.5)	127.5
3′	7.30 (t, 7.2)	128.7	7.30 (t, 7.3)	130.1
4′	7.24 (t, 7.2)	127.7	7.20 (t, 7.3)	128.7
5′	7.30 (t, 7.2)	128.7	7.30 (t, 7.3)	130.1
6′	7.38 (d, 7.2)	126.6	7.39 (d, 7.5)	127.5
1″	4.00 (s)	29.9	3.98 (s)	30.2
2″	-	133.6	-	134.5
3″/7″	7.01 (d, 8.6)	129.2	6.94 (d, 8.4)	130.4
4″/6″	6.68 (dt, 8.6)	115.1	6.63 (d, 8.4)	115.7
5″	-	153.4	-	156.5
5-OH	4.83 (s)	-	-	-
5″-OH	4.63 (s)	-	-	-
3-OMe	3.76 (s)	55.7	3.78 (s)	55.9

[a] Garo *et al*., 2007

Table 15 HMBC spectral data of compound PE09 (in acetone-d_6)

^{1}H signal	HMBC correlations with
H-4	C-2, C-3, C-5, C-6
H-6	C-2, C-4
H-α	C-1, C-2, C-6, C-1′
H-β	C-α, C-1, C-2′/ C-6′
H-2′/ H-6′	C-β, C-3′/ C-5′
H-3′/ H-5′	C-3′/ C-5′
H-4′	C-2′/ C-6′
H-1″	C-1, C-2, C-3, C-2″, C-3″/ C-7″
H-3″/ H-7″	C-1″, C-3″/ C-7″, C-5″
H-4″/ H-6″	C-2″, C-4″/ C-6″, C-5″
5-OH	C-5
5″-OH	C-5″
3-OMe	C-3

4.10. Taxonomic significance

The *trans*-stilbenes isolated in this study represented interesting chemotaxonomic significance. Among these six *trans*-stilbenes, (*E*)-3-methoxy-5-hydroxystilbene (PE03) is the most widely found in nature: it has been reported as a constituent of pteridophytes, gymnosperms, monocots and dicots. Its wide distribution might be due to its basic *trans*-stilbene structure with biosynthetically typical oxygenations at positions 3 and 5 of ring A, matching the carbonyl positions of acetate units used in the formation of the stilbenoid skeleton. The 2-*para*-hydroxybenzyl substitution on ring A, found in both (*E*)-3-methoxy-2-(4-hydroxybenzyl)-5,2′-dihydroxystilbene (PE05) and (*E*)-2-(4″-hydroxybenzyl)-3,5′-dimethoxy-5-hydroxystilbene (PE09), occurs limitedly in some members of Orchidaceae (Garo *et al*., 2007; Starks *et al*., 2012) and gymnosperm (Li *et al.*, 2001). Another common feature in the structures of PE05 and four other *trans*-stilbenes isolated from *P. exul*, i.e. (*E*)-3,2′-dimethoxy-5-hydroxystilbene (PE04), (*E*)-3,5′-dimethoxy-5,2′-dihydroxystilbene (PE07), (*E*)-3,2′-dimethoxy-5,6′-hydroxystilbene (PE08) and (*E*)-3-methoxy-5,2′-dihydroxystilbene (PE11) is the 2′-hydroxy or 2′-methoxy substitution. Based on the phenylpropanoid unit which is the biological origin of ring B and double bond of stilbenes, the position of this hydroxy or methoxy group is more difficult to occur than the frequently found 4′-oxysubstitution. Previously, the presence of compounds PE04, PE05 and PE11 has been reported only once from the genera *Paphiopedilum* (Lertnitikul *et al*., 2007) and *Phragmipedium* (Garo *et al*., 2007), and PE07 has been found twice in orchids of the same subfamily Cypripedioideae. The new compound in this study, (*E*)-3,2′-dimethoxy-5,6′-hydroxystilbene (PE08), also has this feature. It should be noted that the presence of a hydroxy group at this position is suitably located for the formation of furan ring in the 2-phenylbenzofuran stilbenes found in the roots of another *Paphiopedilum* species, *P. godefroyae* (Lertnitikul *et al*., 2007).

Three flavonoids were isolated from *Paphiopedilum exul* roots including galangin (PE01), pinocembrin (PE02) and alpinetin (PE06). PE01 is a flavonol, whereas PE02 and PE06 are flavanones. However, they all possess unsubstituted B ring, which is a feature of flavonoids abundantly found in plants of the families Zingiberaceae and

Asteraceae. Interestingly, alpinetin has 5-*O*-methylation, which is relatively rare in flavonoids and limitedly occurs in some woody plants (Harborne, 1977).

4.11. Cytotoxicity of isolated compounds

Resazurin microtiter assay (REMA) plate method was employed to examine the *in vitro* cytotoxicity of compounds isolated from the roots of *P. exul* against three cancer cell lines including small cell lung carcinoma (NCI-H187, ATCC CRL-5804), epidermoid carcinoma of oral cavity (KB, ATCC CCL-17) and breast adenocarcinoma (MCF-7, ATCC HTB-22) and a normal cell line, African green monkey kidney epithelial cells (Vero). Percent inhibition of cell growth was used for screening of the cytotoxic potential of these compounds. Isolated compounds, at a concentration of 50 µg/ml, were assayed and percent inhibition of more than 50% was considered to be cytotoxic. Only eight isolated compounds (PE01-05, PE07-09) were subjected to cytotoxicity evaluation due to their limited amount and the results are shown in **Table 16**. Compounds PE04, PE05, PE07, PE08 and PE09 were cytotoxic against all three cancer cell lines, and compounds PE01 and PE03 were cytotoxic against only two cell lines: NCI-H187 and KB. All stilbenes were cytotoxic against normal cell line (Vero cells), but galangin, which is a flavonoid, was not cytotoxic to this cell line. Some of their IC_{50} values are shown in **Table 17**.

Topoisomerase I inhibitory activity of these isolated compounds was investigated using the yeast cell-based assay. The budding yeast *Saccharomyces cerevisiae* has been a valuable model for the study of underlying anticancer mechanism including the inhibition of topoisomerase enzyme. For the RS190 strain, wild type *topI* gene of the yeast cell was deleted and *TopI* gene of *Arabidopsis thaliana* was inserted into expression vector containing strong inducible promoter, *pGAL1*, so that galactose can promote the production of topoisomerase I enzyme from this gene (Sirikantaramas *et al.*, 2008). To evaluate topoisomerase I inhibitory activity, survival of the gene-engineered yeast was determined by comparison to the viability of yeast colonies in the vehicle control culture (DMSO plate) and positive control culture (CPT plate) on agar media containing glucose and galactose. The

pharmacological effect of topoisomerase-targeted anticancer drugs results from the formation of enzyme/DNA cleavage complex called topoisomerase poison (Pommier and Osheroff, 2012). Therefore, the parallel models between glucose and galactose cultures were established to compare yeast cell viability in the absence or presence of topoisomerase I enzyme, respectively. In the presence of topoisomerase I poison compound, the enzyme which is plentiful in galactose culture would be trapped in the form of enzyme/DNA cleavage complex. This complex is cytotoxic to yeast cells and can cause cell death. On the other hand, yeast cells in glucose culture do not produce topoisomerase I enzyme, thus the cells can survive in the presence of poison compound (Woo *et al*., 2001). As can be seen from the result presented in **Table 18** and **19**, both compounds PE01 and PE02 caused cell death in galactose culture but not in glucose culture, suggesting their action as topoisomerase I poison. On the other hand, compounds PE03, PE04, PE08 and PE09 inhibited yeast cell growth in both cultures but displayed less potential in the glucose culture. Therefore, these compounds might act as topoisomerase I poison in combination with other mechanisms of cytotoxicity.

Galangin (PE01) has been reported to be cytotoxic against several cell lines including OE33 human oesophageal adenocarcinoma (Wang *et al.*, 2011), A549 human lung carcinoma (Ludwiczuk *et al.*, 2011) and PANC-1 human pancreatic cell lines (Li *et al*., 2010). Many underlying cytotoxic mechanisms of galangin has been investigated. Previous reports revealed that galangin inhibited TNF- alpha gene expression (Ludwiczuk *et al*., 2011), induced apoptotic cell death, decreased Bcl-2 level in cells, caused cell arrest in G0-G1 phase (Tolomeo *et al.*, 2008) and inhibited topoisomerase I activity (Zhao and Zhang, 2015). This study provides supporting data for its topoisomerase I inhibitory activity and suggests that the flavonol could act as an enzyme poison. Furthermore, galangin displayed no cytotoxic effect on normal cell line in this study. Based on numerous reports and the result from this study, among the compounds isolated from *P. exul*, galangin appears to have the most potential to be developed as an anticancer. Other compounds including compounds PE04, PE05, PE07, PE08 and PE09 have been rarely investigated for their biological activities and

these data will hopefully be useful for further study on drug discovery from natural sources.

Table 16 Cytotoxicity of isolated compounds (reported in percent inhibition of cell growth)

Compound	% inhibition			
	KB	MCF-7	NCI-H187	Vero
PE01	90.57	43.27	80.61	15.49
PE02	7.10	-15.87	8.88	n.d.
PE03	92.12	45.60	99.36	97.89
PE04	98.81	66.76	100.05	98.41
PE05	99.00	99.89	100.43	93.71
PE06	n.d.	n.d.	n.d.	n.d.
PE07	98.71	98.83	100.17	99.37
PE08	96.86	92.92	100.00	95.63
PE09	98.95	98.38	99.95	95.42

Table 17 Cytotoxicity of isolated compounds (reported in IC_{50} values)

Compound	IC_{50} ± SD (μM)		
	KB	MCF-7	NCI-H187
PE01	102.25 ± 29.12	Inactive	57.02 ± 2.27
PE02	Inactive	Inactive	Inactive
PE03	124.11 ± 21.54	Inactive	118.67 ± 30.21
PE04	49.08 ± 19.29	74.85 ± 3.71	46.70 ± 11.52
PE05	n.d.	n.d.	n.d.
PE06	n.d.	n.d.	n.d.
PE07	n.d.	n.d.	n.d.
PE08	n.d.	n.d.	n.d.
PE09	n.d.	n.d.	n.d.
Doxorubicin	4.38 ± 1.34	15.69 ± 1.75	0.24 ± 0.06
Ellipticine	2.68 ± 0.41	n.d.	11.41 ± 0.85
Tamoxifen	n.d.	18.52 ± 0.89	n.d.

Table 18 Topoisomerase I inhibitory activity of vehicle control (1% DMSO) and positive control (camptothecin 10 µg/ml) evaluated by yeast cell-based assay

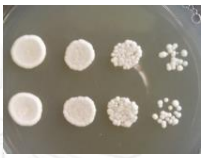
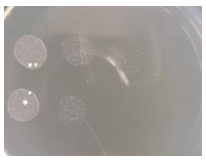
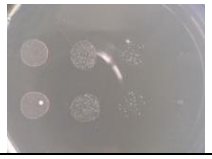
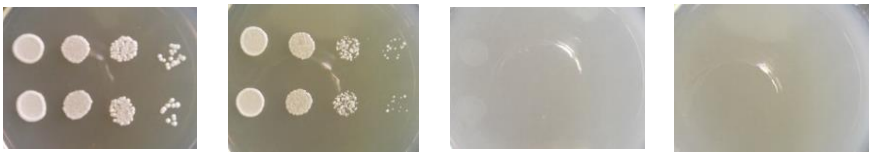
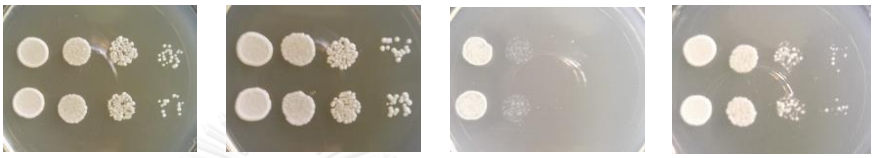
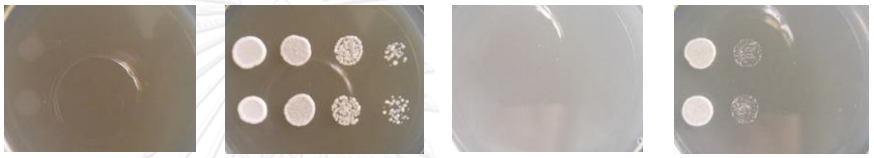
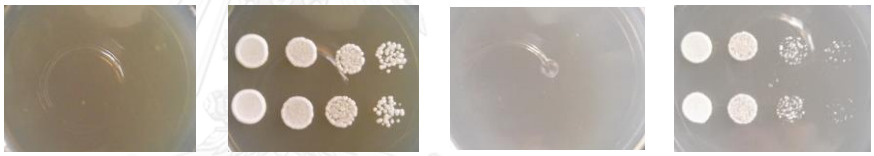
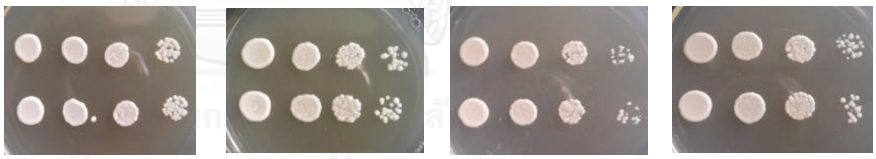
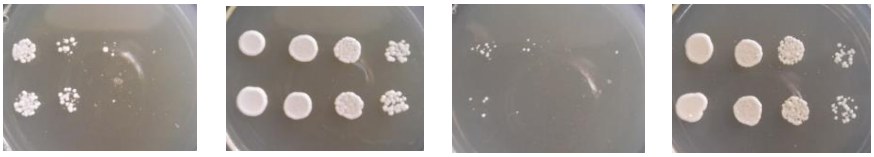
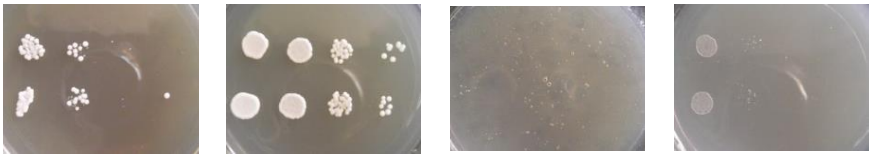
Compound	Glucose-containing culture	Galactose-containing culture
Vehicle control (1% DMSO)		
Positive control (Camptothecin 10 µg/ml)		
Positive control (Camptothecin 5 µg/ml)		
Positive control (Camptothecin 2.5 µg/ml)		

Table 19 Topoisomerase I inhibitory activity of isolated compounds evaluated by yeast cell-based assay

Tested compounds	Glucose-containing culture		Galactose-containing culture	
	100 μM	50 μM	100 μM	50 μM
PE01				
PE02				
PE03				
PE04				
PE05				
PE07				
PE08				
PE09				

CHAPTER 5
CONCLUSION

The methanolic extract of the roots of *Paphiopedilum exul*, a lady's slipper orchid native to the south of Thailand, was cytotoxic against small cell lung cancer cell line (NCI-H187). Isolation process for the determination of its cytotoxic constituents yielded one new compound, (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene, and eight known compounds including five stilbenes, (*E*)-5-hydroxy-3-methoxystilbene, (*E*)-5-hydroxy-3,2′-dimethoxystilbene, (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3,5′-dimethoxystilbene, (*E*)-5,2′-dihydroxy-3,5′-dimethoxystilbene and (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene, and three flavonoids: galangin, pinocembrin and alpinetin. The 2′-hydroxy substitution observable in three isolated *trans*-stilbenes appears to be characteristic of orchids in the subfamily Cypripedioideae and might be chemotaxonomically significant.

In vitro cytotoxicity of the isolated compounds was evaluated by rezasurin microtiter method against three cancer cell lines (NCI-H187, KB and MCF-7) and a normal cell line (Vero). Results of the assay showed that (*E*)-5-hydroxy-3,2′-dimethoxystilbene, (*E*)-2-(4″-hydroxybenzyl)-5,2′-dihydroxy-3,5′-dimethoxystilbene, (E)-5,2′-dihydroxy-3,5′-dimethoxystilbene, (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene and (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene, at 50 µg/ml, were cytotoxic against all three cancer cell lines, whereas galangin and (*E*)-5-hydroxy-3-methoxystilbene were cytotoxic against only NCI-H187 and KB cell lines. All stilbenes were cytotoxic against Vero cells, but the flavonoid galangin, which was cytotoxic to both KB and NCI-H187 cell lines, was not cytotoxic to normal cell line. Furthermore, topoisomerase I inhibitory activity of these compounds was examined using the yeast cell-based assay. The results demonstrated that galangin and pinocembrin might act as topoisomerase I poison, while (*E*)-5-hydroxy-3-methoxystilbene, (*E*)-5,6′-hydroxy-3,2′-dimethoxystilbene and (*E*)-2-(4″-hydroxybenzyl)-5-hydroxy-3-methoxystilbene, in addition to acting as topoisomerase I poison, might possess other mechanisms of

cytotoxicity. This study is the first report of chemical constituents of *P. exul* and their cytotoxicity.

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APPENDIX

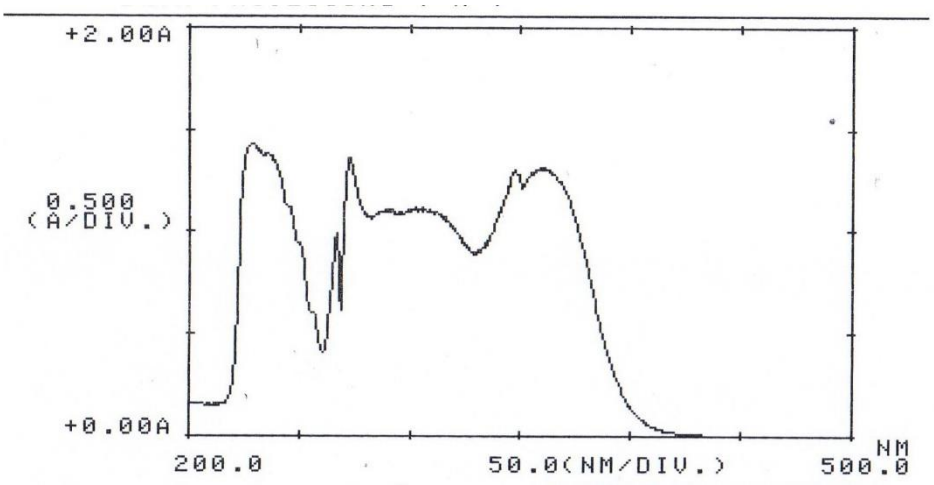


Figure 14 UV spectrum of compound PE01 (galangin)

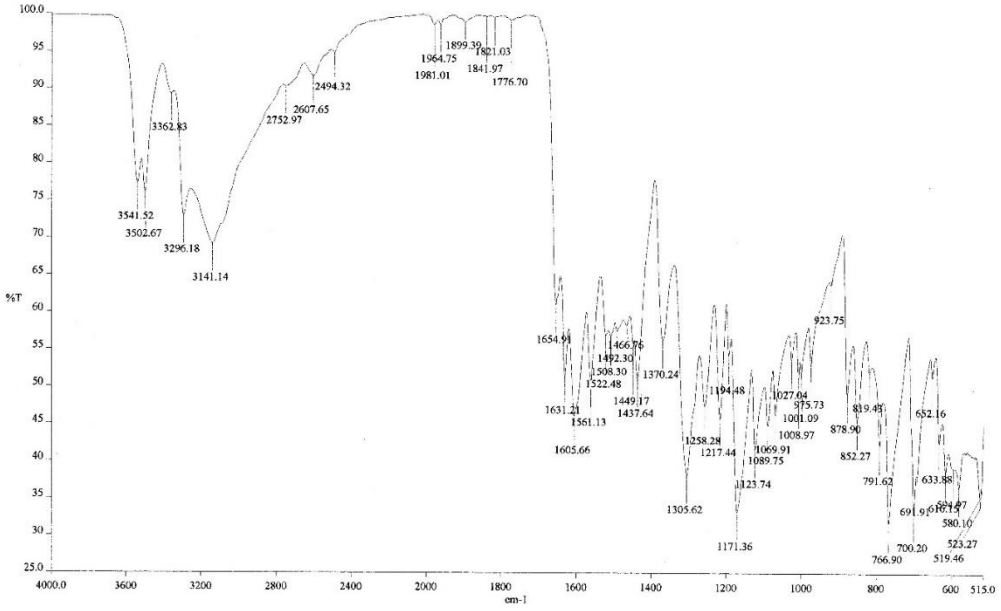


Figure 15 IR spectrum of compound PE01 (galangin)

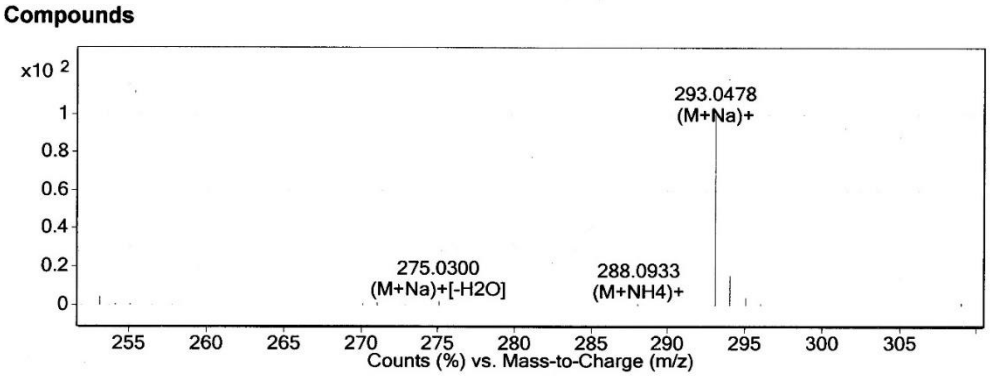


Figure 16 MS spectrum of compound PE01 (galangin)

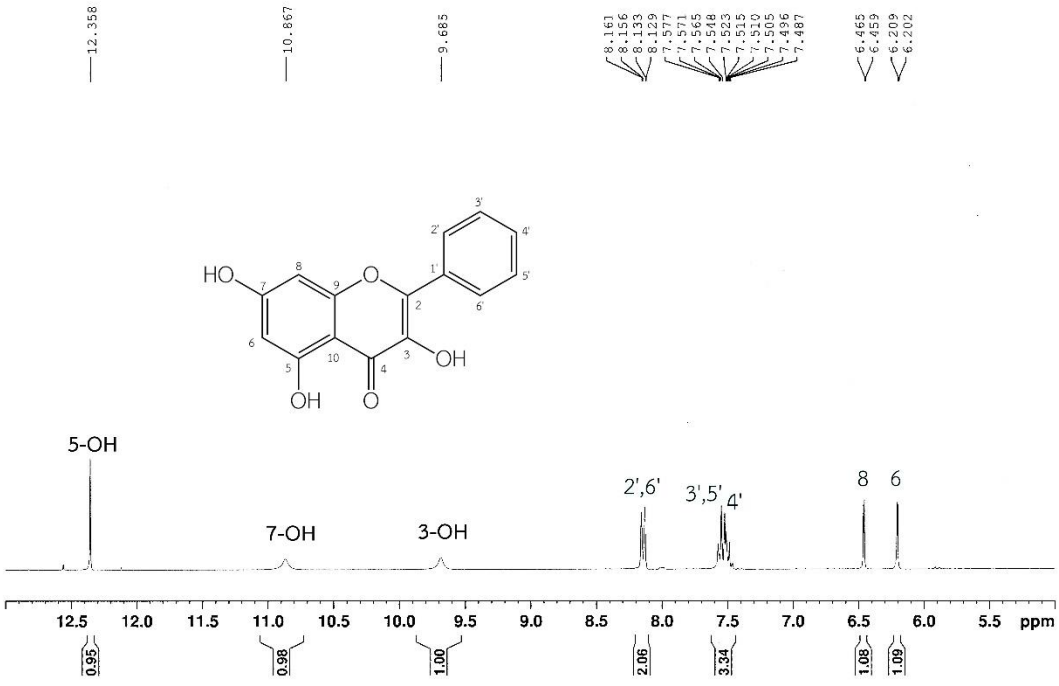


Figure 17 ^{1}H NMR (300 MHz) spectrum of compound PE01 (galangin) in DMSO-d_6

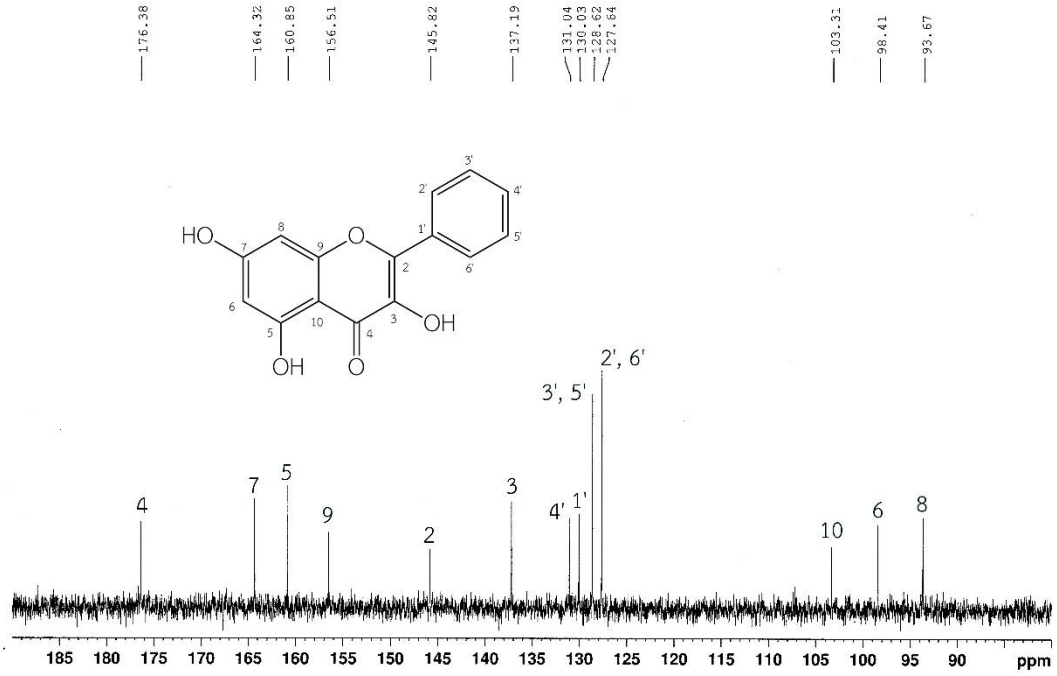


Figure 18 ^{13}C NMR (75 MHz) spectrum of compound PE01 (galangin) in DMSO-d_6

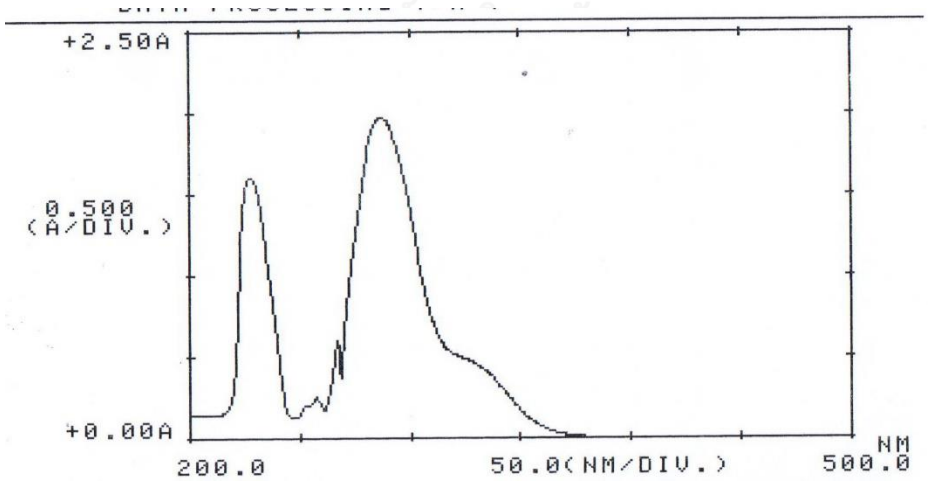


Figure 19 UV spectrum of compound PE02 (pinocembrin)

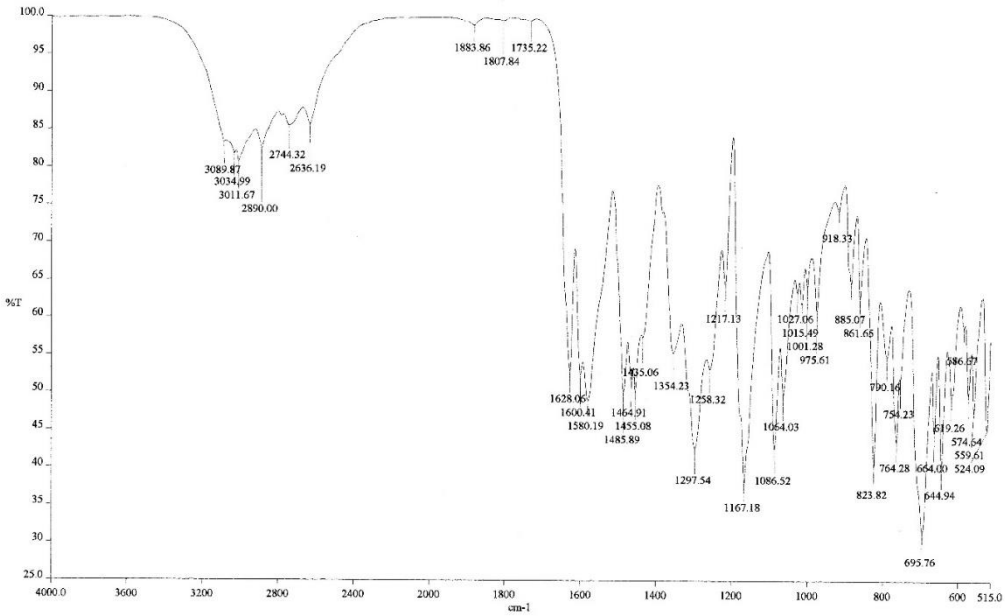


Figure 20 IR spectrum of compound PE02 (pinocembrin)

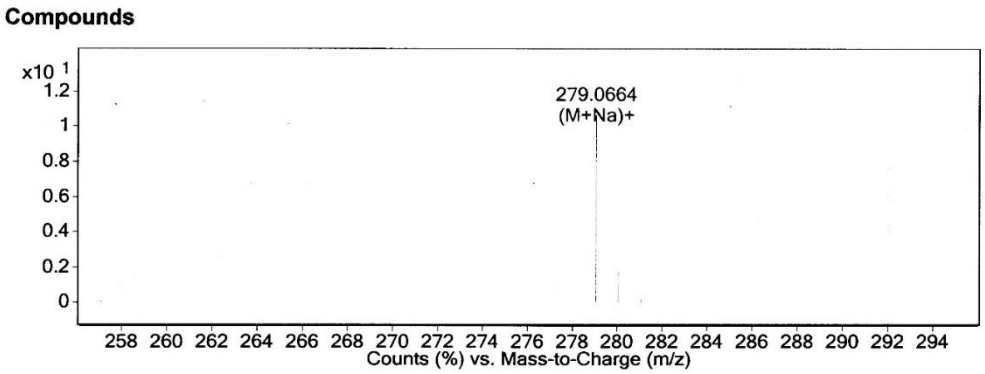


Figure 21 MS spectrum of compound PE02 (pinocembrin)

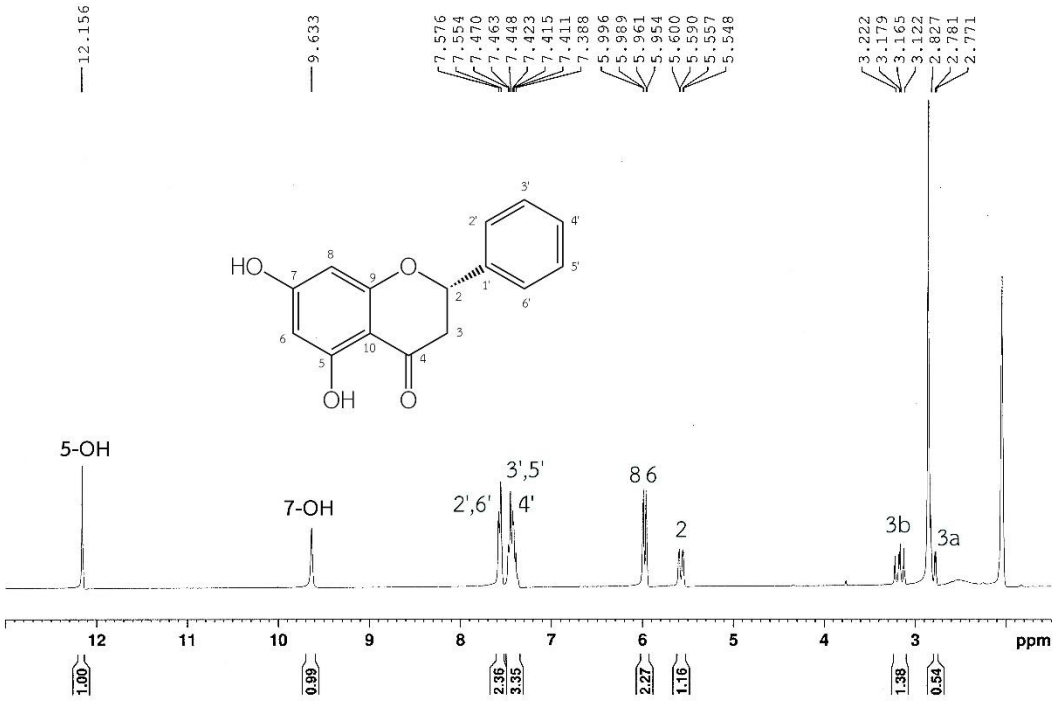


Figure 22 ^{1}H NMR (300 MHz) spectrum of compound PE02 (pinocembrin) in acetone-d_6

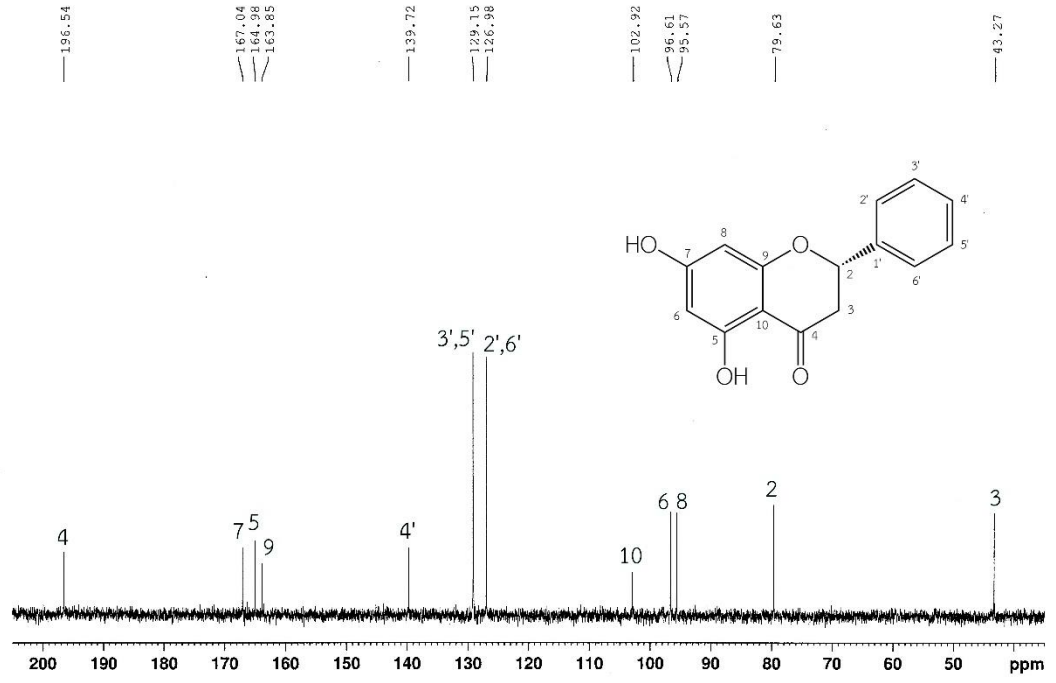


Figure 23 ^{13}C NMR (75 MHz) spectrum of compound PE02 (pinocembrin) in acetone-d_6

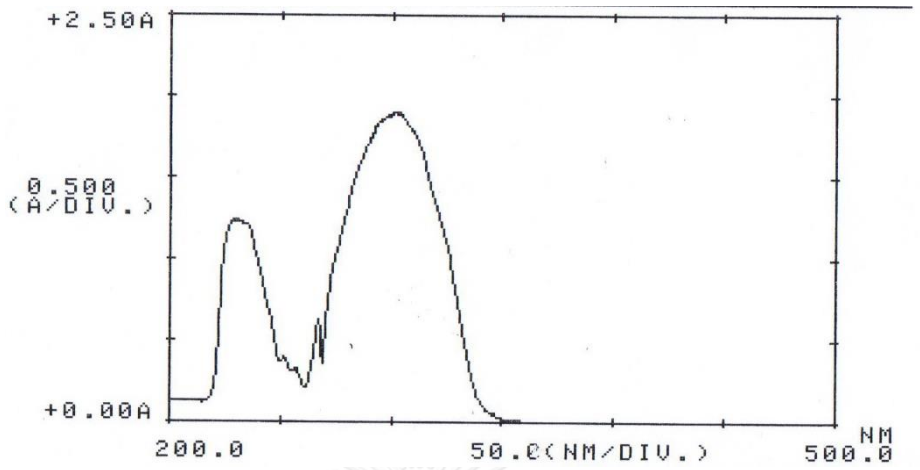


Figure 24 UV spectrum of compound PE03

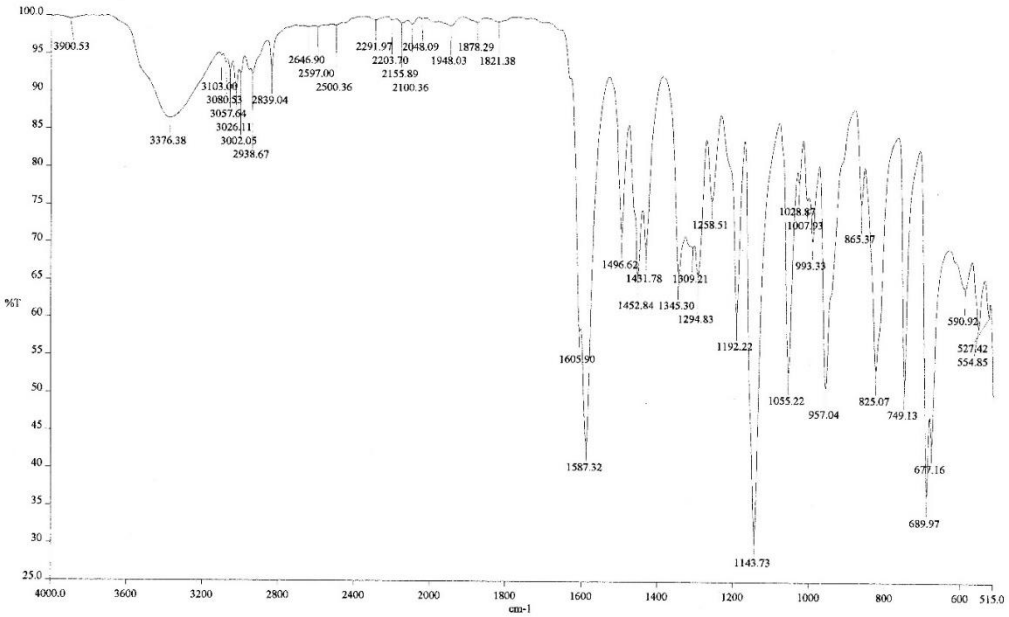


Figure 25 IR spectrum of compound PE03

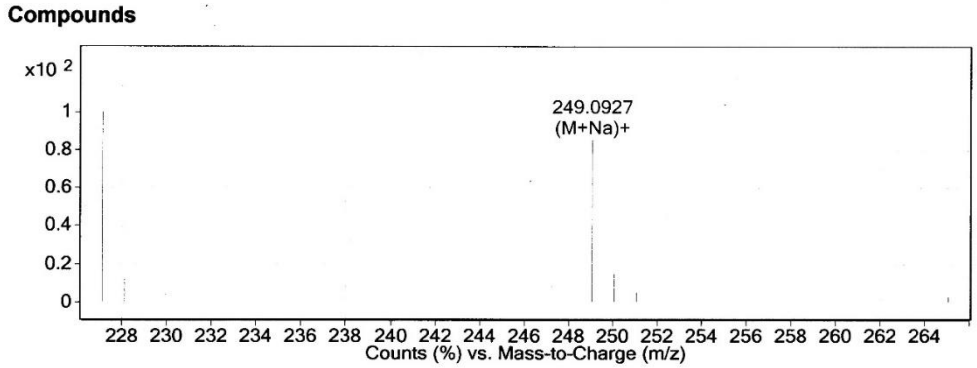


Figure 26 MS spectrum of compound PE03

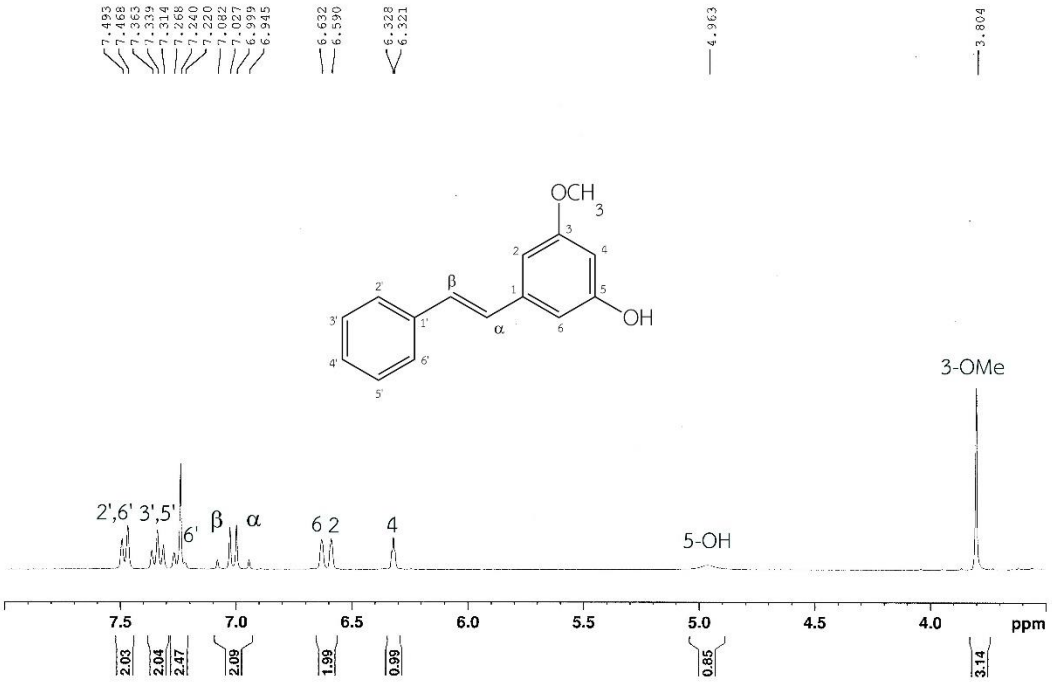


Figure 27 ^{1}H NMR (300 MHz) spectrum of compound PE03 in $CDCl_3$

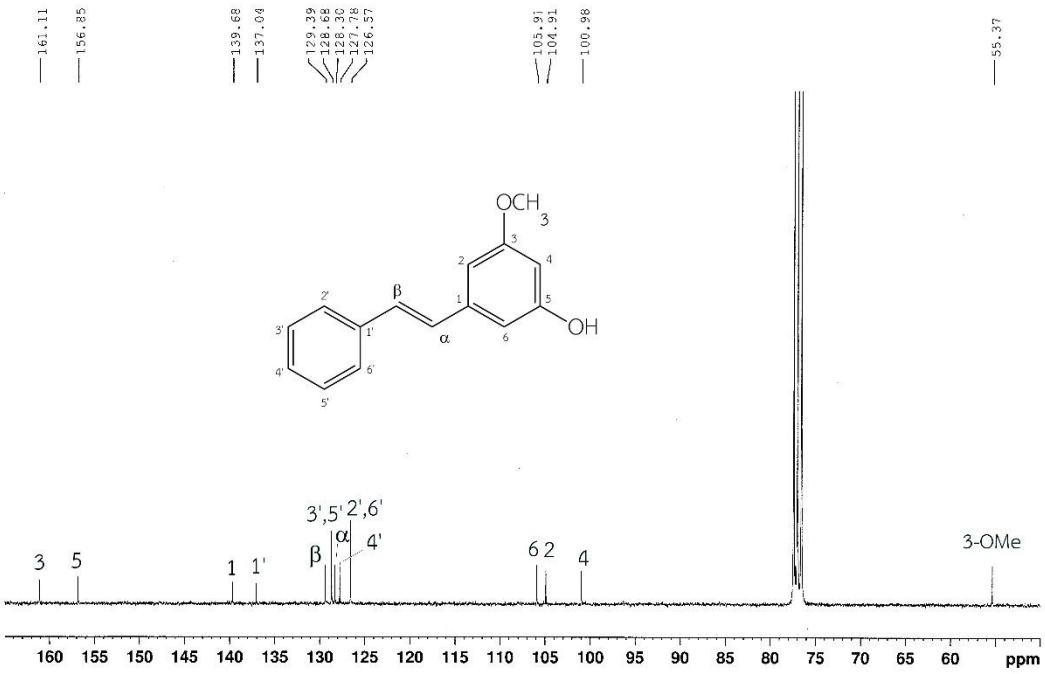


Figure 28 ^{13}C NMR (75 MHz) spectrum of compound PE03 in $CDCl_3$

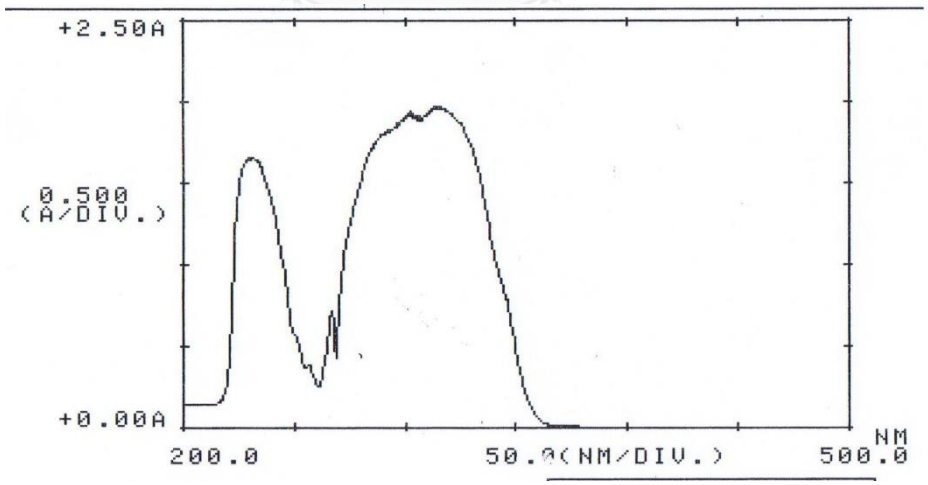


Figure 29 UV spectrum of compound PE04

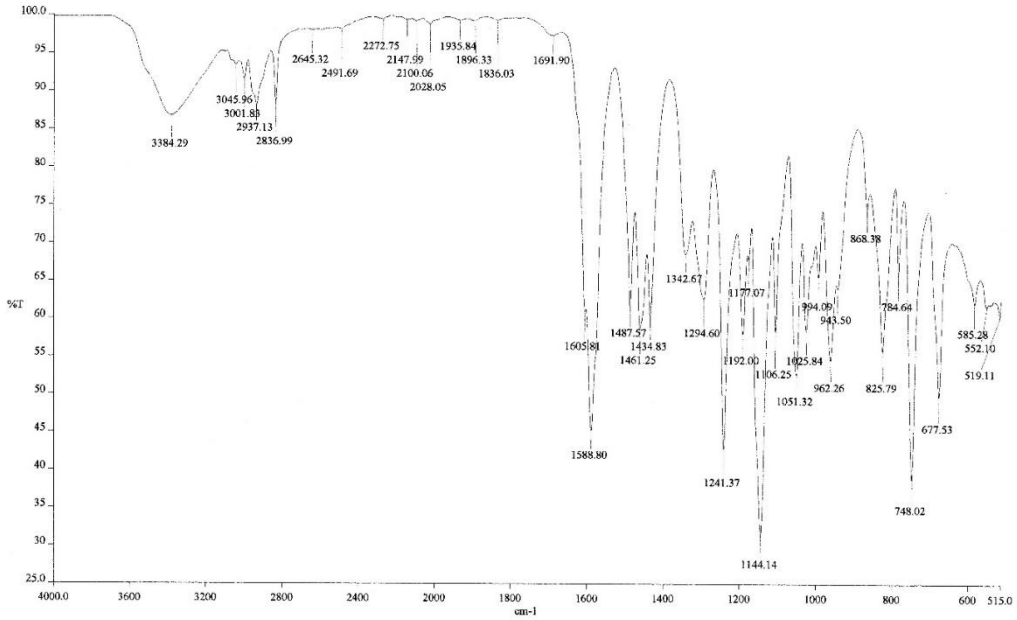


Figure 30 IR spectrum of compound PE04

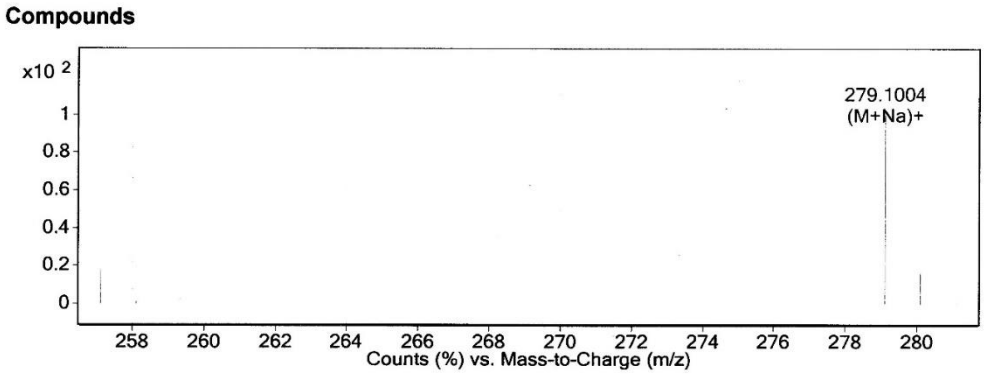


Figure 31 MS spectrum of compound PE04

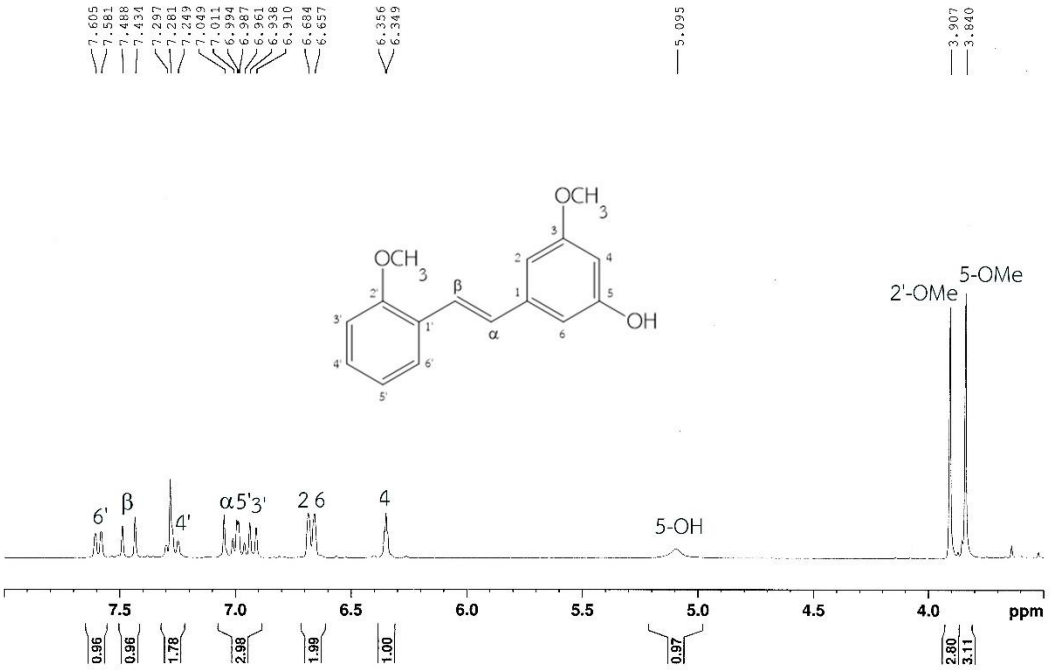


Figure 32 ^{1}H NMR (300 MHz) spectrum of compound PE04 in $CDCl_3$

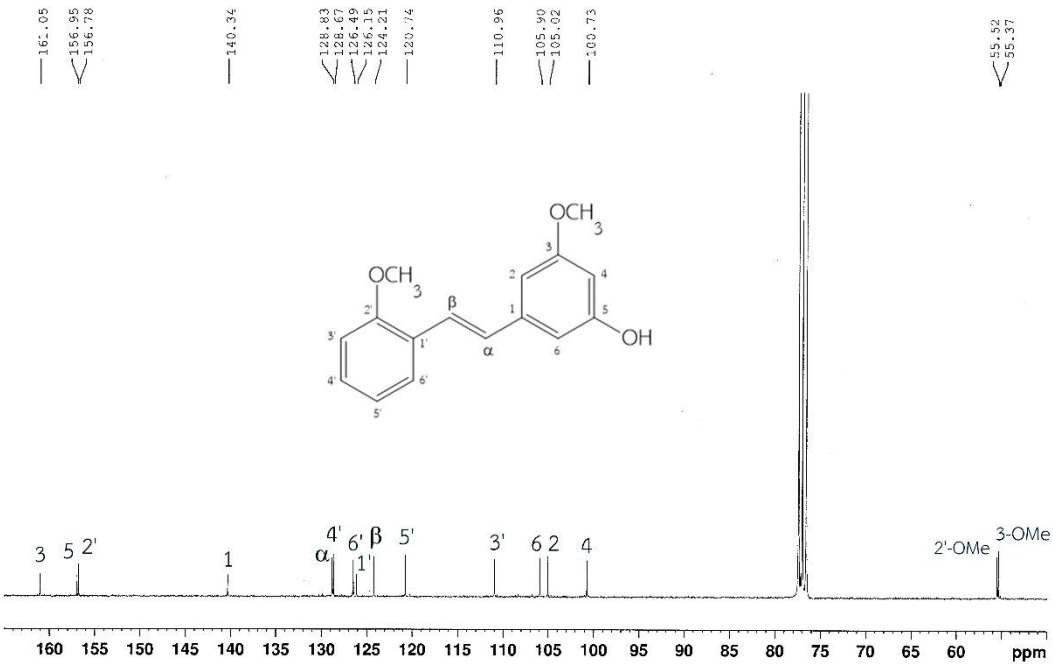


Figure 33 ^{13}C NMR (75 MHz) spectrum of compound PE04 in $CDCl_3$

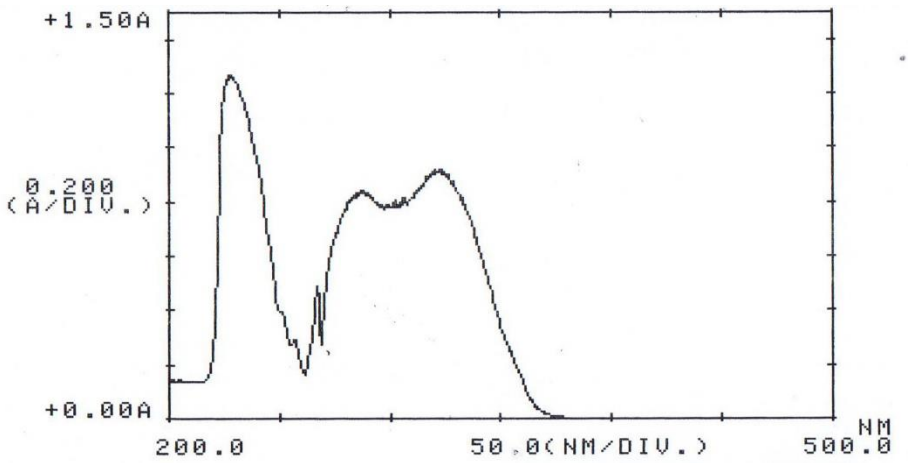


Figure 34 UV spectrum of compound PE05

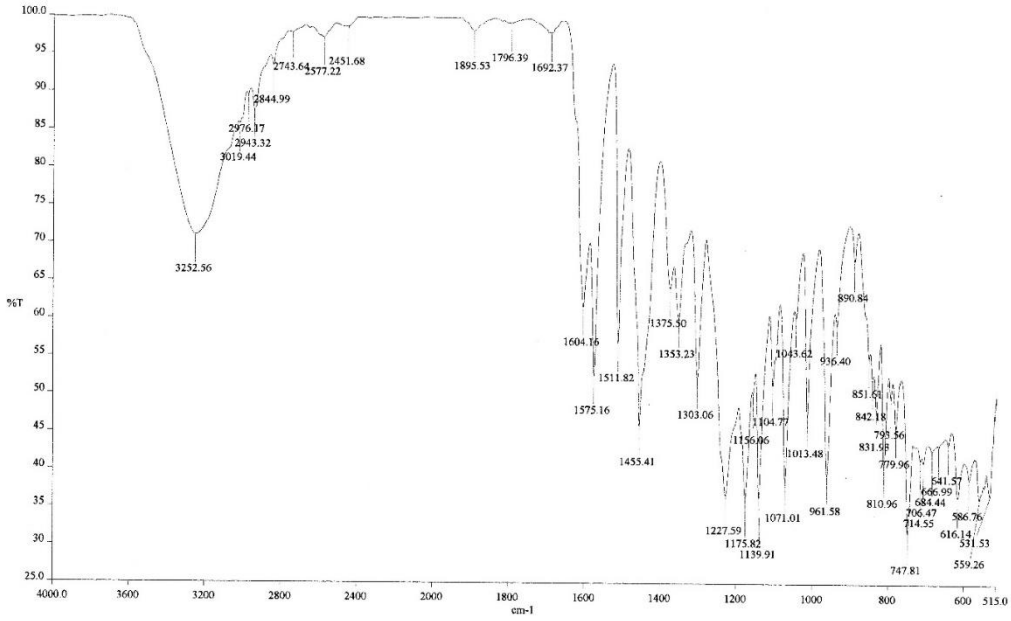


Figure 35 IR spectrum of compound PE05

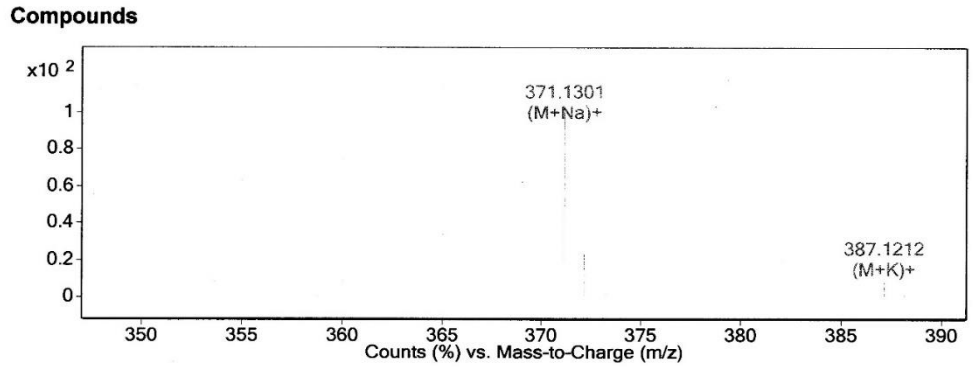


Figure 36 MS spectrum of compound PE05

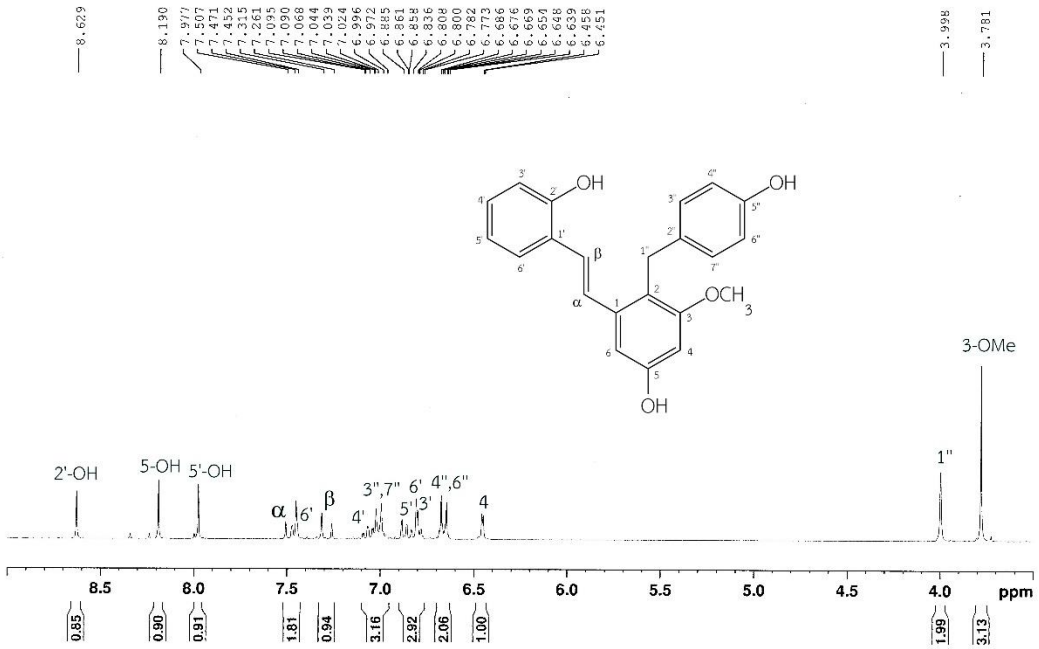


Figure 37 ^{1}H NMR (300 MHz) spectrum of compound PE05 in acetone-d_6

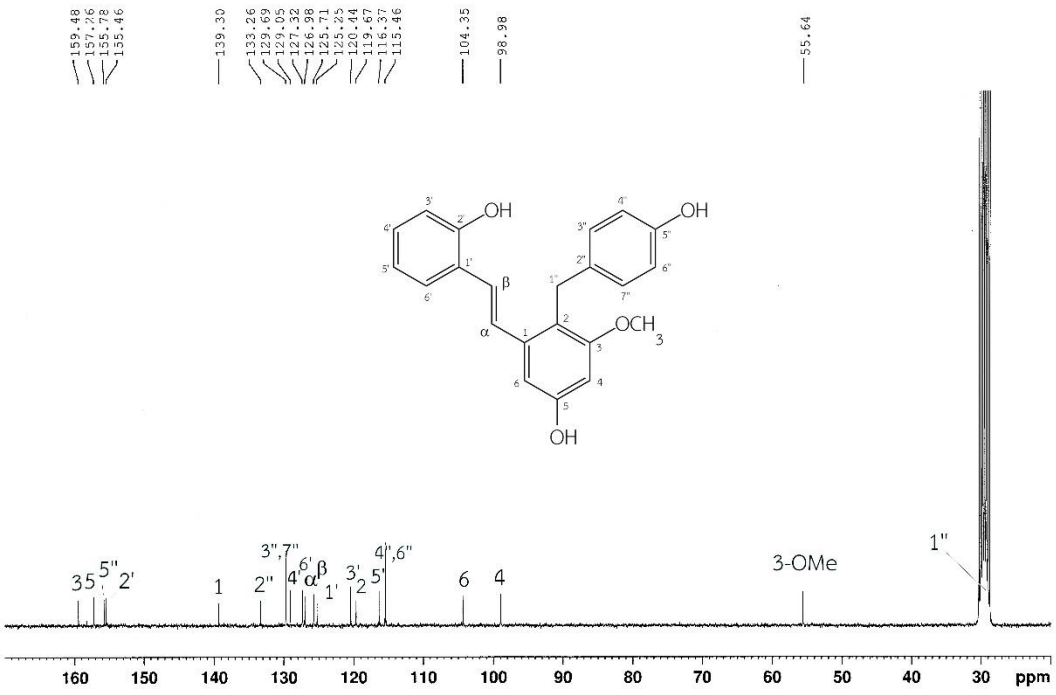


Figure 38 ^{13}C NMR (75 MHz) spectrum of compound PE05 in acetone-d_6

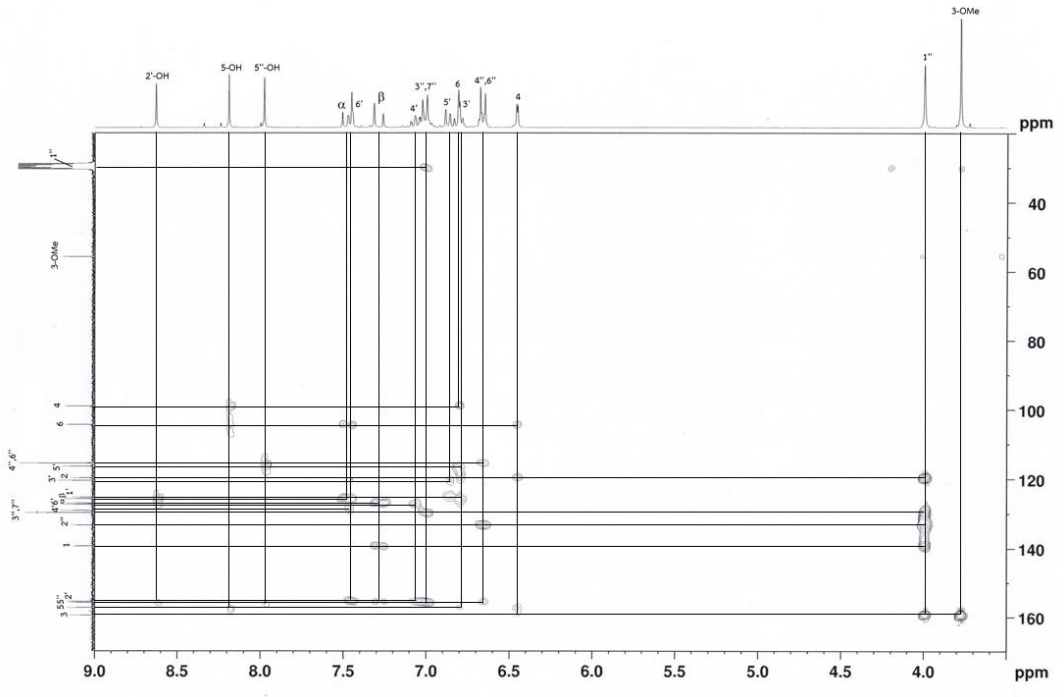


Figure 39 HMBC spectrum of compound PE05 in acetone-d_6

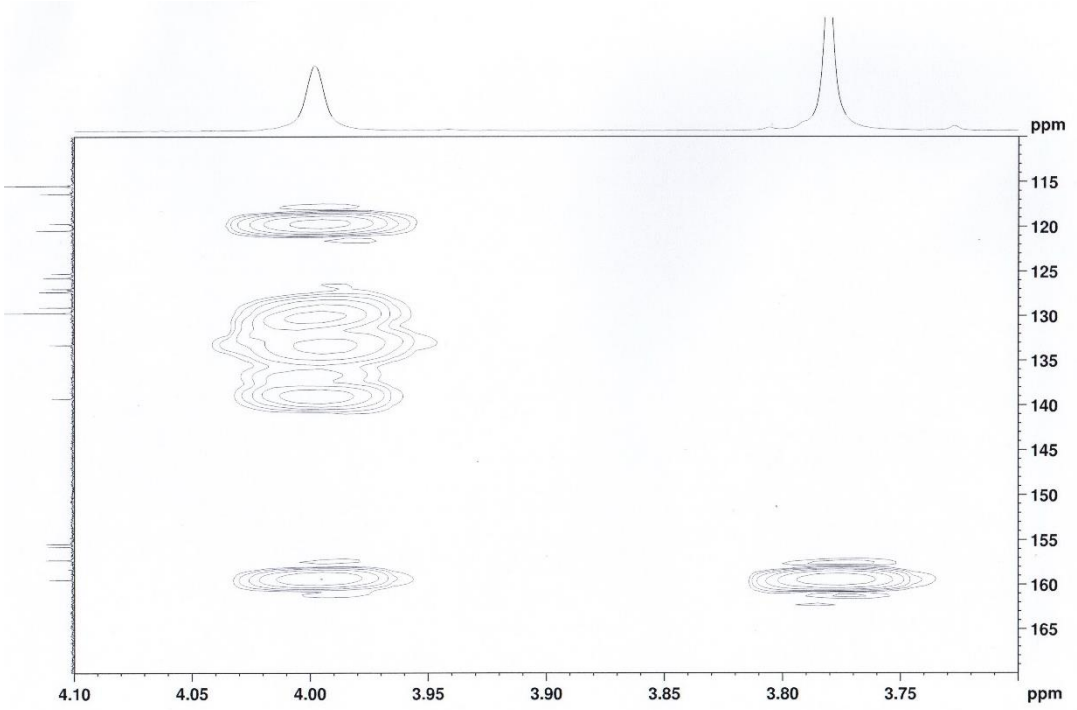


Figure 39 HMBC spectrum of compound PE05 in acetone-d_6 (continued)

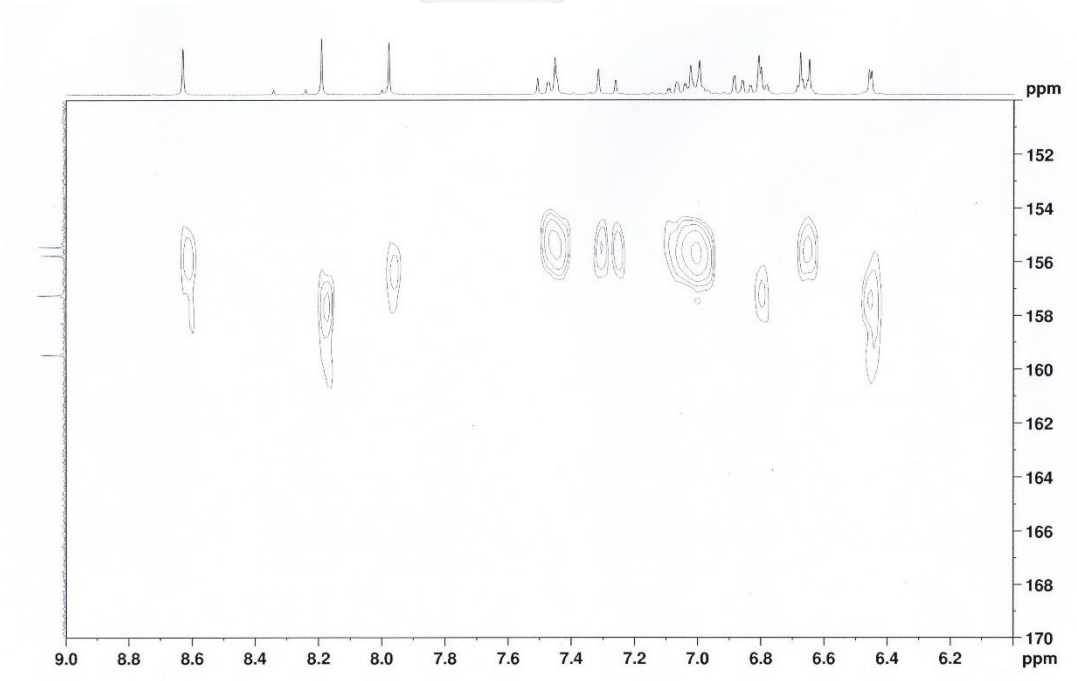


Figure 39 HMBC spectrum of compound PE05 in acetone-d_6 (continued)

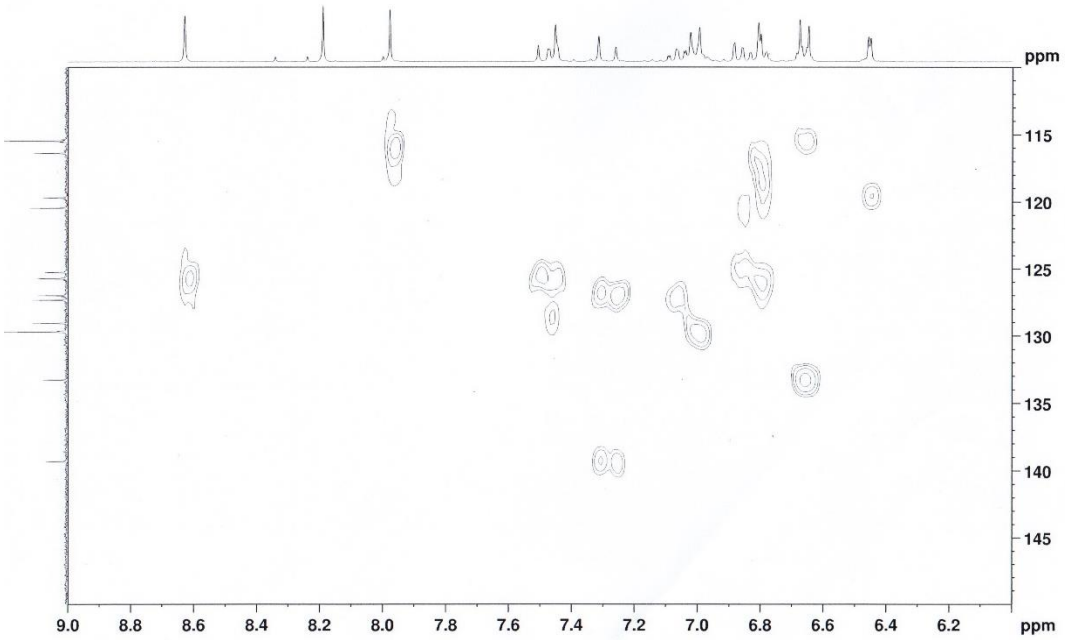


Figure 39 HMBC spectrum of compound PE05 in acetone-d_6 (continued)

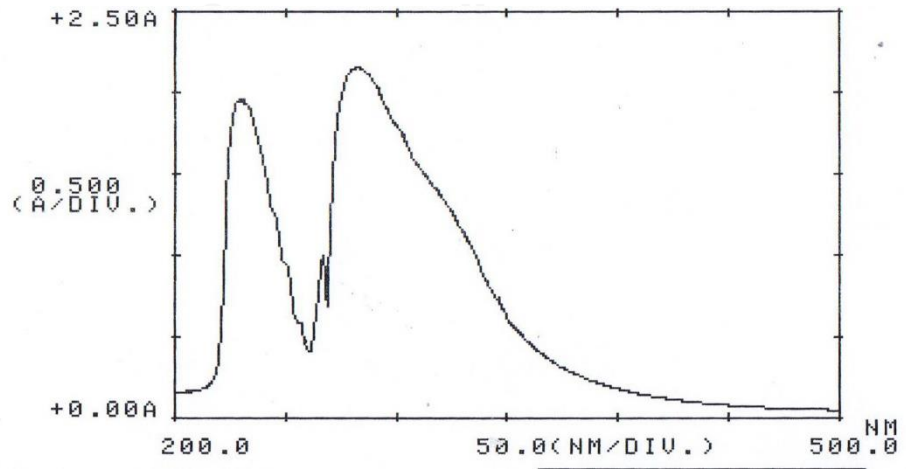


Figure 40 UV spectrum of compound PE06

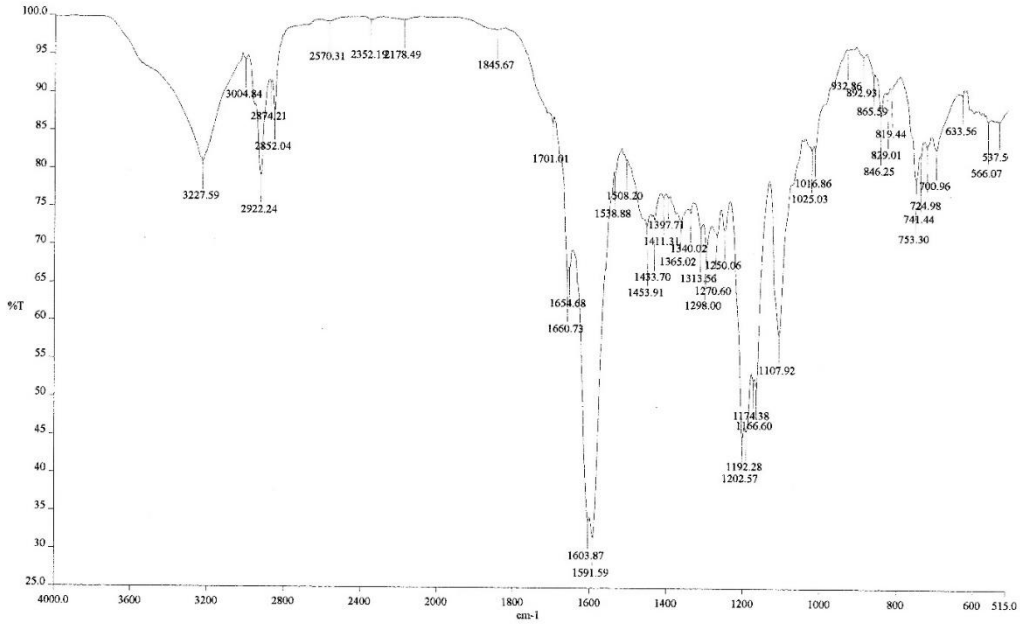


Figure 41 IR spectrum of compound PE06

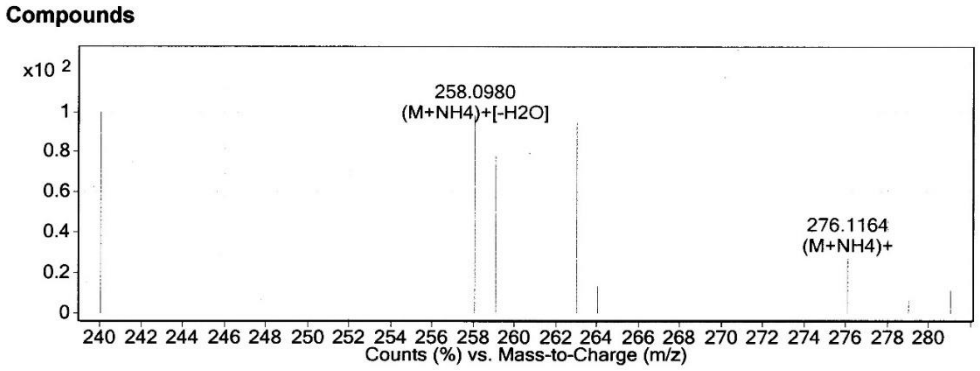


Figure 42 MS spectrum of compound PE06

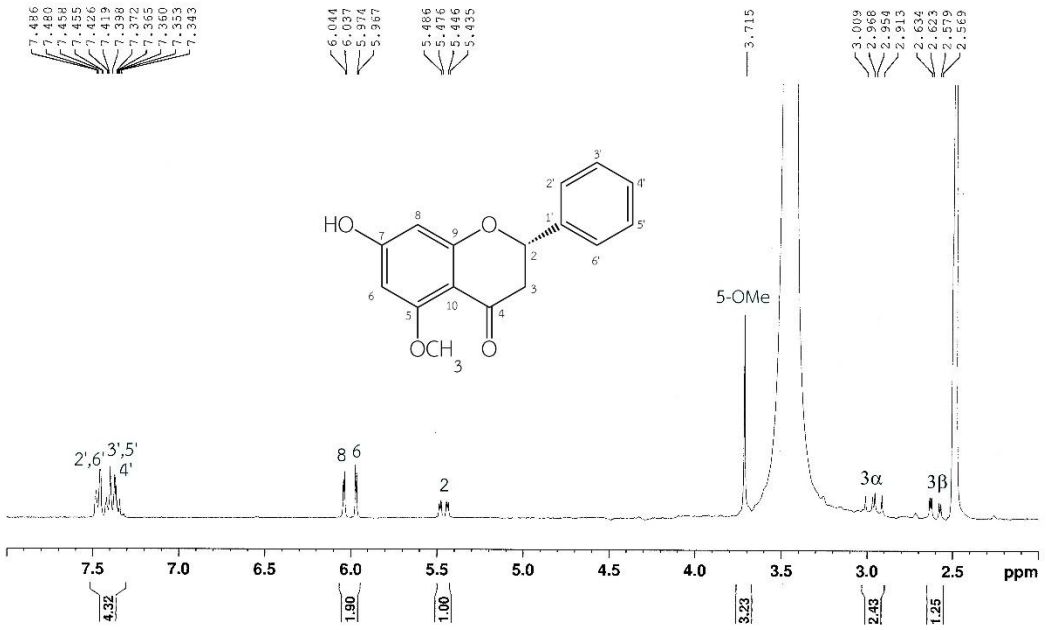


Figure 43 ^{1}H NMR (300 MHz) spectrum of compound PE06 in DMSO-d_6

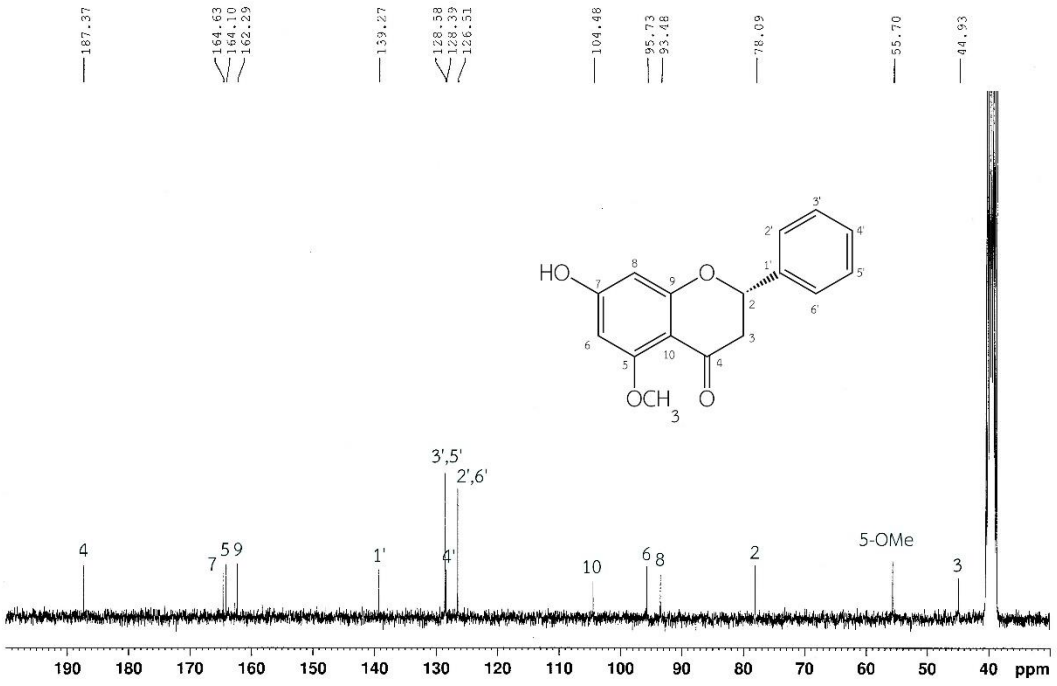


Figure 44 ^{13}C NMR (75 MHz) spectrum of compound PE06 in DMSO-d_6

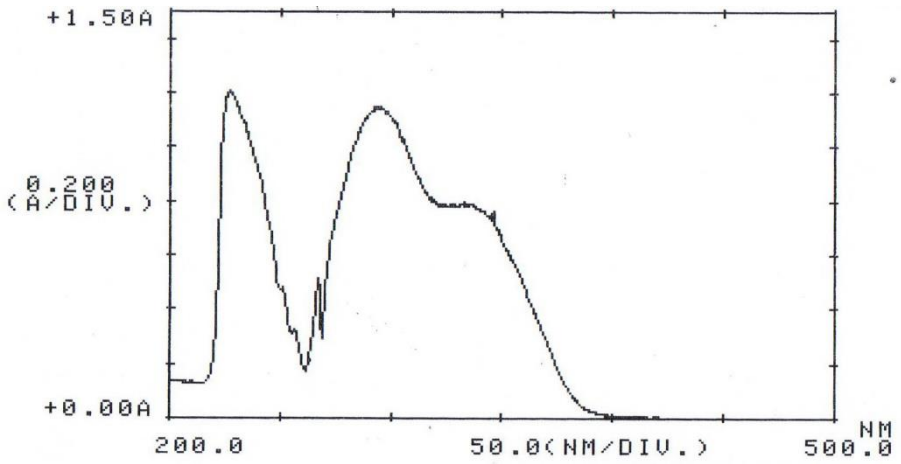


Figure 45 UV spectrum of compound PE07

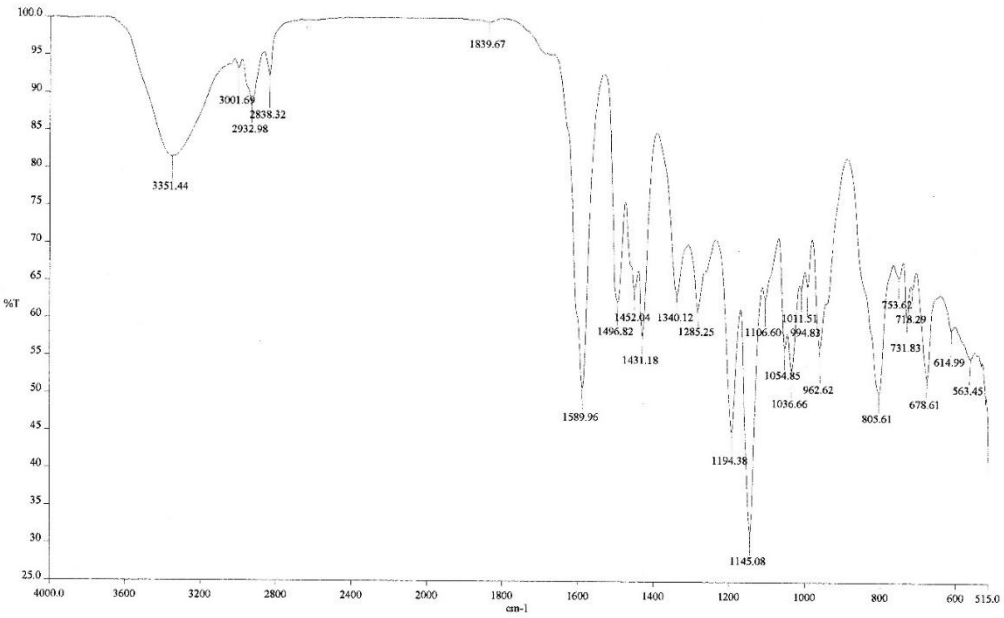


Figure 46 IR spectrum of compound PE07

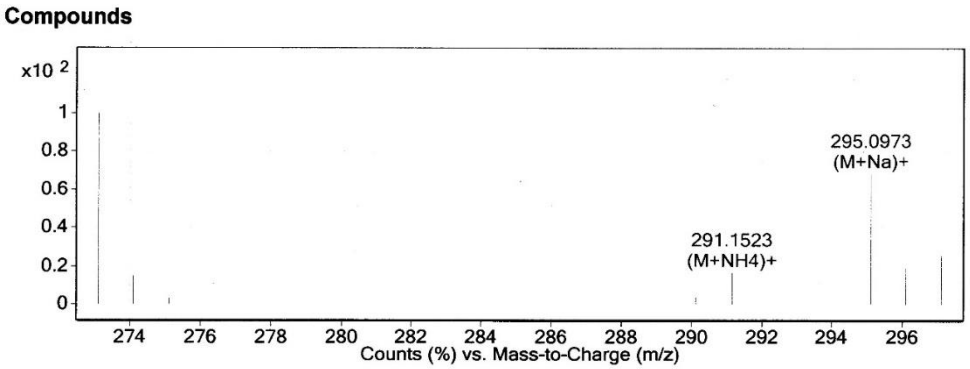


Figure 47 MS spectrum of compound PE07

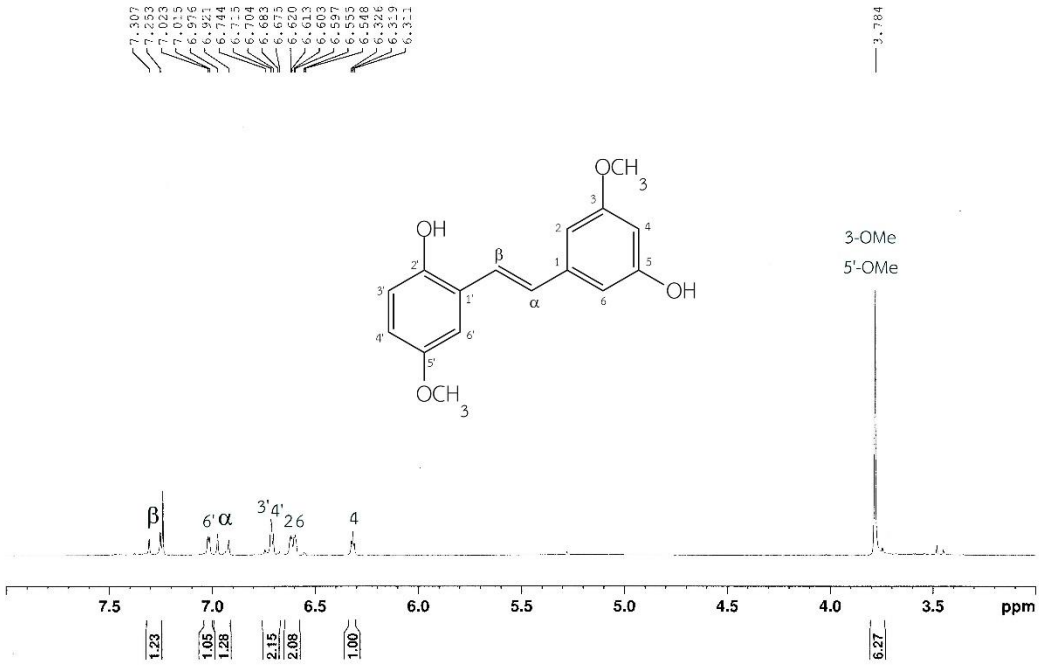


Figure 48 ^{1}H NMR (300 MHz) spectrum of compound PE07 in $CDCl_3$

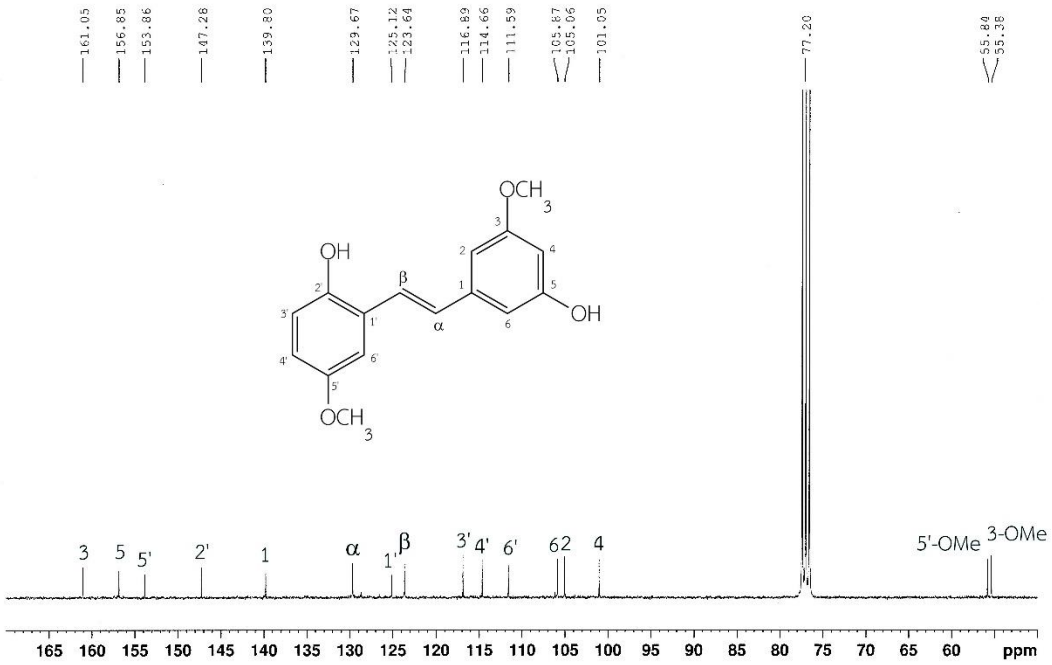


Figure 49 ^{13}C NMR (75 MHz) spectrum of compound PE07 in $CDCl_3$

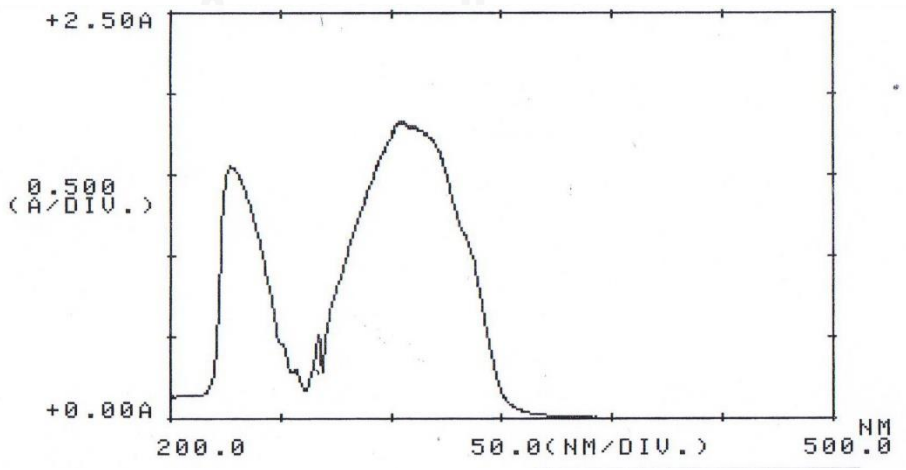


Figure 50 UV spectrum of compound PE08

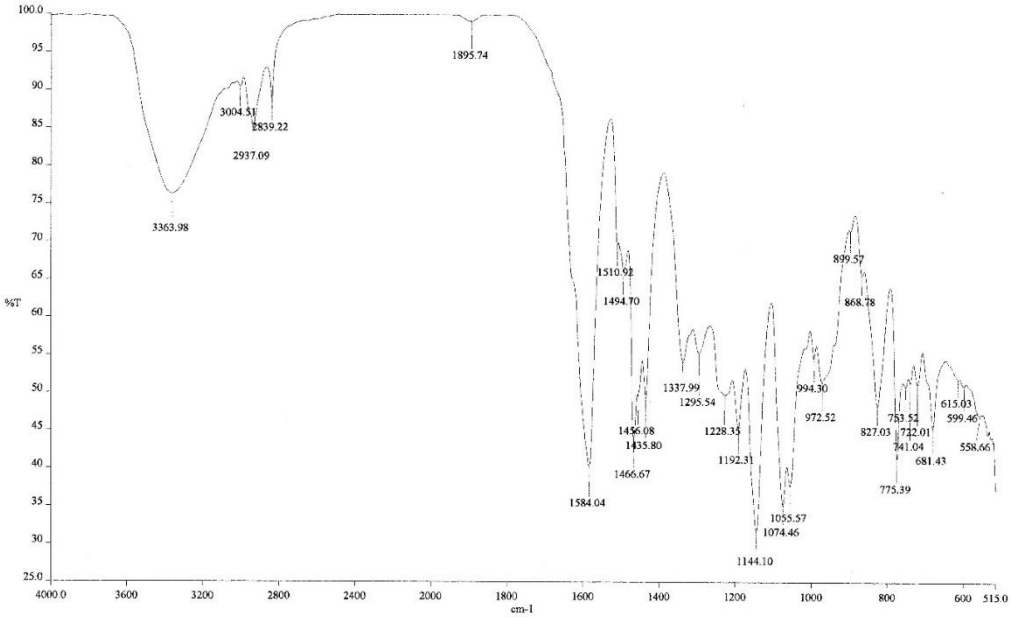


Figure 51 IR spectrum of compound PE08

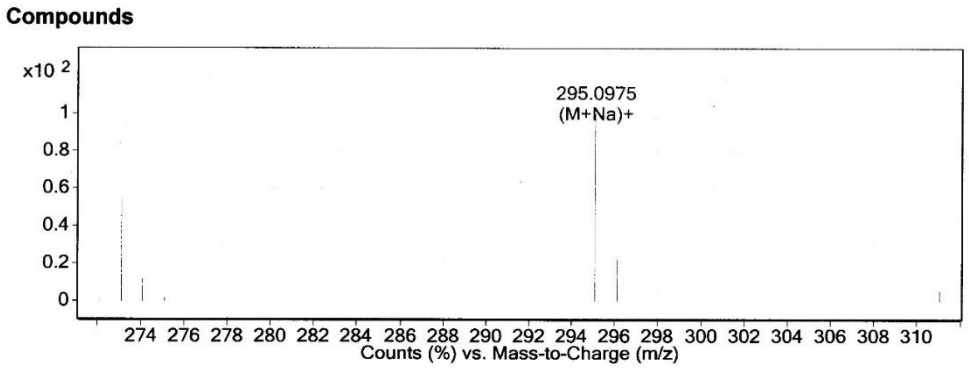


Figure 52 MS spectrum of compound PE08

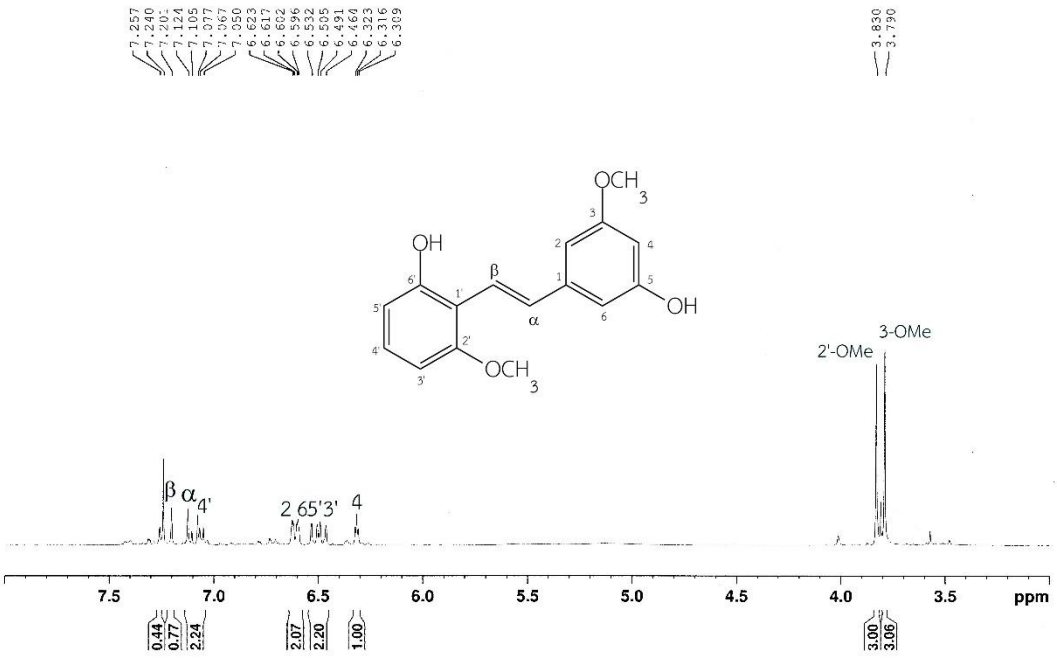


Figure 53 ^{1}H NMR (300 MHz) spectrum of compound PE08 in $CDCl_3$

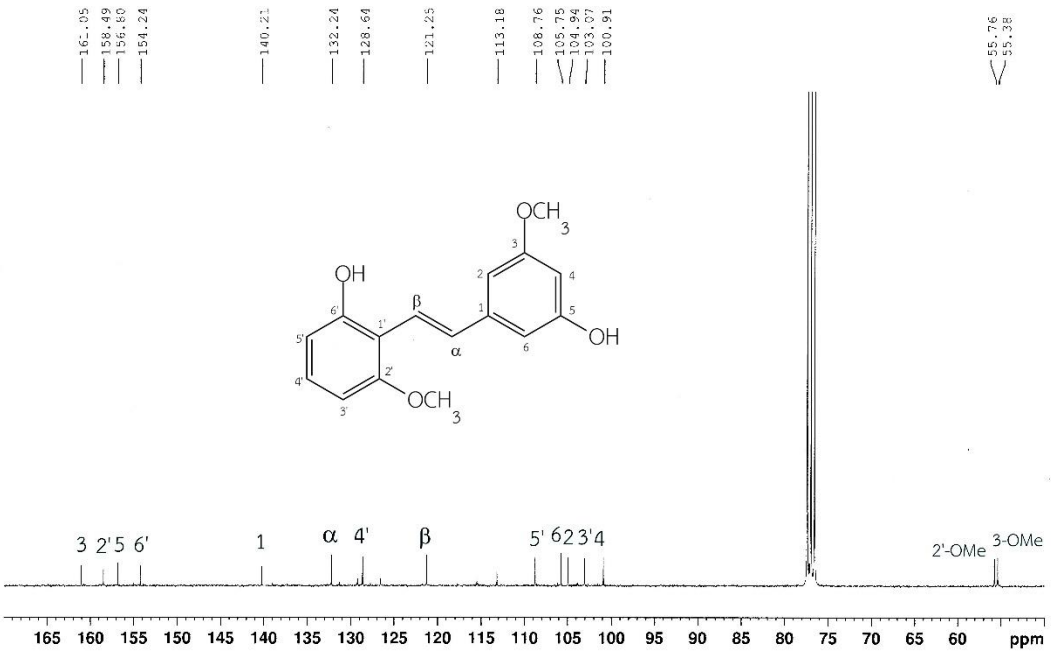


Figure 54 ^{13}C NMR (75 MHz) spectrum of compound PE08 in $CDCl_3$

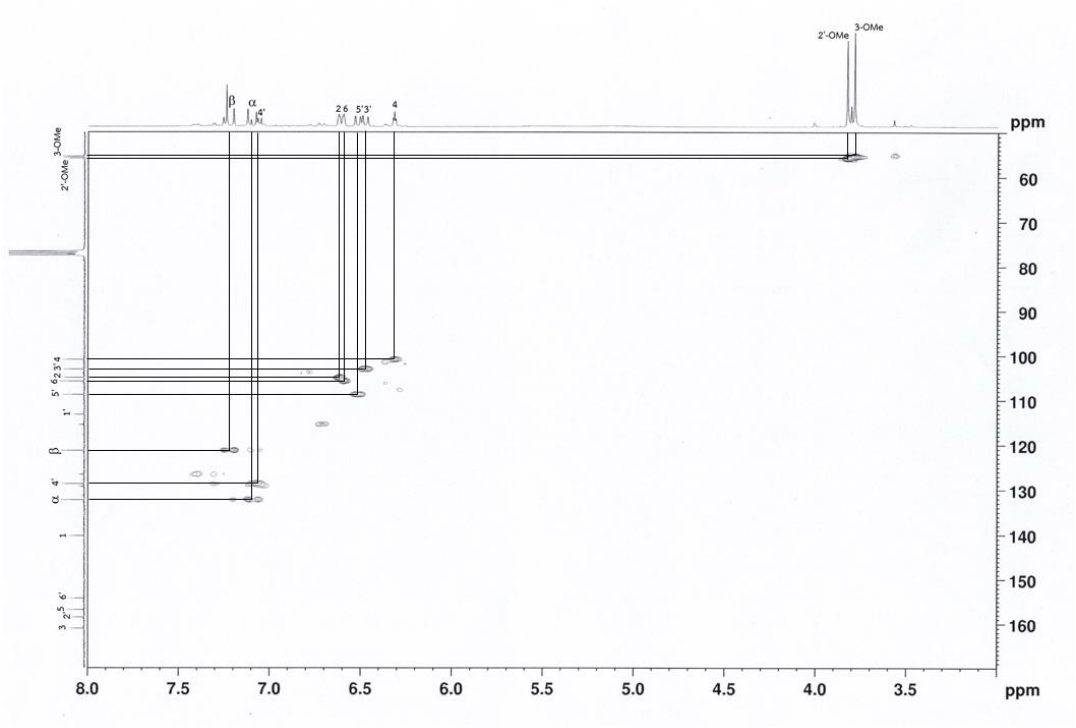


Figure 55 HSQC spectrum of compound PE08 in $CDCl_3$

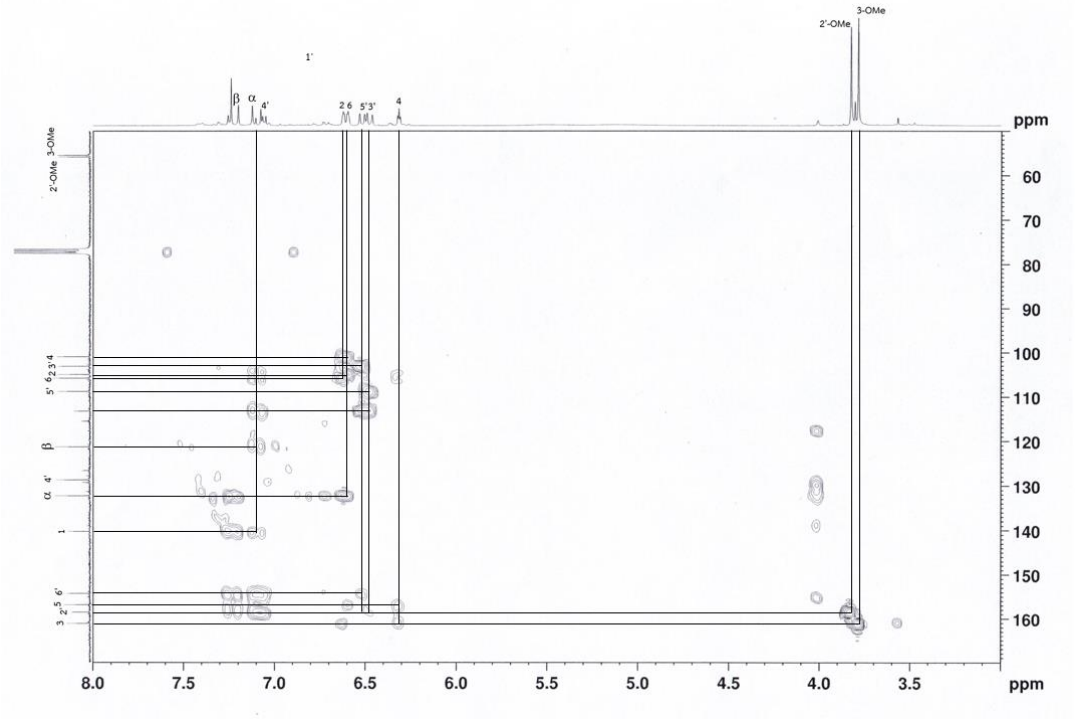


Figure 56 HMBC spectrum of compound PE08 in $CDCl_3$

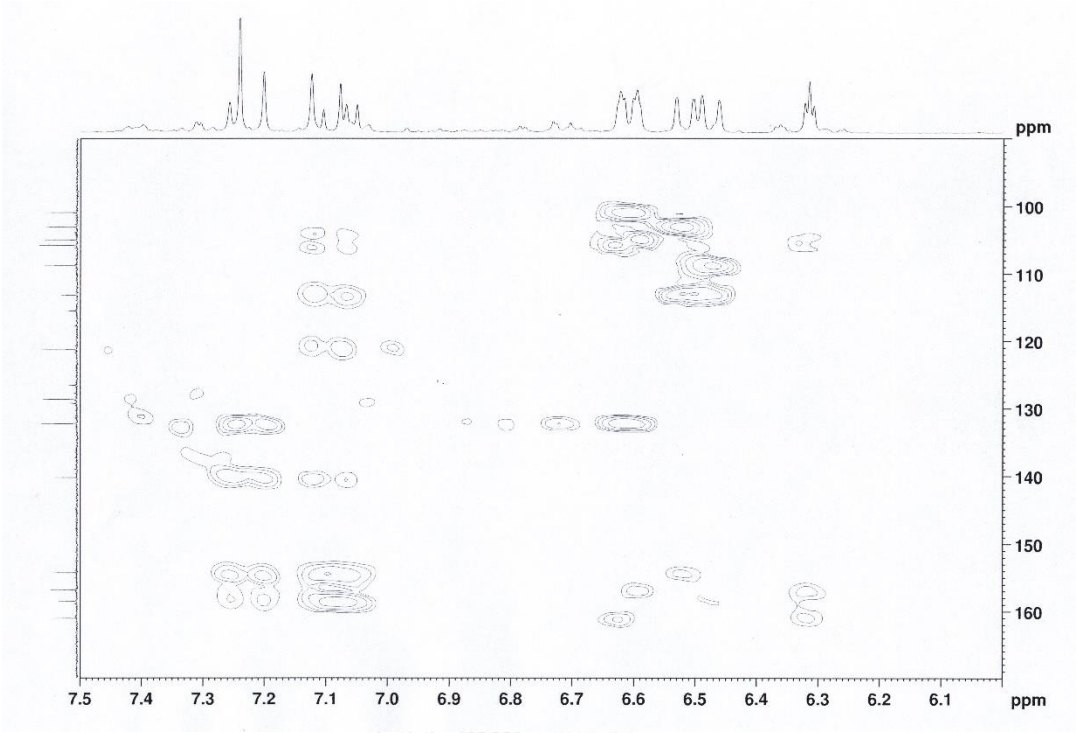


Figure 56 HMBC spectrum of compound PE08 in $CDCl_3$ (continued)

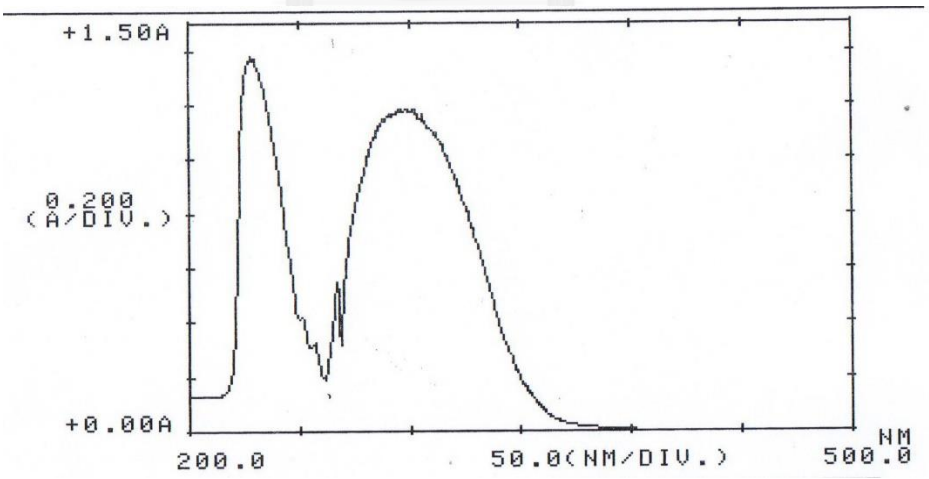


Figure 57 UV spectrum of compound PE09

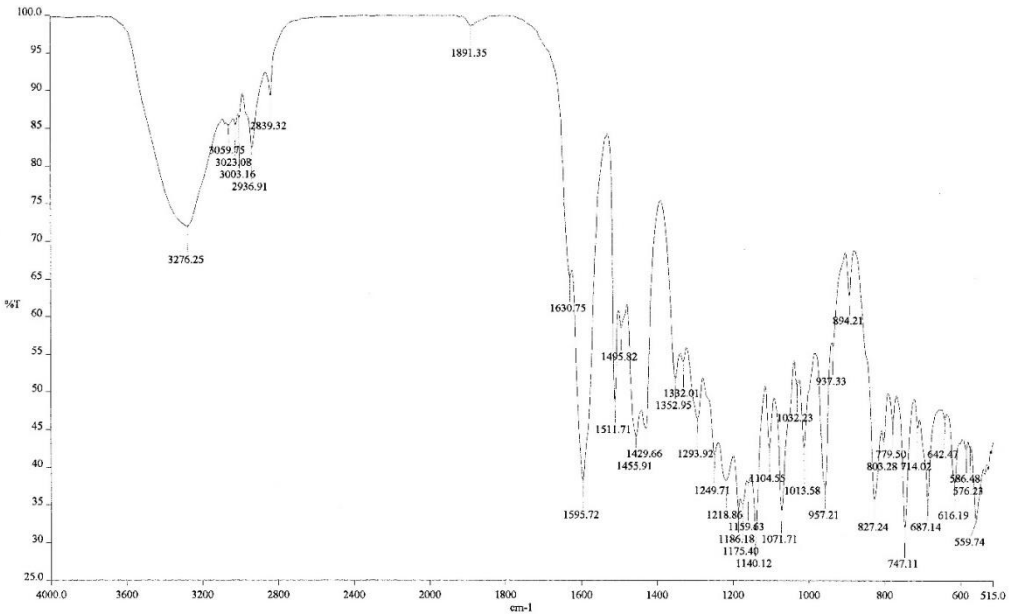


Figure 58 IR spectrum of compound PE09

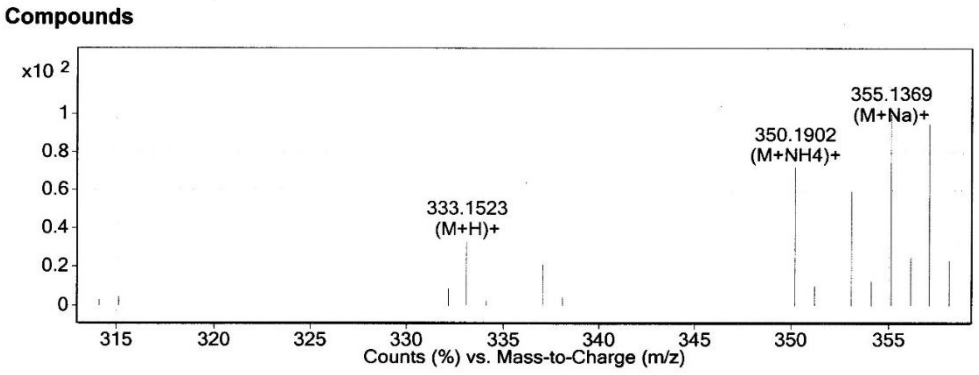


Figure 59 MS spectrum of compound PE09

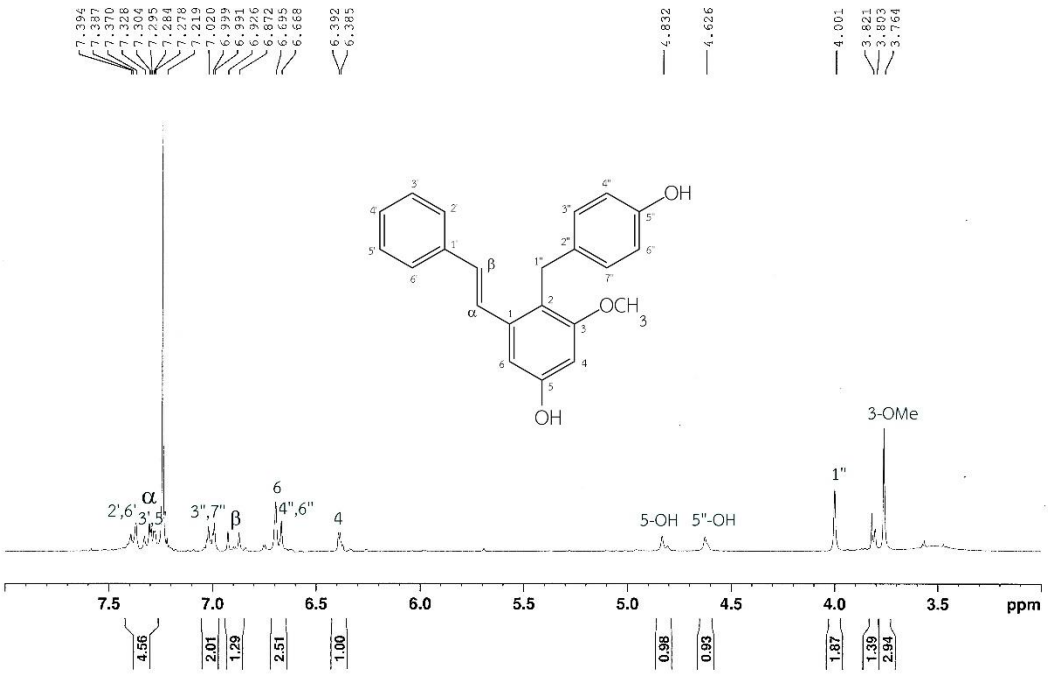


Figure 60 ^{1}H NMR (300 MHz) spectrum of compound PE09 in $CDCl_3$

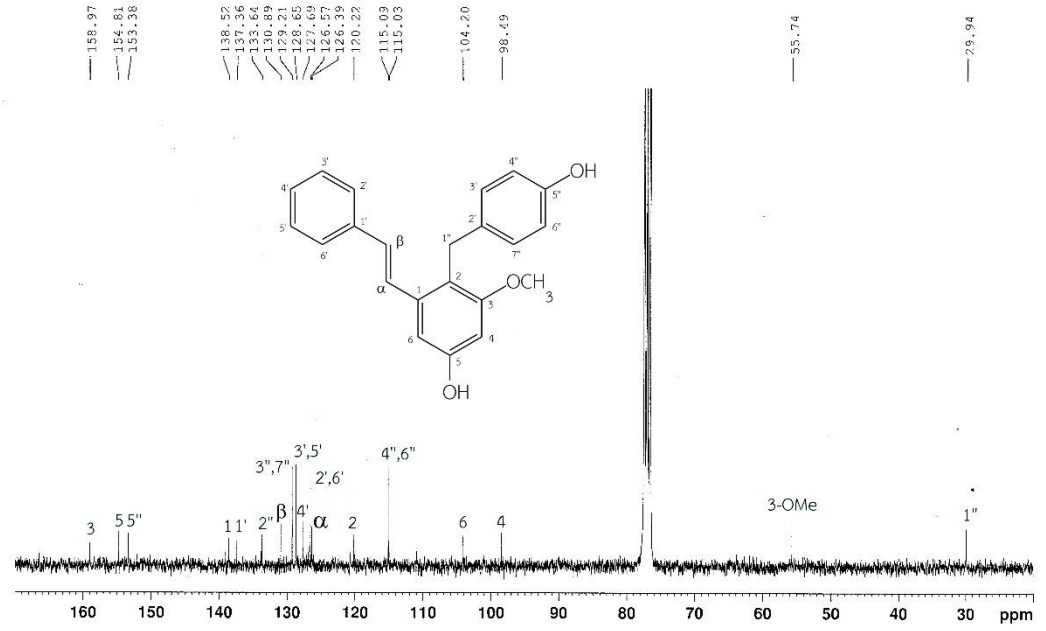


Figure 61 ^{13}C NMR (75 MHz) spectrum of compound PE09 in $CDCl_3$

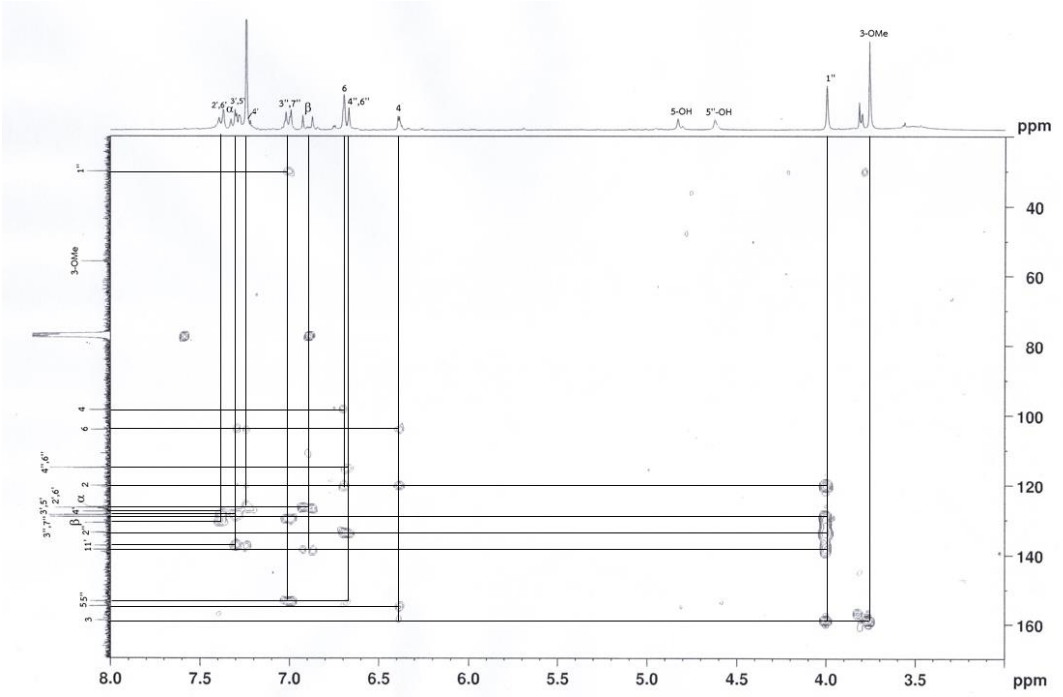


Figure 62 HMBC spectrum of compound PE09 in $CDCl_3$

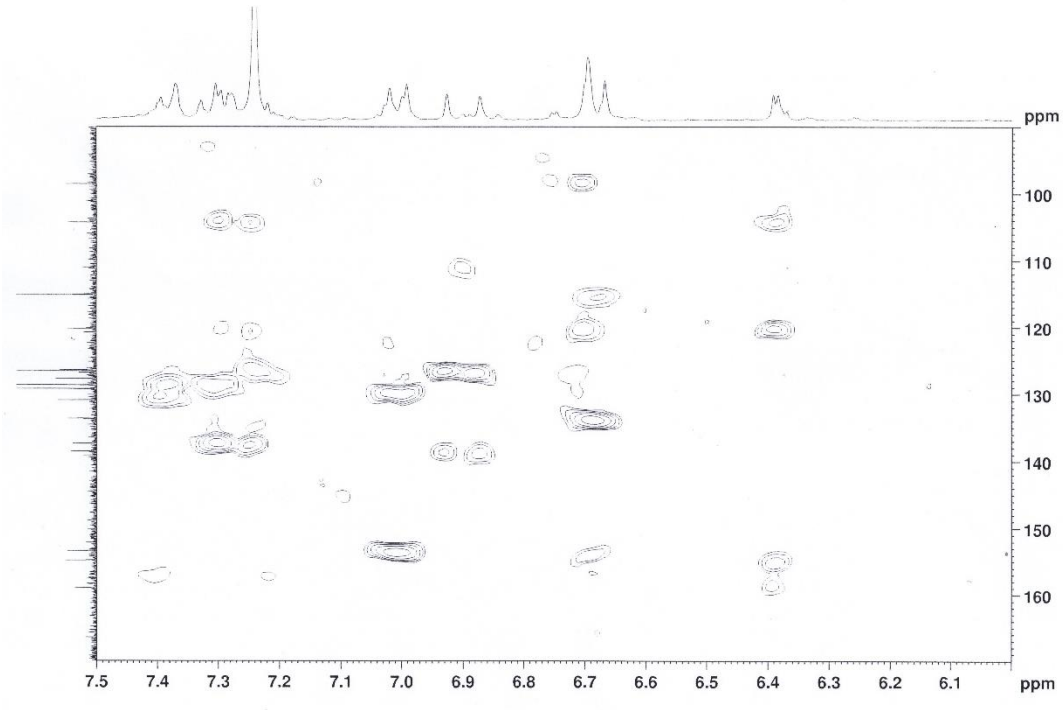
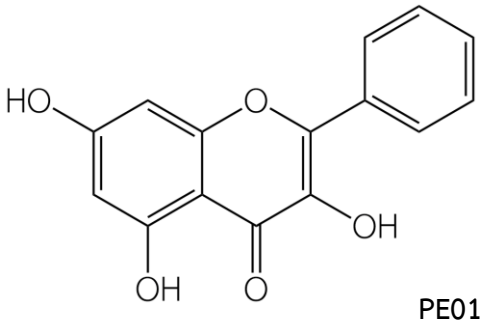
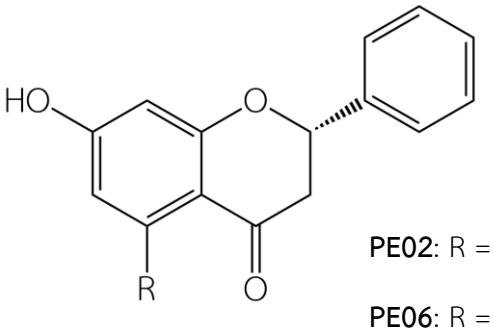


Figure 62 HMBC spectrum of compound PE09 in $CDCl_3$ (continued)

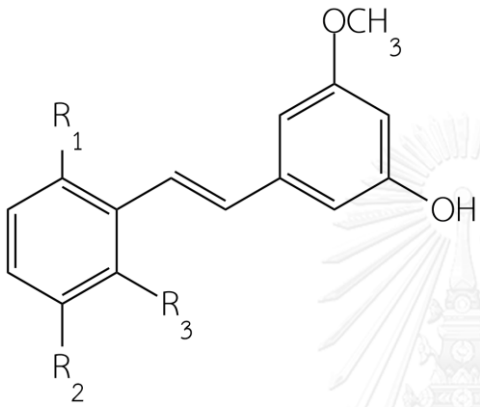


PE01

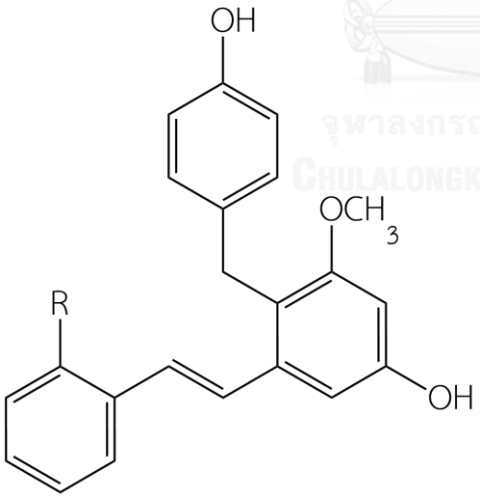


PE02: R = OH

PE06: R = OCH_3



	R_1	R_2	R_3
PE03	H	H	H
PE04	OCH_3	H	H
PE07	OH	OCH_3	H
PE08	OH	H	OCH_3



PE05: R = OH

PE09: R = H

Figure 63 Chemical structures of isolated compounds from *P. exul* roots

Table 20 Media formula for yeast culture

Ingredients	concentration (g/L)
1. Growth media (YPD media) (Difco™)	
1.1. Broth	
Yeast extract	10.0
Peptone	20.0
Dextrose	20.0
1.2. Agar	
Yeast extract	10.0
Peptone	20.0
Dextrose	20.0
Agar	15.0
2. Synthetic complete media lacking uracil (S.C. ura⁻ media)	
2.1. Glucose containing agar medium	
Yeast nitrogen base (Difco)	6.7
Amino acid mixture lacking uracil (Sigma)	1.92
Bactro™ agar (Difco)	20.0
Dextrose (Difco)	20.0
2.2. Galactose containing agar medium	
Yeast nitrogen base (Difco)	6.7
Amino acid mixture lacking uracil (Sigma)	1.92
Bactro™ agar (Difco)	20.0
Galactose (Difco)	20.0

VITA

Miss Naphatsawan Pooreecharirot was born on January 23, 1991 in Bangkok, Thailand. She received her Bachelor's degree in Pharmaceutical Sciences from Chulalongkorn University in 2013.

Poster Presentation:

Naphatsawan Pooreecharurot, Nonthalert Lertnitikul, Rutt Suttisri and Suchada Sukrong. Stilbenes from Paphiopedilum exul roots. 32nd International Annual Meeting in Pharmaceutical Sciences "Pharmaceutical research for the local needs and international collaborations", March 10 - 11, 2016, Faculty of Pharmaceutical Sciences, Chulalongkorn University, Bangkok, Thailand.